Research Topics

Advancing science in support of sustainable bio-innovation: 16th ISBR Symposium

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Advancing science in support of sustainable bio-innovation: 16th ISBR Symposium

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Editorial: Advancing science in support of sustainable bio-innovation: 16th ISBR Symposium

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Editorial on the Research Topic
Advancing science in support of sustainable bio-innovation: 16th ISBR symposium

The ISBR Symposium is an international meeting organized biennially by the International Society for Biosafety Research (ISBR) since 1990 at various locations throughout the world (www.isbr.info). Aim of the symposia is to provide a unique opportunity for public and private sector research scientists, regulators, technology developers, non-government organizations and others to share their experience and expertise and to discuss biosafety related to the sustainable application of biotechnology. As with past symposia, ISBR hosted a Research Topic in Frontiers in Bioengineering and Biotechnology: section Biosafety and Biosecurity, open to the presenters at the most recent 16th ISBR Symposium held in May 2023 in St. Louis, Missouri, USA (Figure 1).

Around the central theme of 'Advancing Science in Support of Sustainable Bio-innovation', the 16th ISBR Symposium program included a series of presentations in four topical plenary sessions: 1) Ensuring social license for bio-innovation; 2) Risk analysis for persistent engineered genetic traits; 3) Fit-for-purpose governance frameworks for sustainable bio-innovation; and 4) Sustainable biotechnologies for a changing world. In addition to these plenary sessions, the symposium included over 20 organized sessions and workshops offered in parallel on a range of topics, and numerous Pecha Kucha and traditional poster presentations. Out of the diverse presentations, the society has assembled a Research Topic of 17 peer-reviewed publications representative of the different topics presented and discussed at the ISBR symposium.

As the ISBR Symposia aspire to delve broadly into considerations of biosafety policy as well as research, included in the Research Topic from the 16th ISBR Symposium are reviews discussing innovative approaches to the regulation of biotechnology. Two of these articles, by Storer et al. on modernizing and harmonizing regulatory data requirements for

FIGURE 1
The 16th ISBR Symposium was held in May 2023 in St. Louis, Missouri, United States.

genetically modified crops and by Koch et al. on improving regulatory efficiency for biotechnology products, cover an important and recurring theme for the ISBR about using the growing years of experience with regulation of GM crops to make the regulatory process and decision-making more efficient and effective. Pence et al. also wrote about applying knowledge and experience from potato to update genetic stability data requirements in the risk assessment for cases of vegetatively propagated biotech crops.

Another review article by Nakai et al. is about the concept and scientific justification for data transportability for confined field trials conducted to support the risk assessment of GM plants. Data transportability, as a concept, considers the use of laboratory and field data generated on GM plants in one country to support the risk assessment of GM plants in another country, minimizing unnecessary duplication of regulatory studies and increasing efficiency in regulatory decision-making. The concept is being adopted in more and more countries and regions as experience with GM crops increases. Candia et al. also touch upon data transportability as a way to improve efficiency in an article describing the biosafety system in Paraguay and how the system might be improved taking experiences with its implementation into consideration.

Several articles also highlighted technical and policy issues related to some of the newer gene-editing techniques which require a different approach to regulation from that established for what is now thought of as 'traditional' genetically engineered organisms with inserted genes. Among these articles is a second paper with insights from Paraguay, by Rios et al., on the regulatory landscape for new breeding techniques (NBT) such as gene-editing. Goralogia et al. discuss rare but diverse off-target and somatic mutations found in field and greenhouse grown trees expressing CRISPR/Cas9. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is one of the most common tools used for gene-editing. One review article by Tripathi et al. discusses the use of CRISPR/Cas-based gene editing for improvement in one specific crop, bananas. Another article related to gene-editing, by McFadden et al., presents the results of a study to determine U.S. public opinion about the safety of gene editing in the agriculture and medical fields and the amount of evidence needed to improve opinions. A related opinion article by Lunshof discusses whether 'social license', as it has been used in the past, is obtainable in the case of novel bioinnovations that will be deployed in a shared environment, and suggests there might be more appropriate scenarios for involving all stakeholders in responsible deployment of novel bioinnovation.

Other articles presented more technical information for biosafety risk assessment. Avisar et al. share a risk assessment evaluation perspective in a research article on GM eucalyptus expressing pesticidal proteins from *Bacillus thuringiensis* for insect resistance. Mukani et al. present the results of a study on the nutritional composition analysis of GM potatoes developed in Kenya with resistance to late blight resistance, nutritional composition being a data set and analysis typically required for food safety assessment of GM crops. Two other studies, both from Bangladesh, present the analysis of baseline information management of fertilizer practices in potato (Nahiyan et al.) and crop weed management (Islam et al.), to inform the development and risk assessment of GM crops in this country.

Ahmed et al. share a perspective on understanding public perspectives on GM brinjal and the adoption of modern biotechnology in Bangladesh. Two more perspective articles in the Research Topic deal specifically with biotechnology and biosafety education. One from Diaz et al. is about Building bio-innovation systems through advanced biotechnology education, and another from Vicien and Rubinstein is about the successful implementation of a graduate certificate on risk analysis for the Agrifood sector at the University of Buenos Aires.

Finally, an opinion from Gray et al. is included in this Research Topic to honour the memory of a treasured member of the ISBR community, the late Professor Alan Raybould. ISBR gratefully acknowledges the contribution from all the authors to this Research Topic. The society has identified an important niche to fill in the scientific community, and the diversity of topics and article types published as part of this Research Topic exemplify the goals and impact of the ISBR Symposium. The society intends to continue to bring together this unique group to share perspectives, learn from experiences and plan for sound scientific global approaches to biosafety in the future. The 17th ISBR Symposium will take place in November 2025 in Ghent, Belgium.

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Improving regulatory efficiency for biotechnology products

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Small procedural changes in how regulatory agencies implement biotech policies can make significant differences in improving regulatory efficiency. This paper discusses how science based, crop specific guidance documents can improve dossier content and the review and approval of biotech varieties. In addition, we describe how the adoption of established risk assessment methodology and applying policy-linked decision making at the agency level can boost both efficiency and developer, public and government confidence in agency decision making and in biotech crops.

KEYWORDS

regulation, risk assessment, decision making, data requirements, regulatory efficiency

Introduction

Biotech plant varieties have the potential to offer society more sustainable food production, improved food security, and more appealing foods. However, before these benefits can reach farmers, markets, and consumers, they rely on an efficient, predictable, and timely regulatory review process. In addition, national decisions on biotech safety evaluations can build consumer confidence when they are linked to national policies that provide clarity on why biotech products are important for the country (Raybould, 2021).

Many improvements in the efficiency of regulatory review processes do not require changes to regulations or rulemaking. Based on a decade of experience obtaining approvals for food safety and environmental release of more than ten biotech potato varieties in twelve countries, we have noted recurring gaps in some national regulatory procedures. These gaps significantly slow down the review, approval, and marketing of biotech products. In this paper we focus on three of these gaps: the absence of crop specific data requirements, poor risk assessment methodology, and delayed decision making. We show that these regulatory delays impact on farmer access to improved planting material, the financial cost of regulation, and consumer acceptance, and we suggest ways to improve regulatory efficiency and decision making without compromising food or environmental safety reviews.

Crop-specific data requirements

To keep regulatory agencies effective and efficient, it is important that data requirements for safety reviews are written in guidelines, not in regulations or Acts. For the last 30 years, agencies have reviewed hundreds of new biotech varieties, many of which are seed crops like cotton, maize, and soybean (ISAAA, 2019). Early biotech products helped to formulate the regulatory procedures and requirements in many countries. In some countries, general data requirements for safety assessments that are appropriate for these

FIGURE 1
Hierarchy of legal instruments and time to implementation.

seed crops, are written into the laws (Acts) that govern release and use of biotech products. Data requirements in these laws make it difficult or impossible for regulators to adapt their review processes to new or different crops where seed crop-specific data requirements are inappropriate.

Generally, Acts are overarching legal instruments that govern broad categories of activities and things, such as biosafety laws that regulate biotechnology and the products of biotechnology (Figure 1). Changing or establishing an Act can take many years depending on the country and political priorities. Regulations are developed under the authority of Acts; they lay out processes for implementation and may narrow the scope of implementation to specific high-risk activities or products. Updating regulations can take several years and generally requires public consultation and interagency review. Guidelines, developed by the implementing department, help provide clarity and navigate stakeholders through the regulatory process. Guidelines are working documents that can be developed more easily and are focused on specific processes that need clarification. They can be updated and edited as regulated activities, new technologies, and science evolve.

Guidelines are most useful when they are crop specific, published proactively, and developed in consultation with stakeholders. Good examples of crop specific guidelines for biotech crops are provided by the Canadian Food Inspection Agency (CFIA; Crop-specific terms and conditions—Canadian Food Inspection Agency (canada.ca)). This agency proactively develops crop-specific guidelines when they are informed that new biotech varieties will be entering the Canadian regulatory system. These guidelines give developers a clear understanding of the terms and conditions that will apply to the design and running of their field trials and improve the developer's ability to provide science-based, well-structured applications to the agency. Similarly, CFIA develops biology guidance documents for specific crops to help developers prepare for environmental safety assessments when the varieties move to regulatory approval for commercial use (Biology Documents—Companion Documents for Directive 94-08—Canadian Food Inspection Agency (canada.ca)).

Simplot has been working with biotechnology to improve potatoes for the past 20 years. In that time there have been requests from regulators for data on analytes that are important in seed crops but are not present at measurable levels in potatoes, genetic stability data for a clonally propagated crop, and grain seed handling standards applied to perishable potato seed tubers.

In our experience, composition analysis of biotech crops runs into the tens of thousands of United States dollars (US$) for each biotech product. Analytes such as lipids and phytase levels are important composition requirements for some crops, such as cotton, canola, and soybean, but these analytes are typically below the level of detection in potato tubers. One way to improve efficiency of the regulatory review process would be to remove data requirements from laws or regulations where they require biotech developers to collect data that is not relevant to safety reviews and can be costly. AgBioInvestor (2022) calculated the average cost to bring a biotech event to the market was US$ 115 million and of this, US$ 43.2 million was spent on regulatory compliance and approval, 37% of the total expense. These costs would be prohibitive for public sector and small to medium enterprises trying to bring improved biotech varieties to the market. In addition, amending regulations for each crop is often impractical and time consuming. However, countries that provide crop specific data requirements in guidance documents can rapidly adapt the requirements based on the crop under review.

Similarly, genetic stability is important for inheritance and breeding in seed crops but has no impact during the clonal propagation of vegetative crops like potatoes, sweet potatoes, bananas, strawberries, and ginger. Because the suggestion to check stability comes from international risk assessment guidance (CODEX CAC/GL 45-2008), some agencies feel obligated to request these data for vegetative crops even though clonal propagation does not involve the major contributors to genetic instability, i.e., meiosis, segregation, or recombination (Pence et al., in this publication). Genetic stability data requirements have no scientific justification for vegetative crops and can be costly, which adds regulatory burden to all developers, including small developers and the public sector. Agencies could use science-based decision making to remove data requirements, such as stability data for vegetative crops, that provide no input on safety of the crop under review.

Some countries have a regulatory requirement that the biotech variety being reviewed must be grown and tested locally in their country for safety assessment, even when the application is specifically for food safety approval for imported food products. This additional testing is duplicative based on identical studies already conducted in the country of origin and is often required even when the crop will not be cultivated in that country either due to environmental conditions or phytosanitary regulations prohibiting the importation or cultivation of the plant. When regulations require the developer to provide planting material, the regulators request that seed is shipped to the agency for field testing. This process is suitable for true seed, such as corn or soybean, which stores well for months at room temperature, but is not feasible for vegetative propagation material, like potato seed tubers, which are bulky and perishable. Shipments of vegetative planting material require specialized transport, equipment, and sufficient storage space in controlled environments, which are rarely available at regulatory offices. In addition, importation of potato tubers is often restricted or prohibited by phytosanitary regulations.

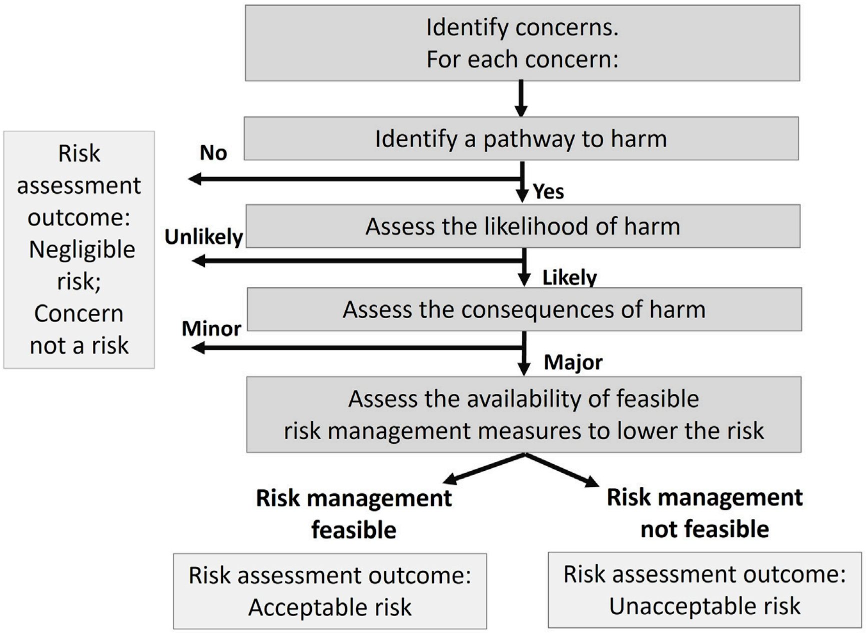

FIGURE 2
Risk assessment methodology.

Unless regulatory requirements are appropriate for different crops, they delay the regulatory process and remain a problem for developers. Other mechanisms to ensure that required safety data are appropriate for specific crops include enabling pre-submission consultations with regulators and enabling the use of data waivers where it is agreed that current data requirements are not suitable for specific products. Pre-submission consultations allow developers to discuss data requirements with the regulators and to request data waivers where there is justification for this allowance.

In addition, there is evidence supporting the transportability of risk assessment data between countries and agroclimatic zones (Nakai et al., 2015; Vesprini et al., 2020; Backman et al., 2021). Laboratory, greenhouse, and field data generated for a biotech crop in one country can be used to support risk assessment for the same crop in other countries (Bachman et al., 2021). This practice is implemented by regulatory agencies in countries like Argentina, Brazil, Canada, and Japan, for environmental risk assessment (Nakai et al., 2015; Vesprini et al., 2020) and in most countries for food safety risk assessments. Policy decisions that enable agencies to consider data generated in other countries for environmental safety and food safety could improve efficiency of regulatory reviews for new biotech products. Using this approach, only the identification of a potential hazard specific to the country and not yet adequately addressed by existing data would require additional assessment and might require country specific data (Bachman, et al., 2021).

Risk assessment methodology

Risk assessment methodology is well established and many guides exist for its application to biotech products, for example, the Australian Office of the Gene Technology Regulator's Risk Analysis Framework (OGTR, 2013). The risk assessment methodology applied in biotech regulatory agencies varies widely from country to country. Even established agencies with years of experience sometimes appear to improperly apply established risk assessment methodology. As a result, developers receive a substantial number of questions with no identified safety concern or pathway to harm.

Risk assessment, which includes risk identification and risk management steps, is used to identify plausible pathways to harm, assess the likelihood that harm will occur, understand the consequences should harm occur, determine whether risk management can reduce the likelihood or consequences of harm, and assess whether the risk of each safety concern is acceptable or unacceptable in the local context. This stepwise risk assessment aligns with problem formulation (Wolt, et al., 2010) and identifies and describes safety concerns and is used to categorize them into i) concerns with no plausible mechanism for harm to occur, ii) concerns with low likelihood or minor consequence, and iii) concerns with unacceptable risk that need further clarification and risk management (Figure 2). Safety concerns with no or low risk, generally do not require additional information from the developer. Concerns with plausible pathways to harm, a likelihood of harm, and potentially major consequences, become the focus of the risk assessment.

Questions from regulators to developers can be formulated to obtain information that will clarify the likelihood and consequences of risk, understand applicable risk management measures, and bring risk to an acceptable level in the local context in order to make a risk assessment conclusion that can be used to guide decision making. Documenting the risk assessment process records all the concerns that were considered and identifies potential hazards that need to be addressed before the product can be approved. A summary of this record in the final decision document provides transparency and helps to build public trust in the regulatory process.

In our experience, use of this methodology varies widely from agency to agency. In agencies where risk assessment is not fully implemented, Simplot has seen a substantial number of questions asked that have no impact on product safety and with no defined decision point. For example, Simplot has a biotech potato variety that has been approved by food safety agencies in nine countries and has been on the market in the United States for over 8 years but has yet to receive food safety approval in some countries. These delays limit commercial release of this product, however, millions of servings have been eaten without any reported safety issue. In one country, the agencies reviewing the product have asked 173 questions during the 5-year review period. Questions for nine previous approvals of this product averaged eight per country. An analysis of the 173 questions shows that 66% have no identified risk basis. In addition, while this application is specifically for food safety approval, 12% of the questions relate to environmental safety of the biotech potato when grown in the import country. Of the questions asked, 31% are duplicate questions asked by different agencies within the same country. Finally, 9% of the questions could be addressed by involvement of a local potato expert on the review committees. Many of the questions are curiosity questions raised by scientists interested in the product or the technology. In biosafety terms these would be considered ‘nice to know’ questions with no bearing on safety. Functioning risk assessment methodology would ensure that all questions reaching the developer were ‘need to know’ with direct bearing on the safety of the product. This example illustrates the regulatory barriers that developers face when regulatory processes are not efficient and are not risk-based.

Global harmonization of risk assessment and regulatory approvals is an achievable goal that would accelerate the approval and deployment of safe biotech crops and products. An example of this is the 2014 policy decision in Vietnam that any biotech product with food safety approval in five developed countries would be eligible for food safety approval in Vietnam without additional review or data requirements (Ministry of Agriculture and Rural Development, 2014). This is a science-based policy decision that makes it easier for developers to move new biotech crops to the market. Facilitated approval of biotech products in import countries ensures that farmers in production countries can benefit from the improved planting material without long delays. As more biotech products are developed by public sector scientists, who may not have the expertise and funding to complete regulatory approvals in multiple countries, regulatory efficiencies will be essential if countries are to benefit from the improvements these crops bring to the sustainability of food production.

Policy-linked decision making

In countries where socio-economic considerations such as the impact on food prices, consumer acceptance, or farmer access to seed, are part of the decision process, the risk assessment outcome is not the final regulatory decision. However, where approvals are based on safety, the risk assessment conclusion determines the decision, and the agency quickly issues a regulatory decision based on safety of the product. These are risk-based regulatory systems and are the most supportive of new biotech variety introduction. In countries where the risk assessment is not the only factor that informs regulatory decisions, decisions can be influenced by the socio-political environment, such as pressure from groups in opposition to biotechnology or concerns that approval of biotech products will impact negatively on careers of the decision makers. For this reason, reaching a safety conclusion at an agency does not necessarily mean reaching a quick decision. An example of this is provided in Table 1 for one potato variety in 12 countries.

There is a clear difference in the regulatory timelines between risk-based decision making and socio-political decision making (Table 1). Average time to risk conclusion with risk-based assessment was 63% faster than socio-political decision making (19.4 months vs 52.7 months). Moreover, time to decision was 79% faster when the assessment was risk based (19.4 months vs. 91 months). These delays restrict farmer access to new potato varieties in production countries and inhibit the ability of developers to release new crop varieties in these countries. Delaying farmer access to new varieties restricts their access to sustainable production benefits, thereby limiting the ability of production countries to address climate challenges.

Raybould (2021) discussed the delays and problems with biotech regulatory decision making and noted that regulatory decisions are inevitably political because decision makers decide whether particular products will help or hinder the delivery of public policy objectives. He suggested to regulators that a way to overcome the political uncertainty of biotech decision making would be to include in decisions the impact products could have on national policies, or locally adopted international policies. Many countries have policies for food security, poverty alleviation, rural development, sustainable agriculture, climate mitigation, and technology innovation that are supported by the regulatory approval and use of improved crop varieties, even those developed using biotechnology. In addition to these national policies, many countries have adopted United Nations Millennium Development Goals (United Nations, 2000) and Sustainability Goals (United Nations, 2015). The Millennium Development Goal #1 is to eradicate extreme poverty and hunger, and the Millennium Development Goal #7 is to ensure environmental sustainability. The Sustainable Development Goal #2 is No Hunger, and #12 is Responsible Consumption and Production. Each of these can support and strengthen biotech decision making when the product supports a policy goal.

Many traits selected for integration into popular and commonly grown biotech varieties directly contribute to policies focused on food security and environmental sustainability. Potatoes with genetic resistance to late blight disease have protection that increases marketable yields, contributes to eradicating hunger in potato growing countries, and would reduce poverty in rural areas where farmers are affected by crop losses due to this disease. Similarly, biotech potatoes that produce more tubers on less land or decrease blackening due to polyphenol oxidase, would improve the sustainability of potato production by optimizing land usage and decreasing waste at harvest, processing, grocery stores, and homes. National development and sustainability policies offer a strong

TABLE 1 Timelines for risk-based vs. socio-political food safety decision making for one biotech potato variety.

Decision Criteria	Country	Time to risk conclusion (months)[a]	Time to decision (months)[b]
Risk based	1	25	25
	2	31	31
	3	9	9
	4	52	52
	5	12	12
	6	12	12
	7	8	8
	8	8	8
	9	18	18
Socio-political	10	36	88+[c]
	11	30	93+
	12	92+	92+

[a] Time until no more questions were received and/or agency noted the risk assessment was complete.
[b] Time until the regulatory decision was issued.
[c] + = decision not yet made.

framework for regulatory agencies as they grapple with socio-political decisions for biotech varieties.

Actionable recommendations

Assuming regulatory reform requires legislative rule making detracts from the many other implementation changes that can improve the efficiency of existing regulatory review processes and shorten the timelines for obtaining regulatory decisions. Our experience with obtaining approvals for biotech potato varieties in 12 countries suggests that three areas of regulatory implementation could have a positive effect on regulatory efficiency. The first is to ensure that data requirements are provided in guidance documents and are crop specific. This will help developers prepare submissions and reduce data collection and analysis by focusing on the safety of their crop variety. The second is to ensure that all reviewers are trained in risk assessment and the review process follows established risk assessment procedure. This will focus the review on questions important for safety and will filter out questions that might be of interest but have no relevance to safety. Part of achieving this risk assessment efficiency is to ensure that crop experts are available to the review committees and that there is good coordination between agencies to reduce duplication. The third area is to base regulatory decisions on the risk assessment outcome: i.e., the product is or is not safe for the local population. Where decisions include socio-political considerations, linking the product to national and locally adopted international policies provides a strong platform for decision making.

Regulatory efficiency is important for the release of new biotech varieties. Without a clear and efficient regulatory review and decision process, both public and private sector products will not be available to help improve farming livelihoods, food security, and environmental sustainability.

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Regulatory landscape for new breeding techniques (NBTs): insights from Paraguay

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Introduction

Regulation of New Breeding Techniques (NBTs)[1] in agriculture has garnered considerable attention and discussion in recent years. These emerging technologies have the potential to greatly impact crop productivity and address a range of global challenges, prompting countries worldwide to grapple with how best to regulate and oversee their use.

Despite the abundance of scholarly literature discussing the regulatory dimensions of these biotechnological advancements (Menz et al., 2020; Entine et al., 2021; Turnbull et al., 2021; Rosado and Eriksson, 2022; Sprink et al., 2022; Wei et al., 2022; Ahmad et al., 2023; Mikhaylova, 2023), detailed information on the specific criteria used by each country is often inaccurate. In the particular case of Paraguay's regulatory framework, precise criteria guiding the regulatory status of products derived from NBTs remain elusive or erroneously construed in the current scientific literature. To enhance the public understanding of this topic and provide an additional perspective, we examine the regulations surrounding NBTs in Paraguay.

Regulatory landscape

Different countries deploy diverse criteria to oversee crops derived from NBTs. Typically, these criteria consist of a definition, such as the Genetically Modified Organism (GMO) definition; triggers, like "novel trait" in the Canadian regulatory

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[1] The term "New Breeding Techniques" (NBTs) has been introduced to encompass the emerging technologies that harness modern biotechnology to create genetic diversity. However, it should be noted that as a class, NBTs lack universally agreed upon definitions (Whelan and Lema, 2015). Previously, the following have been classified as NBTs: genome editing, epigenetic modification, agroinfiltration, cis/intra-genesis, grafting with GMO material, reverse breeding, RNA interference and gene drives. For the purposes of this study, the term "NBTs" has been employed as synonymous with the terms "precision breeding," "new plant breeding techniques," or "new genomic techniques." (Rosado and Eriksson, 2022).

TABLE 1 Overview of various countries that have adopted the Argentinian criteria concerning New Breeding Techniques (NBTs).

Country	Party to the Cartagena protocol	NBTs first regulated (year)	Current NBT regulation	References
Argentina	No	2015	Resolution No. 21/2021 and three annexes[2]	Goberna et al. (2022)
Chile	No	2017	Consultation procedure[3]	Sanchez (2020)
Brazil	Yes	2018	Resolution No. 16/2018[4]	Nepomuceno et al. (2020)
Colombia	Yes	2018	Resolution No. 00022991[5]	Menz et al. (2020)
Paraguay	Yes	2019	Resolution MAG No. 842/2019[6]	Organization for Economic Cooperation and Development (2023b)
Ecuador	Yes	2019	Ministerial Agreement No. 063[7]	Gatica-Arias (2020)
Guatemala	Yes	2019	Resolution No. UA 60–2019: https://visar.maga.gob.gt/visar/2019/20/InMini60-19.pdf and Annex: 65.06.01:18[8]	Hernandez-Soto et al. (2021)
Honduras	Yes	2019	Agreement SENASA 008–2019[9]	Gatica-Arias (2020)
Philippines	Yes	2020	NCBP Resolution No. 001[10]	Entine et al. (2021)
Nigeria	Yes	2020	National Biosafety Guidelines on Gene Editing[11]	Jenkins et al. (2021)
Kenya	Yes	2022	Guidelines for determining the regulatory process of genome editing techniques in Kenya[12]	Sprink et al. (2022)

framework; or a roster of inclusions and exclusions, as utilized in the Australian regulatory system (Duensing et al., 2018).

Argentina was a pioneer in the development and application of regulatory guidelines for NBT crops in 2015, and since 2014 it has earned the distinction of "Reference Center for Biosafety" from the Food and Agriculture Organization (FAO) (Ministerio de Economía de Argentina, 2022). The country's national biotechnology regulatory agency, CONABIA, provides guidance and technical assistance and engages in international cooperation to promote

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[2] Ex-Ministerio de Agricultura, Ganadería y Pesca (2021). Resolución N° 21/2021 S/EX-2020−72811589- -APN-DGD#MAGYP Establece procedimiento para determinar alcance en el marco de la Resolución N° 763/2011 - Nuevas Técnicas de Mejoramiento. Available at: https://magyp.gob.ar/sitio/areas/biotecnologia/conabia/_pdf/Resolution_N21-2021_3%20annexes.pdf.

[3] Servicio Agrícola y Ganadero de Chile (2017). Aplicabilidad de Resolución N° 1.523/2001 en material de propagación desarrollado por nuevas técnicas de fitomejoramiento. Available at: https://www.sag.gob.cl/ambitos-de-accion/aplicabilidad-de-resolucion-ndeg-15232001-en-material-de-propagacion-desarrollado-por-nuevas-tecnicas-de-fitomejoramiento.

[4] Comissão Técnica Nacional de Biossegurança, Brasil (2018). Resolução Normativa N° 16, de 15 de janeiro de 2018. Available at: http://ctnbio.mctic.gov.br/resolucoes-normativas/-/asset_publisher/OgW431Rs9dQ6/content/resolucao-normativa-n%C2%BA-16-de-15-de-janeiro-de-2018?redirect=http%3A%2F%2Fctnbio.mctic.gov.br%2Fresolucoes-normativas%3Fp_p_id%3D101_INSTANCE_OgW431Rs9dQ6%26p_p_lifecycle%3D0%26p_p_state%3Dnormal%26p_p_mode%3Dview%26p_p_col_id%3Dcolumn-2%26p_p_col_count%3D3%26_101_INSTANCE_OgW431Rs9dQ6_advancedSearch%3Dfalse%26_101_INSTANCE_OgW431Rs9dQ6_keywords%3D%26_101_INSTANCE_OgW431Rs9dQ6_delta%3D15%26p_r_p_564233524_resetCur%3Dfalse%26_101_INSTANCE_OgW431Rs9dQ6_cur%3D2%26_101_INSTANCE_OgW431Rs9dQ6_andOperator%3Dtrue.

[5] Instituto Colombiano Agropecuario (2022). Resolución No. 00022991. Available at: https://www.ica.gov.co/getattachment/e4b3a97e-b44e-4974-8bb0-f3b947063e67/2022R22991.aspx.

[6] Ministerio de Agricultura y Ganadería de Paraguay (2019). Resolución MAG N° 842/2019 Por la cual se aprueba el Formulario 3 de consulta previa para productos obtenidos mediante Nuevas Técnicas de Mejoramiento (New Breeding Techniques). Available at: https://conbio.mag.gov.py/media/ckfinder/files/RES.N842%2010%20DE%20JULIO%20DE%202019%20NBT.pdf.

[7] Ministerio de Agricultura y Ganadería de Ecuador (2023). Ministerial Agreement No. 063. Available at: http://servicios.agricultura.gob.ec/mag01/pdfs/aministerial/2023/063-2023_acuerdo_ministerial_guia_tecnica_edicion_genetica_vf_numerado-signed.pdf. Article 401 of The Constitution of Ecuador, as established in 2018, declares that the country is free of transgenic crops and seeds. Although the presence of transgenic crops or seeds in Ecuador is limited, consumption of food from GMOs or their derivatives is permitted as long as proper labeling is provided. However, regulations specific to NBTs are not included in these provisions. Organisms that are genetically improved through genome editing or other NBTs will not undergo risk assessment if foreign or recombinant DNA is not present in the resulting improved organism. Ministerial Agreement No. 063/2023 is consistent with the existing regulatory framework for genetically modified organisms because it contributes to the procedures for determining whether a seed or crop contains foreign genetic material and whether it is part of the seed or crop genome.

the development of sound regulatory frameworks and approaches for biotechnology.

The Argentinian model focuses on regulating NBT crops which involve permanent insertion of foreign DNA. In other words, products obtained through modern biotechnology techniques that do not have a new combination of genetic material are considered conventional (Goberna et al., 2022; Pixley et al., 2022). Several countries have adopted similar approaches based on Argentina's lead (Table 1).

It is important to highlight that multiple regulatory agencies in different countries work closely together to discuss regulations for products derived from modern biotechnology (Organisation for Economic Cooperation and Development, 2023a). Given the evolution of technologies used in the genetic improvement of organisms for agricultural use, these guidelines are subject to periodic updates, resulting in variations in the dates in which they come into force that are reported by the different sources.

Regional collaboration

The Southern Agricultural Council (CAS) was established in April 2003, and comprises the Ministries of Agriculture of Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay. It aims to identify collaborative regional initiatives for short- and medium-term cooperation. To facilitate this process, CAS has established the Agricultural Policy Coordination Network, which includes the Directors of Agricultural Policies of each member country, as well as various technical-scientific groups. One such group is Technical Group 5 (GT5-CAS) on Public Policies on Biotechnology (Rocha-Salavarrieta, 2022).

Recognizing the significance of NBTs for agricultural development, the GT5-CAS stresses the importance of making science-based decisions to facilitate research and development while avoiding unnecessary trade barriers. In light of this, the said Ministries have declared that they would "seek to work together and with third countries to avoid unscientific barriers to trade in gene-edited agricultural products" (Consejo Agropecuario del Sur, 2018) and they have agreed to "promote the development and application of agricultural innovations that will enable our region to sustainably produce more and better food that is safe and nutritious for human consumption in order to contribute to food security and nutrition, ensuring access to such innovations for all producers, thus promoting economic and social development." (Consejo Agropecuario del Sur, 2016). They also aim to enhance capacity-building efforts and encourage collaboration among countries for information exchange regarding product development and regulatory progress. This strong institutional support has contributed to technical and regulatory advancements for NBTs within the Southern Cone region (Rocha-Salavarrieta, 2022; Soerensen and Valdovinos, 2022).

Paraguay NBT regulation

The regulatory system for agricultural biotechnology in Paraguay has made significant progress since its implementation by the Ministry of Agriculture and Livestock (MAG) in 1997. These advancements aim to establish a sound framework based on scientific criteria that include key concepts such as familiarity, history of safe use, substantial equivalence, transportability, and problem formulation (Fernández Ríos et al., 2018; Benítez Candia et al., 2020).

Specifically, in the case of NTBs, in 2019, MAG implemented a process to assess whether crops derived from these new technologies fall within the scope of the GMO regulation (Ministerio de Agricultura y Ganadería del Paraguay, 2019), as outlined in the Cartagena Protocol on Biosafety. The determination resulting from this procedure classified crops as either conventional or non-conventional (Organisation for Economic Cooperation and Development, 2023b). This classification is based on two main criteria: a) the utilization of genetic engineering techniques and b) the creation of a new combination of genetic material achieved through stable and simultaneous integration of nucleic acid sequences that form an identifiable genetic construct (Secretariat of the Convention on Biological Diversity, 2000).

The implemented procedure prioritizes a thorough evaluation on a case-by-case basis by the National Commission on Agricultural and Forestry Biosafety (CONBIO), free from any predetermined list of techniques or classification systems (e.g., SDN-induced variants). This procedure is not a risk assessment, and instead explores whether the NBT could result in genetic changes similar to those achieved through traditional breeding methods or naturally occurring ones. This norm gives certainty to developers about the regulatory status of a product. Furthermore, the analysis takes into account a science-based consideration of NBTs (Figure 1).

The application form consists of different sections, including: a) Applicant information, b) Organism information (taxonomy and the specific cultivar/line that will be introduced to the agroecosystem), c) Molecular biology details (description of the technique employed, target nucleotide sequences, and any functional modifications made; evidence demonstrating absence or presence of recombinant sequences), d) Phenotype considerations (examples of existing

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[8] Ministerio de Agricultura, Ganadería y Alimentación de Guatemala (2019). Resolution No. UA 60-2019. Available at: https://visar.maga.gob.gt/visar/2019/20/InMini60-19.pdf.

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[10] National Committee on Biosafety of the Philippines (2020). Resolution No. 001. Available at: https://bch.dost.gov.ph/downloads/category/8-the-regulation-of-plant-and-plant-products-derived-from-the-use-of-plant-breeding-innovations-pbis-or-new-plant-breeding-techniques-nbts?download=23:pbi-resolution.

[11] National Biosafety Management Agency, Federal Republic of Nigeria (2020). National Biosafety Guidelines on Gene Editing. Available at: https://nbma.gov.ng/wp-content/uploads/2022/03/NATIONAL-GENE-EDITING-GUIDELINE.pdf.

[12] National Biosafety Authority, Kenya (2022). Guidelines for determining the regulatory process of genome editing techniques in Kenya. Available at: https://www.biosafetykenya.go.ke/images/GENOME-EDITING-GUIDELINES-FINAL-VERSION-25th-Feb-2022-03.pdf.

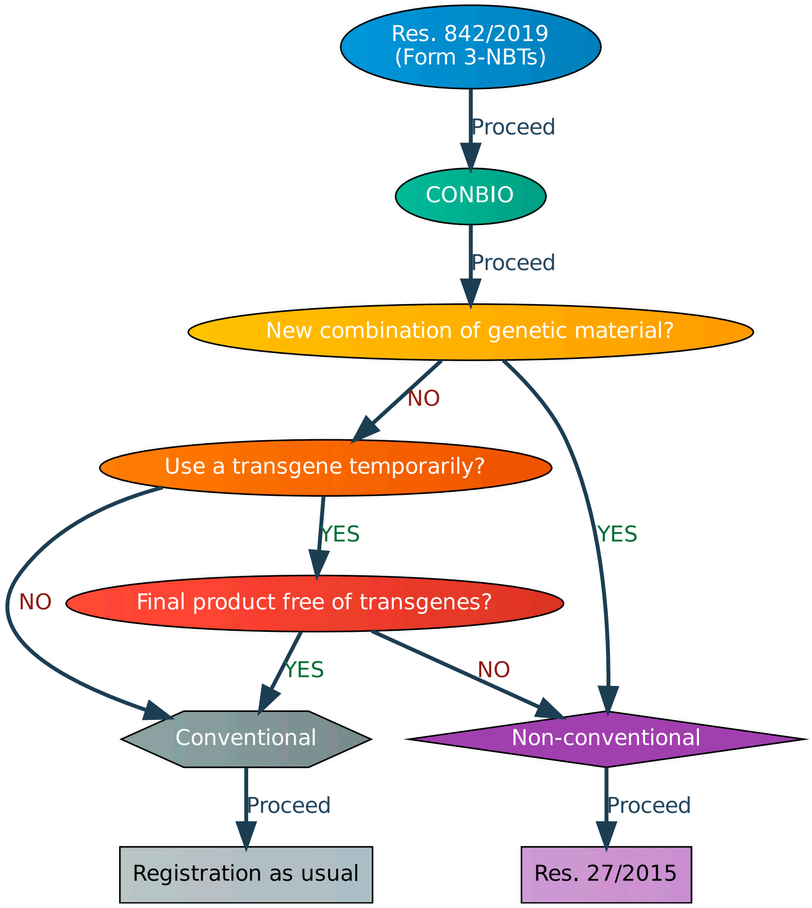

FIGURE 1
Decision-making flowchart that guides through various stages of determining whether a certain crop obtained through NBTs can be classified as "Conventional" or "Non-conventional" in Paraguay[13]. The process starts with the introduction of Form 3, leading to an analysis of the information presented by representatives of CONBIO. The primary determining factor is whether there is a "New combination of genetic material".

crops in the market with similar phenotypes, anticipated changes in proposed uses and management practices, analysis of the possibility of the occurrence of other effects beyond the desired phenotypes) and e) Authorizations (if the propagation material has been authorized by the official agency of any country) (Ministerio de Agricultura y Ganadería del Paraguay, 2019).

Discussion

The regulatory landscape surrounding NBT crops on a global scale is dynamic, with countries continuously adapting their regulatory policies, which presents multiple opportunities not only to implementing countries but also at the regional level (Schmidt et al., 2020). This translates into a joint recognition that this set of techniques is important and relevant for the sustainable innovation of production systems, contributing to global food security and other societal benefits.

Some countries with established approaches to regulating NBT crops share the common practice of conducting analyses on a case-by-case basis and on novel combinations of genetic material as a threshold for regulation. According to this approach, the regulatory framework for NBT-derived crops is consistent with that of conventional breeding, if they are indistinguishable. Furthermore, this ensures that safety regulations prioritize traits, phenotypes, and intended uses (Friedrichs et al., 2019).

There is strong consensus that international regulatory coordination is needed to support the development of effective science-based regulatory systems for NBT crops, ensuring consistent and comprehensive oversight of these innovative technologies (Entine et al., 2021).

Scope statement

The following manuscript is a scholarly contribution to the field of Biosafety. It focuses on New Breeding Techniques (NBTs) and their

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[13] Ministerio de Agricultura y Ganadería (2015). Resolución MAG No 27/2015 Por la cual se aprueban los documentos Formulario 1: Ensayos regulados, 2: Liberación comercial y de la guía para Formulario 2: Liberación comercial Available at: https://conbio.mag.gov.py/media/ckfinder/files/Resolucion%20MAG%20N%2027_15%20Formularios_1.PDF (Accessed November 3, 2023).

regulatory frameworks, with a particular emphasis on Paraguay and its consistency with regional criteria. Furthermore, it investigates regional collaborative initiatives, procedural approaches in Paraguay, and the complexities of NBT regulations. This manuscript provides valuable insights into the broader global landscape of NBTs in agriculture.

Author contributions

DF: Conceptualization, Data curation, Investigation, Project administration, Resources, Software, Supervision, Writing–original draft, Writing–review and editing, Funding acquisition, Visualization. NB: Conceptualization, Data curation, Investigation, Software, Supervision, Visualization, Writing–original draft, Writing–review and editing. MS: Conceptualization, Data curation, Writing–review and editing. MG: Data curation, Investigation, Supervision, Writing–original draft, Writing–review and editing. AA: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

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U.S. public opinion about the safety of gene editing in the agriculture and medical fields and the amount of evidence needed to improve opinions

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Introduction: Implementation of gene editing in agriculture and medicine hinges on public acceptance. The objectives of this study were to explore U.S. public opinion about gene editing in agricultural and medical fields and to provide more insight into the relationship between opinions about the safety of gene editing and the potential impact of evidence to improve opinions about safety.

Methods: Data were from two samples of U.S. respondents: 1,442 respondents in 2021 and 3,125 respondents in 2022. Survey respondents provided their opinions about the safety of gene editing in the agricultural and medical fields and answered questions about the number of studies or length of time without a negative outcome to improve opinions about the safety of gene editing in the agricultural and medical fields.

Results: Results indicated that respondents in both samples were more familiar, more likely to have an opinion about safety, and more positive about the safety of gene editing in the agricultural field than in the medical field. Also, familiarity was more closely associated with opinions about safety than the strength of opinions.

Discussion: These findings add to the literature examining perceptions of gene editing in the agricultural or medical fields separately. Opinions about the safety of gene editing were generally more favorable for respondents who were aware of the use of gene editing. These results support a proactive approach for effective communication strategies to inform the public about the use of gene editing in the agricultural and medical fields.

KEYWORDS

gene editing, biotechnology, public opinion, consumer acceptance, science communication

1 Introduction

Gene editing, the process of precisely changing or deleting a few "letters" of DNA, has already contributed to agricultural and medical advancement, with many more applications in development. However, public perception may hinder implementation and it is unclear what U.S. public opinion about safety may vary across the two fields. It has been argued that the lack of proactive public dialogue surrounding the primary introduction of genetically modified organisms (GMOs) "did irreparable damage to the emerging scientific field of genetic engineering," and that the continued expansion of gene editing in the agricultural and medical fields has led many to call for "broad public dialogue" about the technology (NASEM, 2017). These calls are backed by the desire to "avoid unjustifiably inhibiting innovation, stigmatizing new technologies, or creating trade barriers" (Holdren et al., 2019). At the same time, news reports bring attention and fear to medical uses that can cause the public to question the ethical use, but also present opportunities for conversation about benefits and risks (Zhang et al., 2021). As technologies advance, it is vital to understand and engage the public in conversations about gene editing in agricultural and medical contexts. Opinions about the safety of gene editing in one field may provide the public with context for use in another field. It is, therefore, critical to assess public sentiment and barriers to acceptance.

Public aversion to the use of related biotechnology in agriculture has been well-documented (Lusk et al., 2005), despite support from the scientific community. For example, a 2014 Pew Research survey of U.S. adults and researchers affiliated with the American Association for the Advancement of Science (AAAS) estimated that 88% of AAAS members agreed that *genetically modified* foods were safe to consume compared to only 37% of adults (Funk et al., 2015). It is reasonable to posit that gaps between the opinions of researchers and the public are due to a lack of public understanding of evidence showing that approved biotechnology applications are safe. The public is likely unaware that more than 4,000 science-based risk assessments have concluded genetically engineered crops do not pose greater risks than conventionally bred crops (ISAAA, 2019), or that the National Academies of Sciences, Engineering, and Medicine concluded there had not been any scientifically documented human safety issues after 30 years of evaluation (NASEM, 2016).

Recent research on public opinion toward the use of biotechnology in agriculture has focused on differences in opinions between the use of gene editing and traditional genetic modification (transgenesis). These studies concluded that the public generally supports gene editing in agriculture more than transgenics (Kato-Nitta et al., 2019; Yang and Hobbs, 2020). However, public acceptance of gene editing compared to transgenic technology may differ due to some familiarity with gene editing for medical purposes. When participants in U.S. focus groups were asked what they thought about when hearing the words gene editing, the medical field was discussed more frequently and extensively than agriculture (McFadden et al., 2021a). The announcement of gene-edited twins in China increased public awareness of medical applications, as there was a surge in online searches for gene editing following the announcement (McFadden et al., 2021b). Yet, it is not clear that U.S. adults see a strong connection between gene editing in agricultural and medical fields (Watanabe et al., 2020), nor do we understand how their thoughts may vary across potential uses within fields. In Australia, survey respondents supported the use of gene editing in the agricultural and medical fields for research purposes; however, respondents were more supportive of gene-editing humans to improve health than animals used for food (Critchley et al., 2019).

Other recent research has examined differences in acceptance across agricultural commodities gene-edited for disease resistance (animal vs. plant) and acceptance for gene editing a host or vector (tree vs. insect) to reduce disease pressure (McFadden et al., 2021a; Bush et al., 2022). Respondents were more accepting of gene-editing plants than trees (Bush et al., 2022), and there were similar acceptance levels for trees and insects (McFadden et al., 2021b). Much research examining public attitudes about gene editing in the medical field has focused on which medical changes are acceptable to the public and the demographic characteristics associated with opinions. In general, results from public opinion research indicate support for therapeutic uses of gene-edited and aversion for non-disease uses that are cosmetic or otherwise alter physical characteristics (Gaskell et al., 2017; Treleaven and Tuch, 2018; Critchley et al., 2019; McCaughey et al., 2019; Jedwab et al., 2020; Watanabe et al., 2020; McFadden et al., 2021a; Kobayashi et al., 2022).

Calls for "more research" are made so often that some journals have banned using the phrase (Godlee, 2010). Science engagement must begin asking "how much?" or "what kinds?" of research are needed and or whether a different approach to promoting acceptance is required (Hering, 2016). Science communication promoting technology diffusion is more complex than simply reducing knowledge gaps (Simis et al., 2016), and behavioral responses to evidence can cause rejection of evidence that conflicts with prior beliefs about the safety of genetic engineering (McFadden and Lusk, 2015). Further, affective reactions like disgust can induce absolute moral opposition that devalues any evidence about the benefits of genetic engineering (Scott et al., 2016). Conversely, experience and familiarity with topics can lead to more favorable opinions (Liu and Priest, 2009).

The objectives of this study were to explore U.S. public opinion about gene-editing in the agricultural and medical fields and to provide more insight into the relationship between opinions about the safety of gene editing and the potential impact of evidence to improve opinions about safety. To complete the objectives of this study, data were collected from an online survey distributed to two samples of respondents. Survey respondents provided answers to five questions used as variables in this study. Asked in the following order, the questions measured familiarity with gene editing, the strength of opinion about the safety of gene editing, opinions about the safety of gene editing, and two questions measuring the amount of evidence needed to improve an opinion about the safety of gene editing (i.e., number of studies and amount of time without a negative outcome). Survey respondents answered these questions for gene editing in the agricultural and medical fields separately, with the order of presentation randomized across respondents.

2 Materials and methods

Data were collected at two time periods using surveys distributed by Qualtrics to online samples of U.S. adult respondents. The Institutional Review Board at the University of Delaware approved both surveys (IRB No. 1351707-4 and IRB No. 1351707-5). Collecting data from two samples allowed us to examine the stability of results across groups of respondents and time. The first survey was fielded from 3rd February to

1st March of 2021, and data were collected from 1,442 respondents. The second survey was fielded from 16th June to 25th July of 2022, and data were collected from 3,125 respondents. A quota-based sampling approach was used to obtain samples representative of the U.S. population across respondent characteristics of age, gender, income, and education. Images of the survey questions asked for the characteristics of respondents are shown in Supplementary Figure S1, and the summary statistics for the characteristics in samples 1 and 2 are presented in Supplementary Table S1.

2.1 Survey questions

Survey respondents answered questions about gene editing in the agricultural and medical fields. Questions for gene editing in the agricultural or medical fields were presented in individual blocks so that questions for the different fields were asked separately. Also, the order in which the question blocks were presented was randomized across respondents to minimize possible order effects associated with sequentially responding to questions about the two fields. Images of the survey questions asked about gene editing in the agriculture field are shown in Supplementary Figure S2 and questions asked about gene editing in the medical field are shown in Supplementary Figure S3.

Respondents were asked if they had heard or read about the use of gene editing in the context of "food and agriculture" or "health and medical" to determine awareness before asking if they had an opinion about the safety of gene editing; response options for awareness were a) No, b) Yes, I have heard or read a little, and c) Yes, I have heard or read a lot. Next, respondents were asked if they had an opinion about the safety of gene editing; response options were a) No, b) Yes, I have a weak opinion, and c) Yes, I have a strong opinion. Then, opinions about the safety of gene editing were collected using a five-point response scale (extremely unsafe, somewhat unsafe, neither safe nor unsafe, somewhat safe, extremely safe). For data analysis, these responses were collapsed into three categories (extremely/somewhat unsafe, neither safe nor unsafe, and somewhat/extremely safe).

The following two questions were then asked to examine the amount of evidence needed to change opinions about the safety of gene editing. One question asked what amount of research concluding that gene editing was safe was needed to improve opinions about safety; response options varied by the number of studies and were a) 1–25 studies, b) 26–50 studies, c) 51–75 studies, d) 76–100 studies, e) 100+ studies, and f) No amount of research will improve my opinion. There are often calls for "more research" by the public audiences; however, it is unclear how much is enough. The other question asked about the amount of time without a negative outcome was needed to improve opinions about safety; response options varied by the number of years and were a) 1–3 years, b) 4–6 years, c) 7–9 years, d) 10–20 years, e) 20+ years, and f) No amount of time without a negative outcome will improve opinion.

2.2 Statistical analysis

Within-sample heterogeneity in responses to questions about gene editing in the agricultural and medical fields was estimated using Chi-square tests of independence. These tests determined whether familiarity, opinion strength, opinion about safety, or the evidence needed to improve opinion varied across gene-edited in the agricultural and medical fields.

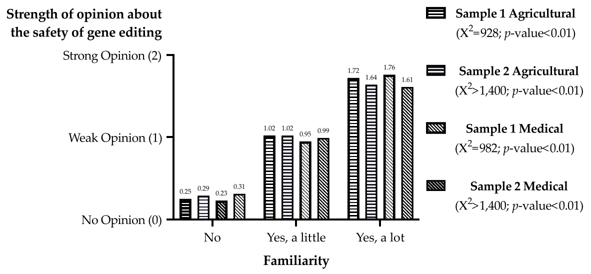

FIGURE 1
Strength of opinion about the safety of gene editing by familiarity with gene editing.

Chi-square tests of independence were also estimated within the agricultural and medical fields to determine relationships between familiarity and opinion strength, relationships between opinion strength and opinions about safety, and relationships between opinions about safety and the amount of evidence needed to improve opinions about safety.

To determine the effects of familiarity and opinion strength on opinions about safety, ordered logistic regression models were estimated for both samples' responses to the agricultural and medical fields. Ordered logistic regression models were selected because the dependent variable, opinion about safety, was categorical and took the value of 0 for extremely/somewhat unsafe, 1 for neither safe nor unsafe, and 2 for somewhat/extremely safe. Indicator variables were created for levels of familiarity and opinion strength, which were used as independent variables in estimation. For familiarity, indicator variables were created for respondents who selected "Yes, I have heard or read a little" or "Yes, I have heard or read a lot" (the "No" responses were used as the base). For opinion strength, indicator variables were created for respondents who selected "Yes, I have a weak opinion" or "Yes, I have a strong opinion" (the "No" responses were used as the base).

Ordered logistic regression models were also estimated to determine the effects of familiarity, opinion strength, and opinions about safety on the evidence needed to improve opinions about safety. The dependent variables for the amount of research and the amount of time necessary to improve opinions were categorical and increased with the amount of evidence required. The same independent variables used for familiarity and opinion strength in the ordered logistic regression models previously described were also used for these estimations, and independent variables were added for opinions about safety. For opinions about safety, indicator variables were created for respondents who selected "neither safe nor unsafe" or "somewhat/extremely safe" (the "extremely/somewhat unsafe" responses were used as the base).

3 Results

Frequency distributions for responses to the five questions used as variables in this study are presented in Supplementary Table S2.

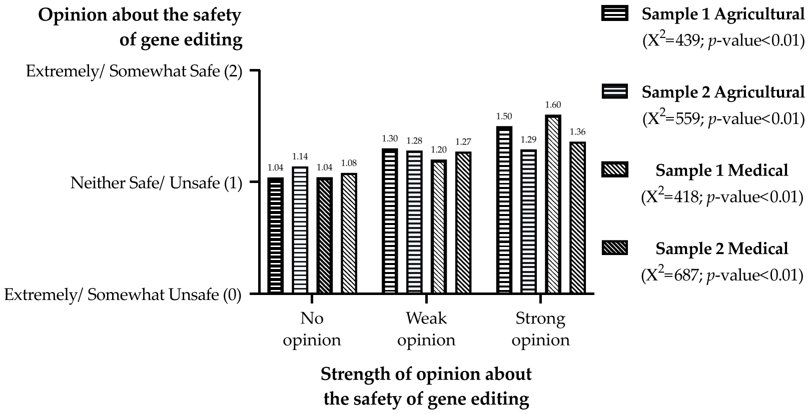

FIGURE 2
Opinion about the safety of gene editing by strength of opinion about safety.

TABLE 1 Ordered logistic regression coefficients for opinions about the safety of gene editing.

	Agricultural		Medical	
	Sample 1	Sample 2	Sample 1	Sample 2
Familiarity				
Yes, a little	0.915***	0.678***	1.043***	0.811***
	(0.142)	(0.089)	(0.142)	(0.086)
Yes, a lot	2.795***	1.505***	2.602***	1.783***
	(0.220)	(0.133)	(0.226)	(0.140)
Strength of Opinion				
Weak opinion	0.014	0.027	−0.269*	0.109
	(0.149)	(0.089)	(0.149)	(0.090)
Strong opinion	−0.033	−0.140	0.427**	0.127
	(0.180)	(0.106)	(0.189)	(0.110)
Log likelihood	−1,327	−3,200	−1,299	−3,098

Note: ***, **, and * denote *p*-value < 0.01, 0.05, and 0.10. Standard errors are reported in paratheses. There were 1,442 observations in the Sample 1 models and 3,125 observations in the Sample 2 models.

Also presented in Supplementary Table S2 are results from Wilcoxon matched-pairs signed-rank tests that were estimated to examine heterogeneity in responses between agricultural and medical gene-edited fields within a sample. Respondents in both samples reported they were more familiar with and more likely to have a stronger opinion about gene editing in the agricultural field. Nearly 66% of sample 1 and 75% of sample 2 had some familiarity with gene editing in agriculture, compared to about 61% and 63% in samples 1 and 2 for medical. About 62% of sample 1 and 66% of sample 2 had an opinion about the safety of gene editing in agriculture, while about 56% and 58% of samples 1 and 2 had formed opinions in the medical field. However, opinions about the safety of gene editing were similar across agricultural and medical fields, with around 40%–44% of respondents stating that gene editing in the two fields was somewhat or extremely safe.

The correlations between question responses are shown in Supplementary Table S3 to provide an understanding of linear relationships between the variables. Linear relationships were generally weak. However, there were moderate correlations between familiarity and strength of opinion, ranging from 0.56 to 0.70, and between the two variables exploring the amount of evidence necessary to improve opinions about safety, ranging from 0.58 to 0.66.

The linear relationships between familiarity and strength of opinion are illustrated in Figure 1. Given the categorical response options for the two questions, Chi-square tests of independence were estimated for each sample and field to determine if opinion strength was independent of familiarity. The null hypotheses of independence were rejected for all tests. Thus, the strength of a respondent’s opinion about safety increased with familiarity with

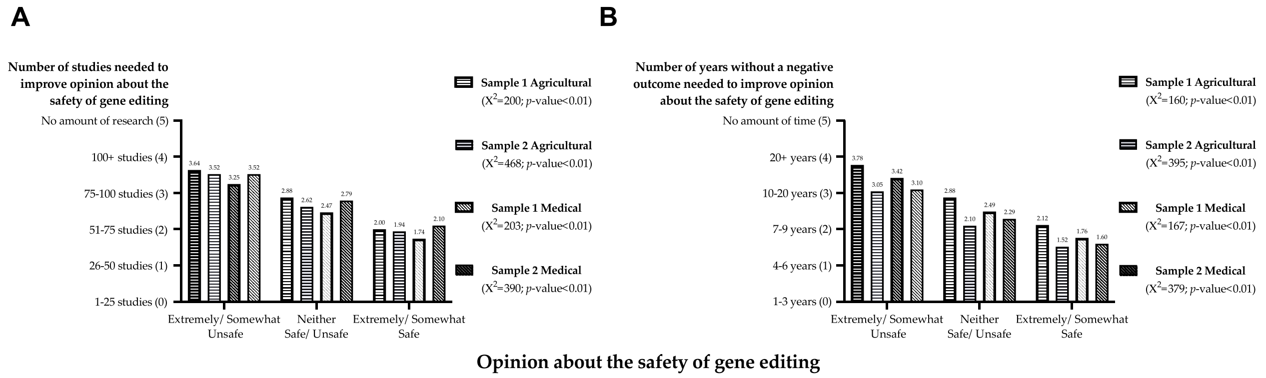

FIGURE 3
Amount of evidence necessary to improve opinions about the safety of gene editing by opinions about the safety of gene editing (Panel **(A)**: Number of studies; Panel **(B)**: Number of years).

TABLE 2 Ordered logistic regression coefficients for evidence needed to improve opinions about the safety of gene editing.

Familiarity	Agricultural				Medical			
	Amount of Research		Amount of Time		Amount of Research		Amount of Time	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Yes, a little	−0.710***	−0.340***	−0.510***	−0.277***	−0.611***	−0.376***	−0.376***	−0.322***
	(0.136)	(0.090)	(0.136)	(0.091)	(0.134)	(0.083)	(0.134)	(0.082)
Yes, a lot	−0.836***	−0.518***	−0.701***	−0.115	−0.831***	−0.524***	−0.486***	−0.277***
	(0.179)	(0.122)	(0.180)	(0.122)	(0.182)	(0.121)	(0.183)	(0.121)
Strength of Opinion								
Weak opinion	−0.416***	−0.141	−0.292**	−0.148*	−0.379***	−0.202**	−0.433***	0.043
	(0.141)	(0.086)	(0.142)	(0.087)	(0.139)	(0.085)	(0.139)	(0.084)
Strong opinion	−0.035	0.117	0.187	0.013	0.040	0.131	−0.030	0.129
	(0.163)	(0.101)	(0.162)	(0.102)	(0.165)	(0.102)	(0.165)	(0.102)
Opinion about Safety								
Neither Safe/Unsafe	−1.110***	−1.143***	−0.884***	−1.104***	−1.159***	−1.017***	−1.076***	−0.961***
	(0.149)	(0.097)	(0.146)	(0.095)	(0.146)	(0.098)	(0.142)	(0.095)
Extremely/Somewhat Safe	−1.605***	−1.740***	−1.374***	−1.680***	−1.652***	−1.605***	−1.608***	−1.670***
	(0.147)	(0.092)	(0.143)	(0.089)	(0.154)	(0.096)	(0.150)	(0.095)
Log likelihood	−2,434	−5,328	−2,440	−5,210	−2,428	−5,280	−2,442	−5,257

Note: ***, **, and * denote *p*-value < 0.01, 0.05, and 0.10. Standard errors are reported in paratheses. There were 1,442 observations in the Sample 1 models and 3,125 observations in the Sample 2 models.

gene editing in the agricultural and medical fields in both samples.

Figure 2 illustrates the relationships between opinions about the safety of gene editing and opinion strength. While the null hypotheses of independence were also rejected by Chi-square tests estimated for these two variables, the linear relationships are much less pronounced than that of opinion strength and familiarity. Respondents with no opinion about safety were, on average, also likely to state an opinion of neither safe/unsafe. In contrast, respondents who did have an opinion about safety generally stated that the use of gene editing was safe. However, there were slight differences in opinions about safety between those with strong and weak opinions in sample 2; this is also highlighted by the correlations between the two variables, 0.08 for agriculture and 0.16 for medical (see Supplementary Table S2).

Ordered logistic regression models were estimated to determine the relative impact of familiarity and opinion strength on opinions about the safety of gene editing. The estimated coefficients are shown in Table 1. Familiarity was strongly associated with opinions about the safety of gene editing in the agricultural or medical fields; all coefficients were positive, indicating that the likelihood of agreeing that gene editing was safe increased with familiarity. After controlling for familiarity, the strength of an opinion about safety had little association with the opinion about the safety of gene editing. The strong opinion coefficient in the medical model for sample 1 was the only significant Strength of Opinion coefficient at a *p*-value less than 0.05, and it was positive, indicating that respondents with a strong opinion about the safety of gene editing were generally more agreeable that the use of gene editing in the medical field was safe (relative to respondents without an opinion).

Both samples required more evidence to improve opinions about the safety of gene editing in the medical field relative to agriculture. On average, respondents with a negative opinion required more than 100 studies or 10 years to improve opinions about the safety of gene editing. The relationships between the amount of evidence needed to improve opinions about the safety of gene editing and opinions about safety are shown in Figure 3. The number of studies needed to improve opinion is shown in Figure 3A; the amount of time without a negative consequence is shown in Figure 3B. The amount of evidence needed to improve opinions was not independent of opinions about safety; respondents who stated that gene editing was unsafe required more evidence to improve opinions about safety.

Ordered logistic regression models were estimated to determine the relative impact of familiarity, opinion strength, and opinion about safety on the amount of evidence needed to improve opinions. The estimated coefficients are shown in Table 2. The coefficients estimated for the Familiarity and Opinion about Safety variables were significant and negative in all models. These results indicate that respondents who were familiar with gene editing or did not hold a negative opinion about safety required less evidence to improve opinions about the safety of gene editing. The coefficients estimated for the Weak opinion variable were significant at a *p*-value less than 0.05 in all models for sample 1 and one model for sample 2, indicating that those with an opinion, but who have not formed a strong opinion, may be more amenable to evidence.

4 Discussion

While the U.S. public is becoming more aware of gene editing in the medical field and may be more aware of the use of gene editing for medical purposes relative to transgenic approaches, results from this study indicate that the U.S. public may associate biotechnology more with agriculture. These findings are a valuable addition to existing literature that has examined perceptions of gene editing in agricultural or medical contexts separately. The prior qualitative study examining awareness about gene editing found uses in the medical field to be mentioned first in focus group discussions (McFadden et al., 2021a). The difference in results could be attributed to a few individuals in each group driving the conversation.

On average, respondents in both samples were more familiar with gene editing in agriculture, more likely to have an opinion about safety, have a more positive opinion, and require less evidence to improve opinions about safety than for medical purposes. The higher familiarity and opinion formation in the agricultural field may result from perception spillover from GMO food conversations. However, compared to the Pew Research study of GMOs, the slightly higher rates of perceived safety may indicate that negative perceptions toward safety do not spill over at a similar rate. This differs from the perception spillover observed in the context of energy innovation (Westlake et al., 2023), but could indicate that a larger percentage of our respondents perceive the safety of GMOs and gene editing in food as dissimilar. These nuances are essential to recognize as we continue engaging consumers in dialogue surrounding gene editing, ultimately impacting future innovation and policy. In particular, the findings suggest opportunities to share evidence of safety with those aware of the use of gene editing in agriculture. Doing so could generate more positive opinions, which individuals may share with others in their trusted social communities and create the opportunity for positive social influence.

Opinions about the safety of gene editing were generally more favorable for respondents who were aware or had formed an opinion about the safety of using gene editing within the agricultural or health field, supporting a proactive approach to messaging for gene editing and other technologies emerging into public consciousness. While familiarity and opinion strength were moderately correlated, familiarity was more closely associated with opinions about safety than opinion strength. The relationship between familiarity and opinions about safety may lead one to conclude that providing the public with more information about gene editing will improve opinions. However, the results of this study show that it may not be a valid conclusion for those with negative opinions about safety, and different approaches overall may be more meaningful for different public audiences. It also has been shown that scientific evidence is not compelling with some segments of the public and that strategies to build trust or rely on trusted messengers such as community leaders are more effective in changing perceptions (James, 2003). Together, these findings present the opportunity to explore strategies for designing tailored messaging and experiences around familiar gene-editing contexts within people's values and belief systems and the potential for engagement to then lean on these community members' positive attitudes to spread to others. Doing so could further enhance positive opinions toward and support for gene editing.

There are limitations to this study. One, no information was provided to respondents before the survey questions about gene editing in the agricultural and medical fields. Results are associated with the opinions that respondents attached to gene editing before participating in the survey, sometimes referred to as "homegrown" perceptions or values (Cummings et al., 1995; McFadden and Malone, 2021). The first question asked about familiarity, and thus, providing information could have primed some respondents. Future research could use a qualitative approach that would allow for follow-up discussions about the factors individuals affiliate with gene editing and what is considered in the scope of gene editing (e.g., transgenesis). Also, there are many nuances among gene editing applications, and familiarity with and opinions about genetic modification have been found to vary by the outcome of an application (Lusk et al., 2015) and even by the organization that developed the application (Lusk et al., 2018). Future research could focus on variations in familiarity with and opinions about gene editing, given specific application nuances. Lastly, there could have also been variations in how respondents interpreted research studies as evidence. For example, low-quality sources are more influential in forming opinions than more traditional sources (Aslett et al., 2023), and confirmation bias may result in individuals rejecting information from reputable sources if the information does not align with opinions before receiving the information (McFadden and Lusk, 2015).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/s/221190b7d727b759dac1.

Ethics statement

The studies involving humans were approved by University of Delaware Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BM: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing–original draft. JR: Conceptualization, Funding acquisition, Investigation, Writing–review and editing. KS: Conceptualization, Funding acquisition, Investigation, Writing–review and editing. KF: Conceptualization, Funding acquisition, Investigation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2024.1340398/full#supplementary-material

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Introduction and scientific justification of data transportability for confined field testing for the ERA of GM plants

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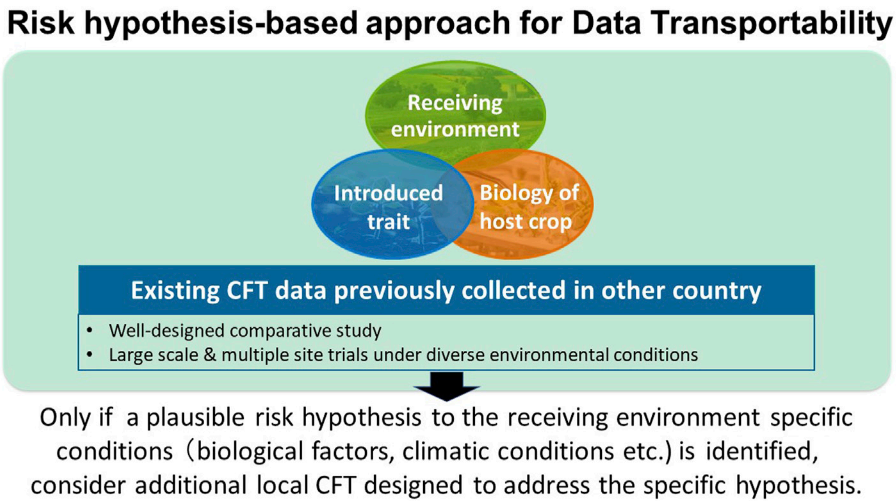
The concept of Data Transportability (DT) of Confined Field Testing (CFT) to support the Environmental Risk Assessment (ERA) of Genetically Modified (GM) plants was first introduced in the literature by Garcia-Alonso et al., in 2014. Since then, DT has been discussed in many countries and regions as a concept to prevent duplication of regulatory studies without compromising quality of the ERA. However, despite its usefulness and scientific justification, DT is not well adopted at this time and many regulatory agencies around the world require additional in-country CFT be conducted before approving GM plants. Based on the current circumstances, the authors organized a parallel session entitled "*Introduction and Scientific Justification of DT for CFT for the ERA of GM plants*" at 16th ISBR (the International Society for Biosafety Research). This session mainly consisted of the following three parts. The first two speakers, Andrew Roberts and Abigail Simmons provided an overview of DT and examples of conditions for the transportability of field data/conclusions advocated in the peer-reviewed scientific journals. Next, the current status of DT adoption in some countries/regions such as Japan and Africa, and a theoretical case study for Argentina were introduced by Kazuyuki Hiratsuka, Douglas Miano, and Facundo Vesprini, respectively. Lastly, a risk hypothesis-based approach for DT which was developed in advance by the five speakers of this parallel session, was introduced. During the discussion, there was a common understanding that transition to the risk hypothesis-based approach for DT was scientifically appropriate, considering the accumulated evidences that several countries have conducted confirmatory local CFT for more than 20 years but they have not detected any differences related to the ERA assessment endpoints in GM crops. The risk hypothesis-based approach for DT introduced here is expected to play an important role in discussions on the implementation of DT in various parts of the world in the future.

KEYWORDS

data transportability, genetically modified plant, environmental risk assessment, confined field testing, problem formulation



GRAPHICAL ABSTRACT

1 Introduction

The cultivation area of GM crops worldwide increased from 1.7 million hectares to over 202.2 million hectares from 1996 to 2021, and the number of cultivation countries also increased from 6 to 27 countries (AgbioInvestor, 2023). The majority of GM crops currently grown commercially are soybean, corn, cotton and canola, but other GM crops such as alfalfa, sugar beet, brinjal, rice, sugar cane, wheat, potato, and tomato have also been commercialized (AgbioInvestor, 2023). Since the beginning of commercial cultivation in 1996, GM crops have been rapidly accepted by farmers around the world, mainly due to improved farmer incomes resulting from increased yield and cost reductions, as well as reduced pesticide usage (Brookes, 2022).

Although GM crops are expected to benefit farmers around the world, there are various barriers to adoption. According to a 2022 study from Agbio Investor, which surveyed four leading biotech crop developers (BASF, Bayer, Corteva, and Syngenta), the average time it takes for a new GM crop to be commercialized has increased by about 26% from 13.1 years for a crop launched 2008–2012 to 16.5 years for a crop launched 2017–2022. Additionally, it was revealed that the regulatory phase accounts for 37.6% of the total cost and takes up 51.1% of the nonconsecutive time (AgbioInvestor, 2022). The extra time taken in the regulatory process delays the access of farmers and consumers to innovations in both cultivation and import countries. Another consequence of this situation is that developers in small companies, startups or from the public sector are discouraged from pursuing interesting projects involving GM crops, limiting innovations to large multinational companies and major commodity crops (Lewi and Vicién, 2020).

Global regulatory data requirements that are fit for purpose and hypothesis-driven, based on the application of adequate problem formulation process, would help overcome these barriers and allow valuable innovations to contribute to food security and reduce environmental impact without compromising biosafety. Laboratory and/or field studies on GM crops are conducted as part of the ERA to determine whether cultivation or incidental release of the GM crops could cause environmental harm. Sometimes, even in the absence of country-specific risk hypotheses, regulatory agencies require local laboratory and/or CFT in a country intending to cultivate GM crops. Some agencies also require local agronomic studies when the GM plant products (e.g., grain) is intended only for import, not for cultivation. For example, regulatory agencies in Japan (Ministry of Agriculture, Forestry and Fisheries, MAFF and Ministry of the Environment, MOE) have required local field studies for import approvals for some GM events, depending on the crop (s) and trait (s). The regulatory agency in China (Ministry of Agriculture and Rural Affairs, MARA) accepts global data as part of the import permit application, but local field studies as well as other laboratory-based studies are then commissioned by MARA to be conducted by a local institution in China. The requirements to repeat studies in different countries without identified risk hypotheses leads to duplication of data and add time and complexity to the regulatory process of GM plant without improving the ERA.

DT has been discussed in many countries and regions as a way to prevent the unwarranted duplication of regulatory studies without compromising the quality of the ERA. However, despite its usefulness and scientific justification, DT is not yet fully adopted, as many regulatory agencies around the world require additional in-country CFT be conducted before approving GM plants. There are primarily two factors why DT is not fully adopted. Firstly, in contrast to controlled environment like laboratories or greenhouses, where testing conditions can be consistently regulated, the growth of GM crops in field, like all crops in the field, may exhibit phenotypic variation in different environment, such as different soil types and weather conditions. Consequently, some regulatory authorities advocate the necessity of conducting CFT specific to the receiving environment. Secondly, there is difficulty of amending the guidelines that regulate GM crops once they have been established. The process of amending guidelines typically entails a complex procedure, which includes a public comment period. Consequently, even in the presence of accumulated scientific evidence, the immediate amendment of these guidelines is generally challenging.

As it was revealed that the regulatory phase accounts for 37.6% of the total cost and takes up 51.1% of the nonconsecutive time (AgbioInvestor, 2022), the extra time taken in the regulatory process including this additional in-country CFT delays the access of farmers to this innovation which could improve farmer incomes resulting from increased yield and cost reductions, as well as reduce pesticide usage. Based on the current circumstance, the authors organized a parallel session entitled "*Introduction and Scientific Justification of DT for CFT for the ERA of GM plants*" at 16th ISBR. In this paper, the authors will introduce the results of this parallel session divided into three items: 1. *Overview of DT and examples of conditions for the transportability of field data/conclusion*, 2. *Current status of DT adoption in some countries/regions such as Japan, Africa and Argentina* and, 3. *A risk hypothesis-based approach for DT*.

2 Overview of DT and examples of conditions for the transportability of field data/conclusion

Andrew Roberts, CEO of Agriculture & Food Systems Institute (AFSI) and a member of organizing committee of 16th ISBR, gave the first presentation entitled "Introduction of Key Concepts for Data Transportability." Dr. Roberts explained DT as the ability to use data collected in one geographic region or legal jurisdiction to inform a risk assessment in another region or jurisdiction. He also cited the avoidance of duplication of regulatory efforts as a key benefit of adopting DT. AFSI began studying the conditions for DT in 2011 and published a conceptual framework paper in 2014 (Garcia-Alonso et al., 2014). This paper provides guiding principles for the practical adoption of DT stating that the environmental and agronomic conditions under which the CFT was conducted in the remote country (ies) must be relevant to the conditions in the local country where the GM plant is intended to be cultivated. One way to demonstrate the relevance of environmental conditions is through the use of agroclimate, an aggregate measure of characteristics of the physical environment over time (Melnick et al., 2023). As a tool to visualize similar agroclimate zone, he introduced "Global Environmental Zones Explorer (GEnZ Explorer) (AFSI, 2023). The GEnZ Explorer can provide scientific justification for planning and location of field testing, as well as demonstrating the relevance of previous trials for consideration in another jurisdiction conducting a risk assessment. Dr. Roberts stressed that having a similar agroclimate is one way to demonstrate that an environment has relevance, but it is not a requirement for DT. Rather GEnZ Explorer is intended to provide simple visual information to support scientific justification by risk assessors who need to present a case to decision makers or skeptical public.

Abigail Simmons, Regulatory Manager of CropLife International, gave a second presentation entitled "Data Transportability for Studies Performed to Support an Environmental Risk Assessment for GM crops." Her presentation was made on the basis of the paper written by Bachman et al. (2021). Dr. Simmons explained the rationale for DT using an ERA framework that relies on problem formulation. Problem formulation refers to the process of developing a testable hypothesis of how a GM plant could affect defined protection goals/values. When an ERA is conducted using problem formulation, a set of data such as the nature of intended traits, the receiving environment and the biology of the unmodified crops is considered first. Additional data should only be requested, if there is a plausible testable hypothesis of a pathway to harm from the GM plant (Anderson et al., 2021). CFT is typically conducted in the country where the GM plant is developed, and specific locations are selected to be representative of the agricultural environments where these crops are grown. The data from these well-designed comparative assessments should be transportable even in the absence of agroclimatic similarity. It was argued that additional data, including local CFT should only be requested when a plausible pathway to harm of a protection goal has been identified as the result of conducting problem formulation.

3 Current status of DT adoption in some countries/regions such as Japan, Africa and Argentina

From Japan, Kazuyuki Hiratsuka, Yokohama National University and a Committee member of the ERA under the Japanese Ministry of Agriculture, Forestry and Fishers (MAFF), and Ministry of the Environment (MOE) made a presentation entitled "Data Transportability of GM corn and cotton for familiar traits in Japan" to introduce the current status of ERA and DT in Japan. In introducing the ERA process in Japan, Dr. Hiratsuka emphasized that the committee members who carry out the ERA are made up of experts from various fields with comprehensive expertise. This allows the ERA of GM crops to be conducted from multiple perspectives. Regarding the examples to adopt DT in Japan, Dr. Hiratsuka explained that Japan adopted DT of GM corn and cotton with familiar traits in 2014 and 2019, respectively. He also introduced that 7 GM corn events have already been accepted for DT until today. As the future prospect, Dr. Hiratsuka mentioned the possibility to expand the scope for accepting DT beyond GM corn and GM cotton with familiar traits with scientific rationale and accumulated evidence.

From Africa, Douglas Miano, University of Nairobi, made a presentation entitled "Evolving dialogue and policy considerations on biosafety data transportability for advancing agricultural biotechnology in Africa." In Africa, the number of countries with functional Biosafety frameworks increased from 6 to 11 between 2011 and 2022. However, even though there are unpublished reports of the use of DT in some African countries like Ghana and Nigeria, widespread use of DT has not been adopted yet, and currently CFT is required for each country planning to cultivate GM crops. In other regions, CFT is usually conducted for 1 year, but in Africa each country requires an average of about 3 years of CFT. Even though useful CFT data has already been available in some other countries, it is not possible to transport that data, resulting in additional time required for obtaining approval of commercialization of GM crops, making it difficult for African farmers to access this innovation. In the presentation, Dr. Miano referenced the African Union Development Agency-NEPAD (AUDA-NEPAD) which has a flagship biosafety program that was established to support Africa Union (AU) member states on matters of biosafety regulation and to facilitate technical cooperation among them. In recent times, AUDA-NEPAD, Michigan State University (MSU) and Bayer Crop Science established a Science Fellowship Program to design and implement research on existing and potential barriers to the transportability of biotech efficacy data towards timely and cost-effective decision making in Africa. This ongoing research (Miano et al., 2023) explores pragmatic, science-based and fit-for-purpose solutions to help overcome these barriers. By adopting DT, this group is aiming to eliminate redundancies in local (efficacy) testing requirements by leveraging existing data.

From Argentina, Facundo Vesprini, Bayer CropScience, Argentina, made a presentation entitled "Transportability of conclusions from Confined Field Trials." He presented a theoretical experience exploring the transportability of conclusion of the ERA from Brazil to Argentina, applied to a GM bean which was developed by Brazilian Agricultural Research Enterprise (EMBRAPA), that confers resistance to the Golden Mosaic Virus. This exercise has already been published in 2019 and tests the transportability of conclusions from EMPRAPA 5.1 field trials to bean growing regions in Argentina (Vesprini et al., 2020). To assess the transportability of conclusions of agro-phenotypic (for ERA) and compositional studies (for food and feed safety assessment) carried out in Brazil, three main criteria were established: "appropriate experimental design and methodologies," "relevance and consistency of measured endpoints across studies" and "diversity of environmental conditions selected for the CFT within the crop production zones." It is worth noting that similarity of agroclimatic conditions is not included in the criteria. However, it was mentioned that if plausible risk hypotheses were to be identified in a particular environment, local CFT in Argentina may be required, or similarity of climatic conditions become relevant to address the concerns at the particular environment with the available studies. Given the criteria on DT described above, the conclusions of the agro-phenotypic and compositional studies conducted in Brazil were considered transportable to Argentina.

4 A risk hypothesis-based approach for DT

To conclude the session, a risk hypothesis-based approach for DT developed in advance by the authors, was introduced (Graphic abstract). For this approach, it is recommended to conduct an ERA with existing information related to the receiving environment, the introduced trait and the biology of host crop, in addition to existing CFT data, when available, before asking for local CFT in other countries by default. In this case, existing CFT data should meet the following two conditions: a) testing should be well-designed comparative study; b) testing should be of an adequate scale, and multiple sites under diverse environmental conditions should be selected. If the previously collected CFT data meets these conditions, regulatory authorities should consider requesting local specific CFT only when a plausible risk hypothesis specific to the receiving environment (such as biological factors, climatic conditions or others) is identified.

This risk hypothesis-based approach for DT introduced during discussion of this parallel session is not entirely new but was developed by integrating the DT criteria and conditions already proposed by several authors during the session. Specifically, this approach is structured around a concept of the ERA based on problem formulation as proposed by Anderson et al, (2021), DT of the data collected in well-designed CFT that test a clear risk hypothesis proposed by Bachman et al. (Bachman et al., 2021) and with transportability of conclusions from confined field trials proposed by Vesprini et al. (2020). These first two papers played a central role in structuring Dr. Simmons's session and are also part of a "Special Issues on Genetically Modified Organisms" (Molins, 2021). This special issue contains seven reports developed by CropLife International (CLI) to contribute to the development of a scientific and internationally harmonized regulatory system based on 25 years of experience since the start of commercial GM crop cultivation. Two of the seven reports summarize recommendations regarding the ERA. Anderson et al. (2021) explains that the ERA should be based on problem formulation and only require relevant data. Bachman et al. (2021) describes the conditions for DT within the framework of problem formulation. These papers conclude that if no biologically relevant differences between a GM plant and its conventional counterparts are observed in one country or region, data from well-designed studies can be transportable for ERA to another country regardless of agroclimate zone.

The paper (Vesprini et al., 2020) introduced by Eng. Vesprini also recommends local CFT, only if there is a risk hypothesis identified for the GM crops in a particular environment, and pointed out that "appropriate experimental design and methodologies," "relevance and consistency of measured endpoints across studies" and "diversity of environmental conditions in CFT locations within the crop production zones" are the three key criteria proposed for transportability of conclusion which are basically the same idea as the risk hypothesis-based approach for DT.

Dr. Roberts introduced a paper written by Garcia-Alonso et al. (2014) which propose four conditions for practical DT. One of these criteria is that evaluators must have scientific justification for the use of CFT data from other places, and one way provide this justification is to demonstrate similarity in agroclimate (Melnick et al., 2023). As previously mentioned, he emphasized that the concept of similarity of agroclimatic zone may be helpful for rational selection of CFT sites, but it is only one way to demonstrate that the environment where data was collected is relevant for consideration during risk assessment, not a requirement for DT.

As introduced by Dr. Hiratsuka, Dr. Miano and Eng. Vesprini in this session, there are different degree of adoption of DT, depending on factors like the experience with ERA process for GM crops, public acceptance of GM crops in each country and region, and others. Even in countries that have accumulated experience with the ERA of GM crops, one of the reasons for low adoption of DT is the idea that GM crops will grow differently in different growing environments. In such case, it is important to recognize the purpose of CFT for the ERA, that is, not to characterize GM crops in as much detail as possible in each of different environmental conditions. Rather, the purpose of CFT for GM crops is to identify whether any unintended and adverse changes occurred related to the ERA assessment endpoints by comparing the GM crop to the conventional crop under highly controlled testing conditions (Anderson et al., 2021).

Many papers comparing the results of CFT conducted in different countries and regions have been published and confirmed that no unintended or adverse changes occurred related to the ERA assessment endpoints in GM crops which do not have unique risk hypothesis in the receiving environment. Clawson et al. (2019) shared the results of agronomic characterization of GM corns performed in five regions (Argentina, Brazil, Mexico, Pakistan, and the United States) from 2004 to 2014. It was demonstrated that risk assessment outcomes from agronomic characterization of the 3 GM corn events (MON 89034, NK603, and MON 89034 × NK603) were consistent across multiple global regions. Additionally, Nakai et al. (2015) compared selected plant characteristics data which related to weediness potential from the CFT performed in Japan and multi-locations in the US for already approved 3 GM corn events (LY038, MON 89034, and MON 87460), expressing diverse traits such as nutritional improvement, lepidopteran insect-protection and drought tolerance, respectively. The study showed the differences related to weediness potentials that were not detected in the US CFT were similarly not detected in the Japan CFT for all of the 3 GM corn events. Finally, Matsushita et al. (2020) compared agronomic data from the CFT performed in Japan and multi-locations in the US for already approved 11 GM soybean events and demonstrated the similarity of results obtained in Japan and the US.

As described above, existing conditions for DT that have already been advocated to date have fallen into three categories. The first is Agroclimate similarity-based approach, as introduced by Dr. Robert. The second is Familiarity-based approach, as introduced by Dr. Hiratsuka from Japan and the third is Risk hypothesis-based approach, as introduced by Dr. Simmons and Eng. Vesprini. While each condition maintains scientific validity, a great deal of scientific knowledge has accumulated over more than 20 years, including the result of CFT conducted in various countries and regions subsequent to some of conditions of DT were proposed. In this context, the authors, who had previously advocated different DT conditions, convened for this DT parallel session. After considering the latest scientific insights, they collectively endorsed the Risk hypothesis-based approach as one of the most scientifically valid DT conditions currently available.

Based on the accumulated evidence, the discussion of this parallel session was concluded with an agreement that the proposed risk hypothesis-based approach for DT is scientifically reasonable. The authors believe that the results of local confirmatory CFT, accumulated over the past 20 years and the science-based criteria established for transportability, will contribute the discussions in countries and regions contemplating the adoption of DT in the future, even for GM crops with new traits.

Author contributions

SN: Writing–original draft, Writing–review and editing. AR: Writing–review and editing. AS: Writing–review and editing. KH: Writing–review and editing. DM: Writing–review and editing. FV: Writing–review and editing.

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Conflict of interest

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Graduate certificate on risk analysis for the Agrifood sector at the University of Buenos Aires

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Argentina has an extensive experience in the biosafety assessment of transgenic crops. The regulatory framework celebrated 30 years of existence in 2021 and has pioneered the establishment of the biosafety systems in Latin America. During this period, Argentina's regulatory framework evolved to keep up with the advancements in plant and animal biotechnology and in risk assessment criteria, as new knowledge and experience was being gained. However, despite the country's agricultural tradition and experience in the adoption of innovations by the productive sector, dedicated, formal academic offerings training is lacking and this is also true for most countries in the world. Responding to this perceived need and going beyond biotechnology to include other regulated inputs used along the food production chain (chemicals, biologics, food additives, etc.), we developed a program to introduce graduates from diverse disciplines to the principles and practice of Risk Analysis (Assessment, Management and Communication) with focus on the Agrifood sector. In 2020, the School for Graduate Students of the School of Agriculture—University of Buenos Aires, approved two Certificates on Risk Analysis for the Agrifood Sector: *Conceptual Bases of Risk Analysis* and *Methodological Tools.* The first edition of the certificates was completed in December 2022 and the second one is presently ongoing. The fundaments, rationale, structure and objectives of these certificates are presented.

KEYWORDS

risk analysis, graduate course, continuing education, Agrifood, biosafety, regulatory science, University of Buenos Aires

1 Introduction

The production, distribution and consumption of food involve different actors and is a strongly regulated sector. Crop protection products-including biologicals-, transgenic seed, food additives and adjuvants for the food industry, are the main inputs subject to regulatory oversight and therefore need to go through authorization processes before entering the market.

Biosafety risk assessment is focused on the potential impacts that these products or technologies might have on health or the environmental, or both. The risk assessment process is the technical analytical stage of risk analysis that regulatory agencies carry out. In general, this process is based on scientific criteria, country policies and analytical methodologies that are internationally accepted (OECD, 2005), but fundamentally, is a way of thinking and approaching problem resolution.

In many countries, there is a need for professionals trained and experienced in these methodologies in the fields of food safety, crop protection products (including biologicals) or transgenic organisms. Academic programs like the one described here provide a formal context to develop capacities in both public and private organizations. Career opportunities for professionals with these capacities are diverse, as regulatory affairs specialists for private sector developers in the agricultural, chemical or biotechnology fields, as risk assessors in the public sector, and also in academia.

In a risk averse society, where concerns about food and environmental safety are part of our everyday lives, the role of regulatory agencies is central and professional risk assessors that are skilled and can clearly communicate, are key to develop fit for purpose, solid risk-based regulations that build credibility and trust (OECD, 2021).

The availability of an educational space dedicated to fulfill this need, in Spanish, was considered a valuable contribution to our region, that would complement the formation of professionals in the different disciplines related to risk analysis for the agrifood sector.

The School of Agriculture of the University of Buenos Aires hosts the School for Graduate Students "Agronomy Eng. Alberto Soriano." Along its 36 years of existence, the school formed over 1,000 professionals and currently offers over 100 Graduate Programs (Masters, PhDs, Specializations, Continuing Education courses and others) in different disciplines related to the Ag Sciences field, from Genetics and Crop Physiology, to Economy, Agribusiness and Natural Resources. Back in 2020, the School added two new correlative Certificates on Risk Analysis for the Agrifood Sector to their academic offerings (EPG -FAUBA).

These Certificates intend to introduce professionals to the general principles of Risk Analysis, familiarizing them with the analytical thinking process required to identify risks and address their management, under the Regulatory Science framework. These competences are key to identify, estimate or quantify risks and define acceptable levels, as well as deal with the management or mitigation measures that might be necessary.

But beyond strictly technical skills, another equally important set of competences involves other abilities like risk communication, consensus building and diplomacy, as risk assessment and regulatory decision making involve not only conducting a technical analysis but also informing risk managers, policy makers and, increasingly, responding to societal demands.

As mentioned, regulatory science is at the foundation of this course, as a scientific discipline on its own, which proposes plausible risk hypotheses that result from the Problem Formulation exercise (Wolt et al., 2010), and tests them applying appropriate methodologies and also generating innovative methods and standards.

Risk assessment it's a multidisciplinary endeavor that requires experts from different fields (chemists, biologists, geneticists and toxicologists, medical doctors, agronomists, microbiologists and animal scientists) and is a scientific procedure based on regulatory science (Deluyker, 2017).

2 Why two parts? topic areas and curriculum

The first, introductory part "Conceptual Bases," gives an overview of risk assessment principles and approaches (like problem formulation and the identification of risk hypotheses) and the scientific rationale used in their development.

The first module provides a detailed overview of the surrounding context (productive, economic, societal perception, regulatory). The second module, focuses on the different disciplines that are key in the generation of experimental evidence and its interpretation for risk characterization: Toxicology, Food Technology, Biotechnology, Regulatory Toxicology, Epidemiology, Food Composition and Nutrition and Statistics are part of the curriculum. Although this first part is more theoretical and informative, examples and cases are discussed and practical situations are presented to the students for their debate and resolution.

The second part, "Methodological Tools," focuses on the practice of Risk Analysis and the important factors to consider when going through the process, from the risk characterization stage and the decision-making process, to the normative aspects. Given Argentina's experience on the subject, regulation of gene editing has been included as a topic, as well as the regulatory approach for agricultural biologicals.

As mentioned above, this part includes contents that have not traditionally or formally been part of risk assessment trainings but that, in our view and experience, are very important aspects to consider. An example of this is the Communication module, discussing the importance of adequately communicating decisions and management considerations, if any, to different stakeholders: risk managers, end users and society at large.

Both parts have considerable time dedicated to lectures, invited speakers, discussion with experts and group work, as one of the objectives of these courses is to foster interactions and consensus building, as it happens in real life. A general scheme of the Certificates is summarized in Figure 1.

3 Faculty, students' profile and first experience

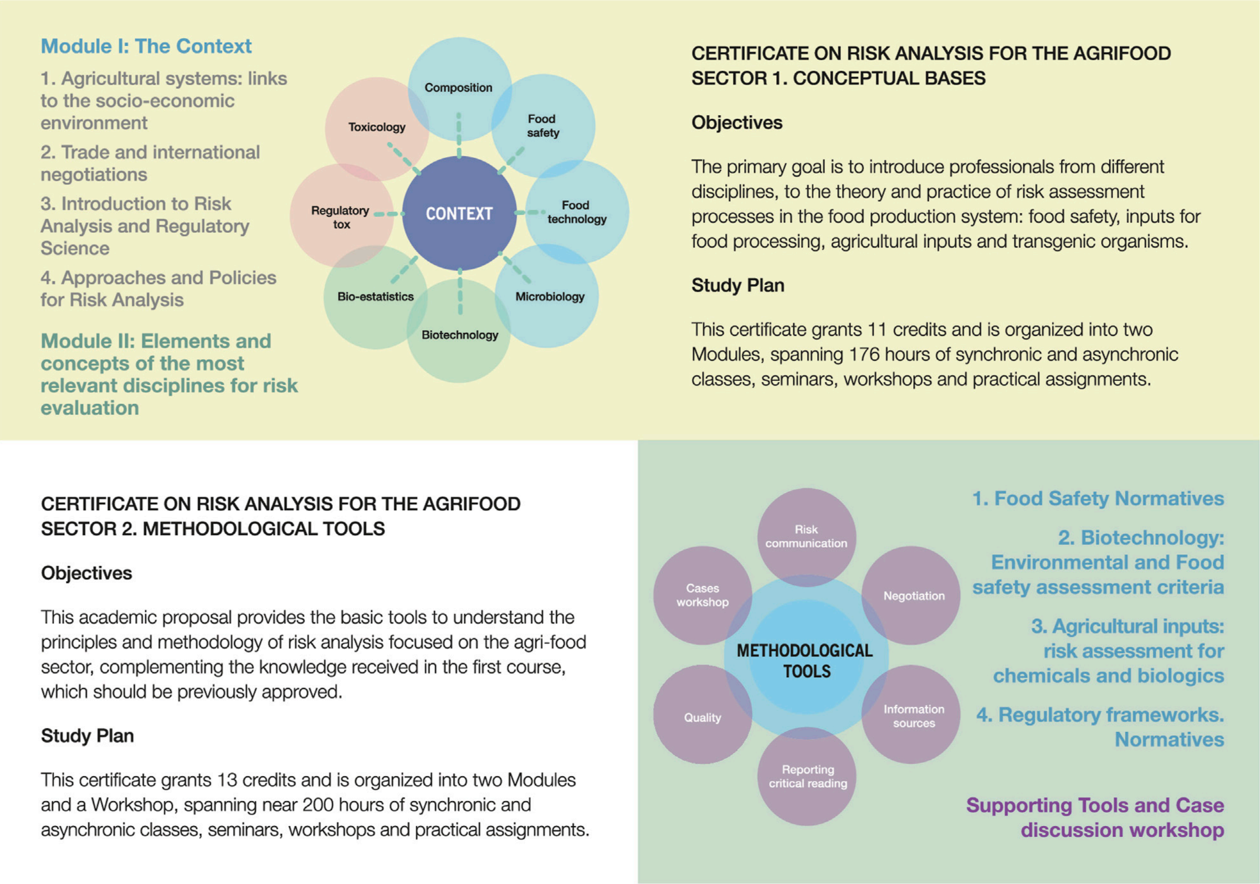
These certificates include over 20 lecturers, all of them specialists in their fields, plus invited experts that share experiences, participate in discussions with the students, or speak about specific topics of interest. Agronomists, molecular biologists, breeders, biochemists, toxicologists and food technologists teach "Conceptual Bases." Additionally, experts in topics like Communications, Quality systems, Research Integrity, Ecotoxicology and Regulations, teach "Methodological Tools."

Our Faculty comes from different sectors, government regulatory agencies, private companies or academia, bringing a high level of expertise and diversity to the courses, as our intention is to give students the opportunity to learn about the concepts and the practice of risk analysis first hand from the experts, and understand how the processes work under real life conditions, as far as possible.

Classes are 3 h long, once a week, with some additional days along the year dedicated to group examinations and complementary lectures or case discussions.

The certificates are open to university graduates from diverse disciplines (Agronomy, Biology, Chemistry, Biochemistry, Genetics, Medicine, Animal Science, Biotechnology, Microbiology, Toxicology, etc.), and the online modality allows to have students from different Spanish speaking countries.

The first cohort was composed of 15 experienced professionals in the fields of Agronomy, Toxicology, Biology, Biotechnology and



Module I: The Context

1. Agricultural systems: links to the socio-economic environment
2. Trade and international negotiations
3. Introduction to Risk Analysis and Regulatory Science
4. Approaches and Policies for Risk Analysis

Module II: Elements and concepts of the most relevant disciplines for risk evaluation

CERTIFICATE ON RISK ANALYSIS FOR THE AGRIFOOD SECTOR 1. CONCEPTUAL BASES

Objectives

The primary goal is to introduce professionals from different disciplines, to the theory and practice of risk assessment processes in the food production system: food safety, inputs for food processing, agricultural inputs and transgenic organisms.

Study Plan

This certificate grants 11 credits and is organized into two Modules, spanning 176 hours of synchronic and asynchronic classes, seminars, workshops and practical assignments.

CERTIFICATE ON RISK ANALYSIS FOR THE AGRIFOOD SECTOR 2. METHODOLOGICAL TOOLS

Objectives

This academic proposal provides the basic tools to understand the principles and methodology of risk analysis focused on the agri-food sector, complementing the knowledge received in the first course, which should be previously approved.

Study Plan

This certificate grants 13 credits and is organized into two Modules and a Workshop, spanning near 200 hours of synchronic and asynchronic classes, seminars, workshops and practical assignments.

1. Food Safety Normatives

2. Biotechnology: Environmental and Food safety assessment criteria

3. Agricultural inputs: risk assessment for chemicals and biologics

4. Regulatory frameworks. Normatives

Supporting Tools and Case discussion workshop

FIGURE 1
General Structure of the Graduate Certificates. Part I and II are correlative and grant credits that can be accepted by other Graduate Programs of the School.

Biochemistry, including two regulators. This group came from both the private and public sectors, from different states in Argentina, and also from Paraguay. Eleven students completed both certificates.

After each module, surveys were conducted among students to gather opinions and use the feedback to introduce improvements. Results have been very positive so far, highlighting the quality of the contents and lecturers and the usefulness and applicability of the courses to their professional practices.

The second cohort completed the first certificate in 2023 and will continue to take the second part in 2024. After our first experience, this second edition will introduce some modifications, redefine specific contents and include international invited speakers, which will give students additional perspectives.

Group discussions are a central part of both courses, as these promote inter-disciplinary exchange and consensus building, the two main pillars of risk assessment processes under real life conditions around the world. Diverse backgrounds are welcome and have not prevented performance or participation so far.

4 Discussion

Functional risk assessment bodies in the biosafety field require continued education and training efforts to form professional profiles. The lack of formal intra-agency processes that ensure continuity in this formative process, is a challenge that is not exclusive of a particular country or region, but is an extended problem.

Capacity building initiatives that have been and are being implemented are numerous and highly valuable experiences, in particular for developing countries, however, the lack of continuity, high staff rotation or insufficient funding, undermines these efforts.

As stated in the recent “Anticipatory regulation in an age of disruption” Nesta report: “ . . . regulation and regulatory practice need to be recognised as crucial elements of the industrial strategies that are being developed and implemented in the United Kingdom and elsewhere. Indeed, the quality of regulatory practice in relation to innovation will be an increasingly important source of competitive advantage in the global economy” (Armstrong et al., 2019).

In fact, science based regulatory frameworks are not static, on the contrary, this is a dynamic field that needs to evolve and adapt as new knowledge, innovation and societal demands increase (Vicién and Trigo, 2017). These demands (like the reduction in animal testing), create new requirements to adapt criteria and methodologies, develop new methods and predictive tests. In turn, this means continuing education and updates to regulators and risk assessors is and will continue to be a permanent need.

Academic offers that provide access to high quality, updated formation and to experts in the different fields, will contribute to develop capacities for risk assessment. In our case, certificates are open to graduates interested in this field, that will incorporate problem solving tools and criteria applicable to the private or public sectors.

Our first experience with the graduate certificates in Risk Analysis for the Agrifood sector is the first of its kind in the region and is held in Spanish. Both certificates provide tools and criteria to introduce students to the principles and practice of risk analysis, from problem formulation to risk characterization and management, including risk perception and communication considerations.

Professional, transparent and efficient regulatory systems, applying state of the art criteria and methodologies, have the highest credibility and build societal trust. This is true at the global level, but specially so in developing countries, some of which still find barriers for innovations to reach farmers and consumers. This is particularly true for local innovations by public institutions or small innovative companies, and one reason is the lack of highly trained assessors. Regulatory systems that are up-to-date, fit for purpose and adaptable to current and future innovations will be increasingly needed in order to bring safe, beneficial products and technologies to consumers.

Going forward, we plan to turn both certificates into a Master's Degree, compiling both parts into one and adding a final work (Master's thesis). Students from previous editions, will be able to use their credits to obtain this degree.

We are in contact with other institutions beyond Argentina, with similar approaches to higher education in the Risk Analysis field, and look forward to establishing agreements to collaborate and exchange students and faculty in the near future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genetically engineered eucalyptus expressing pesticidal proteins from *Bacillus thuringiensis* for insect resistance: a risk assessment evaluation perspective

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Eucalyptus covers approximately 7.5 million hectares in Brazil and serves as the primary woody species cultivated for commercial purposes. However, native insects and invasive pests pose a significant threat to eucalyptus trees, resulting in substantial economic losses and reduced forest productivity. One of the primary lepidopteran pests affecting eucalyptus is *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae), commonly referred to as the brown looper caterpillar. To address this issue, FuturaGene, the biotech division of Suzano S.A., has developed an insect-resistant (IR) eucalyptus variety, which expresses Cry pesticidal proteins (Cry1Ab, Cry1Bb, and Cry2Aa), derived from *Bacillus thuringiensis* (Bt). Following extensive safety assessments, including field trials across various biomes in Brazil, the Brazilian National Technical Commission of Biosafety (CTNBio) recently approved the commercialization of IR eucalyptus. The biosafety assessments involved the analysis of molecular genomics, digestibility, thermostability, non-target organism exposure, degradability in the field, and effects on soil microbial communities and arthropod communities. In addition, *in silico* studies were conducted to evaluate allergenicity and toxicity. Results from both laboratory and field studies indicated that Bt eucalyptus is as safe as the conventional eucalyptus clone for humans, animals, and the environment, ensuring the secure use of this insect-resistant trait in wood production.

KEYWORDS

eucalyptus, genetically modified, *Bacillus thuringiensis*, Cry pesticidal proteins, insect resistance, *Thyrinteina arnobia*, biosafety

Introduction

Genetically modified (GM) crops expressing *Bacillus thuringiensis* (Bt) proteins have led to major advancements in crop protection and productivity (Brookes and Barfoot, 2020; ISAAA, 2020). Since their commercialization in 1995, Bt crops have reduced chemical insecticide usage by over 50% while maintaining high yields and reducing greenhouse gas emissions (Brookes and Barfoot, 2018). Integrating Bt genes into crops represents a deviation from the reliance on chemical insecticides, which often cause environmental and health concerns and allow initial pest damage since application occurred after monitoring (Koul, 2020). Bt is a naturally occurring Gram-positive bacterium that resides in the soil. One of the remarkable characteristics of Bt is its ability to produce insecticidal proteins, known as delta endotoxins (δ-endotoxins) or Cry pesticidal proteins. These proteins are highly effective against a wide range of targeted insect pests, including lepidopteran pests. Over 1,100 of them have been identified and classified based on their structure, sequence homology, and activity (Crickmore et al., 2023). They often target specific insect species within the same taxonomic family or order, possessing a relatively narrow activity spectrum (Schnepf et al., 1998; Crickmore et al., 2021). For decades, Bt spores have been widely used as a biological pesticide safe for humans, animals, non-target invertebrates, and the environment. In parallel, more than 300 genetically engineered events in various crops (maize, cotton, soybean, rice, eggplant, sugarcane, tomato, cowpea, and poplar) expressing Bt proteins have received regulatory approvals (ISAAA, 2023).

According to the Food and Agriculture Organization of the United Nations, eucalyptus farms cover approximately 0.5% (~22.57 million hectares) of the world's forested areas (FAO, 2020) but fulfill approximately 10% of the current global demand for roundwood. Its significant contribution to wood supply renders eucalyptus a crucial species for present and future wood production. This helps in protecting native forests by reducing the utilization of wood from these ecologically important areas. Brazilian eucalyptus farms are managed using modern agricultural practices akin to other row crops, and like any other crop, they are challenged by pests, including exotic pests introduced by eucalyptus originating from Australia and native Brazilian pests that have rapidly adapted to a eucalyptus diet (Paine et al., 2011; Mota et al., 2022).

Brazilian eucalyptus farms typically experience sporadic instances of lepidopteran pest infestations. However, there is a significant likelihood of a major outbreak to occur during the 6–7 years of the rotation cycle. Even a single infestation of defoliators can inflict substantial damage, estimated to cause a loss of 13%–40% in the annual yield (Barbosa et al., 2022), which translates to a reduction of 9%–19% in the wood volume during harvest. Additionally, when the tree's defense mechanism is activated, it leads to undesirable changes in the wood's properties and an increase in the lignin content. Consequently, these cumulative effects result in a decline in pulp production by 15%–24% (Zanuncio et al., 2020).

In Brazil, the brown looper moth *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae) is a major lepidopteran pest of eucalyptus trees. Originally infesting native Myrtaceae hosts, like guava (*Psidium guajava*) (Holtz et al., 2003; Barbosa et al., 2022), *T. arnobia* has expanded its range to include eucalyptus. Since 2015, over 413,000 hectares of eucalyptus fields in Suzano S.A. have been reported to being infested by *T. arnobia*. The two main control methods involve releasing pupal parasitoid wasps, such as *Tetrastichus howardi* (Olliff, 1893) and *Trichospilus diatraeae* (Cherian and Margabandhu, 1942) (Hymenoptera: Eulophidae) (Barbosa, et al., 2022), and applying Bt biopesticides, such as DiPel®. Bt biopesticide applications are carried out after the manual monitoring and detection of caterpillars and their damage in the field (McDowell and Mann, 1991). However, this treatment does not fully prevent damage, and each biopesticide application increases the environmental footprint. Therefore, additional control strategies are needed to protect eucalyptus farms better.

As part of a sustainable, eco-friendly initiative, FuturaGene, the Biotech Division of Suzano S.A., has developed the insect-resistant genetically modified eucalyptus event 1521K059, expressing three Bt Cry pesticidal proteins, Cry1Ab, Cry2Aa (also in DiPel®), and Cry1Bb, targeting *T. arnobia*, as well as the selectable marker kanamycin resistance gene *nptII* (Beck, et al., 1982; Toth, et al., 2007). The three pesticidal protein genes and the selectable marker were cloned adjacent to each other on a single-transfer DNA (T-DNA), resulting in a single genomic insertion site after the transformation using *Agrobacterium tumefaciens* (Prakash and Gurumurthi, 2009).

Under the Brazilian legislation, Normative Resolution 32 (CTNBIO, 2021) governs the standards for the commercial release and monitoring of genetically modified organisms. This resolution does not specify the required or excluded studies, and it does not differentiate between the processes associated with the events intended for human or animal consumption. The responsibility lies with the petitioner to generate scientific data, demonstrating to the National Technical Commission on Biosafety (CTNBio) that the specific genetically modified organism (GMO) event in question poses no risks to the environment, human health, or animal health. Typically, studies are conducted based on the precautionary principle, often following the precedents of the previously approved submissions in Brazil and globally. Brazil's regulatory system is considered one of the most robust worldwide, having conducted risk assessment evaluations for over 25 years without detecting any adverse effects from commercially approved GMOs.

After thorough biosafety assessments in the laboratory and field for over 2 years, the event 1521K059 was approved by the CTNBio for commercial use in Brazil. This manuscript presents the key findings from the extensive biosafety evaluations of the eucalyptus event 1521K059 in the field and laboratory safety tests of transgenically expressed Cry1Ab, Cry1Bb, and Cry2Aa pesticidal proteins. The safety assessments of the NPTII protein were published before (Fuchs et al., 1993; Avisar et al., 2023). Although Cry1Ab has a well-established history of biosafety studies in other commercial GM crops (Federici and Siegel, 2008; Wolt et al., 2008; ILSI, 2011), we present the accumulated data for this pesticidal protein alongside Cry2Aa and Cry1Bb. Cry2Aa and Cry1Bb pesticidal proteins have limited biosafety data in the literature and worldwide regulatory applications since they have been rarely used commercially thus far (AgbioInvestor, 2023). Furthermore, this work emphasizes the potential of insect-resistant eucalyptus as a new tool in pest management and in the promotion of sustainable practices and environmentally friendly solutions in the tree crop sector.

Materials and methods

Genomic insertion site identification

Genomic DNA was extracted from 1521K059 IR GM eucalyptus using the cetyltrimethylammonium bromide (CTAB) protocol (Richards et al., 1994). Fresh leaf tissue (2 g) was frozen in liquid nitrogen and finely powdered. Subsequently, 15 mL of the extraction buffer (2% CTAB, 100 mM Tris at pH 8, 1.5 M NaCl, 0.2 mM EDTA at pH 8, 1% β-mercaptoethanol, and 0.1% PVP) was added. The mixture was then incubated at 65°C for 60 min, periodically swirled, and then cooled to room temperature. Next, the sample was thoroughly mixed with 15 mL chloroform–isoamylic alcohol and then centrifuged at 10,000 *g* for 15 min at 22°C. Then, the supernatant was carefully transferred to a new tube, and the chloroform isoamylic alcohol step was repeated twice. Then, an equal volume of ice-cold isopropyl alcohol was added, and the tube was incubated at −20°C for 30 min. The samples were then centrifuged at 20,000 *g* for 20 min at 4°C. The resulting supernatant was discarded, and the pellet was resuspended in 500 µL of 70% ice-cold ethanol and then centrifuged at 20,000 *g* for 2 min at 4°C. After the aspiration of 70% ethanol, the tubes were left open at room temperature to allow complete ethanol evaporation. The pellet was then resuspended in 250 µL RNase (10 ng/µL; Sigma R6513) in the Tris–EDTA buffer and stored at 37°C until complete dissolution of the pellet (final DNA concentration of 20 ng/µL).

The extracted DNA (0.5 µg) was sequenced on the Illumina HiSeq 2500 platform, utilizing a single individual lane, which generated raw read data (150PE, 80 gigabytes). Read mapping was performed using Geneious Prime software version 11 (http://www.geneious.com). Reads that successfully aligned with both T-DNA and genome DNA sequences were used to determine the specific location of the insert within the genome. The insertion within the genome was located using a published eucalyptus genome reference, BRASUZ 2.0 (Myburg et al., 2014).

Bioassays with *Thyrinteina arnobia*

A laboratory population of *T. arnobia* was maintained in a temperature-controlled room (25°C ± 2°C), with a relative humidity of 70% ± 10% and a 12-h light phase, as described by Oliveira et al. (2005). For the bioassays, the natural leaf diet was replaced with an artificial diet. To prepare one batch of the diet, the following steps were followed: boiling water (544 mL) was combined with 12.5 g of wheat germ (Jasmine, Campina Grande do Sul, PR, Brazil), 9 g of the yeast extract (local market, purchased by kilogram without brand definition), 67.3 g of white corn flour (produced in the laboratory, white corn from Embrapa, Brazilian Enterprise of Agriculture and Livestock Farming Research), 25.5 g of the soybean meal (Ecobio, Coronel Bicaco, RS, Brazil), 5.3 mL of soy oil (Cargill, Uberlândia, MG, Brazil), and 5.3 g of skimmed milk (La Serenissima, Barueri, SP, Brazil). The mixture was stirred for 10 min. Water (300 mL) and agar (12.5 g) (PhytoTech Labs, Lenexa, KS, USA A296) were boiled and then mixed with the above mixture. The mixture was allowed to cool to 45°C, and the Vanderzant vitamin mixture for insects (0.5 mL) (Sigma-Aldrich, Darmstadt, HE, Germany V1007), Nipagin (1.35 g) (Sigma-Aldrich, Darmstadt, HE, Germany H5501), sorbic acid (0.68 g) (Sigma-Aldrich, Darmstadt, HE, Germany S1626), ascorbic acid (3.6 g) (Sigma-Aldrich, Darmstadt, HE, Germany A4544), Wesson's salt (2 g) (Lab House, Belo Horizonte, MG, Brazil 16,632), V8™ tomato and vegetable juice (50 mL) (Campbell Soup Company, Camden, NJ, USA), and any substances being tested were added and mixed. The diet was dispensed into testing plates or tubes for immediate use.

To assess the individual activity of each pesticidal protein, the *Cry1Ab*, *Cry1Bb*, and *Cry2Aa* genes were individually cloned between the *cauliflower mosaic virus* (CaMV) 35S promoter and the T-Nos terminator. These genetic constructs were then introduced into eucalyptus plants using the *A. tumefaciens* transformation method (Prakash and Gurumurthi, 2009). The bioassays involving single pesticidal protein Cry1Ab or Cry1Bb, or Cry2Aa-expressing eucalyptus and the wild-type (wt) FGN-K, were conducted using 3-month-old plantlets. These plantlets were approximately 60 cm tall and had approximately 10 leaves each. They were produced from cuttings from a polycarbonate greenhouse equipped with a pad-fan cooling system. The greenhouse-maintained temperatures ranged from 18°C to 28°C, and the relative humidity was between 70% and 90%.

The bioassays were conducted in a temperature-controlled room (25°C ± 2°C), with a relative humidity of 70% ± 10% and a 12-h light phase. For each experiment, 10 second-instar *T. arnobia* caterpillars were placed on each plantlet (five replicates per treatment). Each plantlet was carefully placed between two 1-L clear polypropylene deli containers, which were then securely taped together. The upper lid of the container was swapped with a 50-mesh net. A caterpillar's survival was assessed after a period of 6–7 days (Tukey's test was employed at a 1% significance level to enhance the sensitivity of the test in detecting differences).

The evaluation of event 1521K059 plantlets (Supplemental Figure S8) was conducted within 50-mesh-net cages under the same controlled conditions, as mentioned above. In each cage, 10 second-instar *T. arnobia* caterpillars were introduced, and both mortality and leaf damage were documented after 6 days. Field bioassays with *T. arnobia* were conducted in separate locations from the regulatory field trials mentioned below to avoid any potential harm to the trees of the regulatory trials.

The experiments were carried out in two farms in São Paulo and one farm in Mato Grosso do Sul. The test on each site involved 6-month-old trees arranged in five linear blocks, with each block containing six trees. The spacing between the trees and rows was 3.0 m. The bioassays included event 1521K059, which expresses all three pesticidal proteins and the FGN-K wild-type clone. Insect-proof cages made of mesh bags were used, and each cage was placed on a branch. A total of 30 neonate caterpillars, hatched on the same day, were introduced into each cage. The branches were thoroughly inspected and cleaned before the release of the caterpillars to ensure the absence of predators inside the cages. The cage bases were securely sealed with cord and tape, to prevent the entry or exit of insects. After a period of 7 days, the cages were opened and the number of surviving caterpillars was counted (Tukey's test at the 1% significance level).

Laboratory bioassays were performed using mature leaves collected from a designated field site in the State of São Paulo (SP). The fresh leaves which were not diluted or mixed with any other part of the diet, served as the undiluted control sample. These leaves were then lyophilized and diluted in the diet. For each dilution, the ratio of the weight of lyophilized leaves to their original fresh weight was multiplied by 150 (the final volume of the diet in ml) and divided by the dilution factor. This calculation determined the quantity of lyophilized leaves in grams to be combined with 150 mL of the artificial diet. Dilutions of 25, 50, 100, 200, 400, 800, 1,200, 1,600, and 2,000 of leaves from both the event 1521K059 and the wild-type eucalyptus FGN-K were prepared. Each dilution was tested on 20 microtubes, each containing one *T. arnobia* neonate caterpillar. After 7 days of exclusive feeding on the diet containing diluted leaves, the surviving caterpillars were transferred to the original growth container (Oliveira et al. (2005) and were provided with a food source devoid of pesticidal proteins. They were allowed to develop and complete their life cycle until reaching adulthood. A qualitative analysis in each group was employed to document the highest life stage attained by individuals for each dilution, without statistical tests.

In silico allergenicity and toxicity analyses

Allergenicity and toxicity analyses are the integral components of all biosafety assessment studies submitted in Brazil, as mandated by the FAO guidelines for any commercially planted GM crop (Hautea, 2009). To assess the potential allergenicity of Cry1Ab, Cry1Bb, and Cry2Aa, their amino acid sequences (Supplemental Figure S1) were analyzed using the COMPARE allergen database (van Ree et al., 2021). The known allergen profilin (GenBank: AGA84056.1) was used as a positive control. Three different types of sequence comparisons were performed using the FASTA search (Pearson, 1990) to identify any similarities between known allergenic proteins. The first comparison involved searching for similarities in the full-length sequence, with a specific emphasis on detecting highly distinctive resemblances that cannot be attributed to a random chance. In protein alignment searches, the accepted threshold for a random chance is less than 1 in 1,000, denoted by a parameter called the E-value that should be lower than 10e-4 (0.001) (Karlin and Altschul, 1990). The second comparison utilized an 80-mer sliding window search to identify instances where the identity exceeded 35%. Finally, an 8-mer sliding window search was conducted to identify any peptides of the complete identity.

The BLASTP tool (version: 2.11.1+), a program that finds protein sequences similar to a given target sequence, was utilized (Altschul et al., 1997) to assess the potential toxicity of Cry1Ab, Cry1Bb, and Cry2Aa in humans and animals. The known human toxin ricin (UniProt P02879) was used as a positive control. The NCBI nr database was employed for this purpose, using the following configuration settings: max target sequence = 5,000; E-value threshold = 0.001; word size = 6; matrix = BLOSUM62; gap costs = existence: 11; extension: 1; filter for low complexity = off. Moreover, a search was conducted on the UniProt database using the BLASTP tool, using the following configuration parameters: target database = UniProtKB reference proteome plus Swiss-Prot; E-value threshold = 0.001; matrix = BLOSUM62; filter for low complexity = off; gap penalty = yes; hits = 1,000. In both instances, the search outcomes were screened for the presence of the terms "toxic," "toxin," "anti-nutrition," "agglutinin," "trypsin inhibitor," and "protease inhibitor" in their descriptions. Additionally, the Toxic Exposome Database (T3DB) was employed to identify any homology to the known toxins (Lim et al., 2010; Wishart et al., 2015). The homology search was conducted using the BLASTP tool with the following configuration parameters: cost to open a gap = −1, cost to extend a gap = −1, penalty for the mismatch = −3, reward for the match = 1, and expectation value = 0.00001.

Recombinant proteins

Recombinant Cry1Ab, Cry1Bb, and Cry2Aa pesticidal proteins (>5 g each) were produced as needed in *Pseudomonas fluorescens* bacteria, by Fraunhofer-Gesellschaft (Fraunhofer-Gesellschaft, Munich, BY, Germany), and analyzed by Schafer Scientific Solutions LLC (Schafer Scientific Solutions LLC, Carmel, IN, USA). In order to stabilize the proteins, the potential trypsin cleavage site at the N-terminal was eliminated by introducing mutations (R28del + I29Q for Cry1Ab and R5Q + R34Q for Cry1Bb). For Cry2Aa, a His6 tag was attached at the C-terminal to simplify purification in a nickel column. These modifications are located outside the active domains of the pesticidal proteins (Sanahuja et al., 2011), indicating that their impact on the protein activities should be minimal. The inclusion bodies containing these proteins were collected from cells that were grown in a standard 50L fermentation process for 48 h. Trypsin was used to activate Cry1Ab and Cry1Bb pesticidal proteins by the cleavage of the C-terminal site. The purity levels (protein/protein) and concentrations (% of the active ingredient per powder mass) were as follows: Cry1Ab purity was greater than 80% with a concentration of 28%; Cry1Bb purity was greater than 35% with a concentration of 20%; Cry2Aa purity was greater than 90% with a concentration of 20%. The activity of the purified proteins was validated by the manufacturers using *Helicoverpa armigera*

(Lepidoptera: Noctuidae) larvae for Cry1Ab, *Manduca sexta* (Lepidoptera: Sphingidae) for Cry1Bb, and *Spodoptera exigua* (Lepidoptera: Noctuidae) for Cry2Aa. The immunoreactivity of the proteins was assessed using enzyme-linked immunosorbent assay (ELISA); Cry1Ab was assessed using the Agdia ELISA Kit PSP 06200 (Agdia, Inc. IN, USA), Cry2Aa was assessed using the Agdia ELISA Kit PSP 05801 (Agdia, Inc. IN, USA), and Cry1Bb was assessed using the Eurofins-Abraxis ELISA Kit PN 599100 (Eurofins Abraxis, Warminster, PA, USA).

Simulated gastric fluid and simulated intestinal fluid digestibility

The digestibility assays were conducted as an additional safety measure based on a tiered approach (Garcia-Alonso et al., 2006). The objective was to demonstrate human safety under any worst-case scenario involving the ingestion of plant parts or pollen. Protein susceptibility to degradation by digestive enzymes was evaluated in the simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF). The study followed the protocols described by Thomas et al. (2004) and Fu et al. (2002). Cry1Ab, Cry1Bb, and Cry2Aa (0.1 mg/mL each), along with bovine serum albumin (BSA, 0.247 mg/mL) [Sigma-Aldrich, Darmstadt, HE, Germany cat#: A7638] and β-lactoglobulin (β-lac, 0.272 mg/mL) [Sigma-Aldrich, Darmstadt, HE, Germany cat#: L7880], were incubated with SGF or SIF at 37°C for varying intervals of time. For SGF, the digestion intervals were approximately 30 s and 1, 2, 4, 8, 16, and 32 min. For the SIF, the digestion intervals were approximately 30 s; 1, 2, 4, 8, 16, and 32 min; and 1, 2, 4, 24, and 48 h. Proteins were analyzed by SDS-PAGE, with gels stained with SimplyBlue SafeStain (Invitrogen, Frederick MD, USA, LC6060) for approximately 1 h and then destained in ultrapure water with agitation at 50 rpm for approximately 16 h. Gel images were captured and analyzed using an iBright 1500 system (Thermo Fisher Scientific Inc. MA, USA) using iBright Analysis Software (version 4.0.0).

Thermostability assessment

Thermostability tests were conducted with the aim of demonstrating that, during the heat stage of pulp production (>140°C; Tran, 2002), the transgene protein products undergo degradation. The thermal stability of Cry1Ab, Cry1Bb, and Cry2Aa was evaluated by subjecting the proteins (200 ng/μL) to increasing temperatures (20, 40, 60, 80, 90, 100, 110, and 120°C) for 20 min, in 50 mM of the CAPS buffer, and pH 10.0. Following the heating process, the samples were centrifuged at 20,000 *g*, 4°C, for 2 min. The soluble portions were subsequently subjected to SDS-PAGE and ELISA, as described above. Gel images and densitometry analyses were captured and processed using Bio-Rad's Image Lab software version 6.1 (Bio-Rad Laboratories, Inc. CA, USA).

Regulatory field trials

The eucalyptus event 1521K059 and the wt clone FGN-K were planted at four sites in Brazil: two in the State of São Paulo, one in the State of Bahia (BA), and one in the State of Maranhão (MA). The planting design consisted of square plots, each containing 16 plants. Five square plots of each clone/event were randomly distributed in blocks within the field, alongside other plots of unrelated clones that were not part of the experiment (Supplemental Figure S2).

Pesticidal protein expression levels

Tissue samples of young and mature leaves, stems, roots, flower buds, and pollen were collected from 6-, 12-, and 24-month-old eucalyptus event 1521K059 trees across the four regulatory trial farms. The tissues were ground in liquid nitrogen using a Thermo Scientific TissueLyser II and then lyophilized for 96 h using a Labconco FreeZone 1 L Benchtop Freeze Dry System (Labconco Corporation, MO, USA) set to −56°C. Cry1Ab, Cry1Bb, and Cry2Aa concentrations were determined using the ELISA kits listed above, following the manufacturers' protocol. The recombinant proteins described above were used for the standard curves. The highest pesticidal protein concentrations found were used in the margin of exposure calculations.

Margin of exposure calculations for non-target indicator species

Exposure studies were conducted on honey bee *Apis mellifera* (Hymenoptera: Apidae) (larvae and adults), earthworm *Eisenia fetida* (Opisthopora: Lumbricidae), springtail *Folsomia candida* (Collembola: Isotomidae), and aquatic invertebrate *Daphnia magna* (Anomopoda: Daphniidae), following OECD protocols 239, 245, 222, 202, and 232, respectively (OECD, 2004a; OECD, 2004b; OECD, 2016; OECD, 2017; OECD, 2021). The diet, growth conditions, and experimental procedures' result analyses and statistics were as described in the protocols.

The no-observed-effect concentration (NOEC) for each organism or the equivalent no-observed-effect dose (NOED) in the case of *A. mellifera* was the highest soluble recombinant Cry1Ab, Cry1Bb, or Cry2Aa pesticidal protein concentration (one high concentration per species) that was incorporated into the diet or liquid habitat (for *D. magna*), according to the OECD protocols, for each indicator species.

The estimated environmental concentrations (EECs) of pesticidal proteins for earthworm *E. fetida* and springtail *F. candida* were the highest measured concentrations (in μg/g) of each protein found in the tissues of the event 1521K059 (Table 4).

To calculate the estimated environmental dose (EED) for *A. mellifera*, the maximum pollen intake per larval development stage of 2.04 mg/pollen (Babendreier et al., 2004) and the maximum daily pollen intake for adult worker bees of 4.3 mg/pollen (Crailsheim et al., 1992) were each multiplied by the highest measured concentration of each pesticidal protein found in pollen from the event 1521K059. Since the Cry1Bb protein is regulated by a promoter specific to green plant tissues (Figure 1), it was not detected in the pollen of the event 1521K059. Therefore, the limit of quantification (LOQ) of 1.24 μg/g, stated in the Eurofins Abraxis detection ELISA kit (PN 599100), was used as a conservative estimate of the maximum Cry1Bb concentration in 1521K059 pollen.

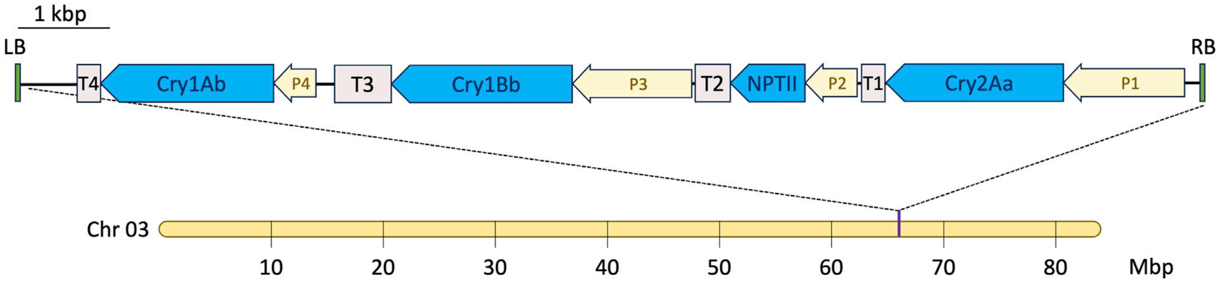

FIGURE 1
T-DNA insertion site in the transgenic eucalyptus event 1521K059. A single insertion was identified on chromosome 3 at approximately 66 Mbp. P1—*cauliflower mosaic virus* (CaMV) 35S promoter fused to the eucalyptus translation elongation factor EF-1 alpha intron (Eucgr J01112); P2—CaMV 35S promoter; P3—eucalyptus ribulose bisphosphate carboxylase small subunit promoter (Eucgr J01502); P4—CaMV 35S promoter; T1 and T4—*Agrobacterium tumefaciens* nopaline synthase terminator; T2—CaMV 35S terminator; and T3—eucalyptus ribulose bisphosphate carboxylase small subunit terminator (Eucgr J01502).

To conservatively estimate the EEC for *D. magna*, a model was used that assumed the living tissue biomass from 10 ha of transgenic eucalyptus drains into a 20,000-cubic meter pond (Carstens et al., 2012). The amount of the living tissue biomass per hectare of eucalyptus was set to 25,200 kg based on published data (Ludvichak et al., 2022). The EEC was calculated by taking 10 ha of the biomass at 25,200 kg/ha, multiplying it by the maximum measured concentration of each protein in the tissues of the event 1521K059, and dividing it by the 20 million-liter pond volume.

Margin of exposure (MoE) values were calculated for each of the four model non-target organisms and each protein using the ratio NOEC/EEC or NOED/EED, respectively.

Soil microbial community analysis

Twenty four months after planting, microbial diversity and density studies were conducted across the four regulatory trial farms, following the methods outlined by Avisar et al. (2023). In summary, soil samples were obtained from each of the event 1521K059 and FGN-K plots (five per plot) in all four field trials (five plots per field), using a clean auger, reaching a depth of 15 cm. Prior to collection, the sampling locations were carefully cleared of any weed and plant remnants. An amount of 1.0 g of the soil was diluted in 10 mL of phosphate-buffered saline, comprising 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. The mixture was thoroughly stirred and then centrifuged at 1000 *g* for 5 min. Subsequently, 100 µL of the resulting solution was inoculated into the appropriate culture medium, as outlined in Avisar et al. (2023). Microbial density was expressed as the logarithm (base 10), with colony-forming units (CFUs) per gram of soil. To evaluate microbial diversity, ribosomal RNA sequencing was employed, and the analysis utilized the "alpha diversity" tool from QIIME software (Caporaso et al., 2010). The principal coordinate analysis (PCoA) was conducted to compare sample groups based on the phylogenetic and count-based distance metrics (Hammer et al., 2001; Podani and Miklós, 2002).

Arthropod collection and analysis

Arthropods were collected and examined by following the methods outlined by Avisar et al. (2023). In summary, in all four regulatory trial farms, a total of five distinct sampling techniques were utilized at various points during the plant growth cycle, when the plants were approximately 4, 10, 12, 19, and 23 months old. These methods included the following (Supplemental Figure S3): modified "beating sheet/net," where the branches were vigorously shaken for 30 s inside a plastic bag (10 samples per plot, at only 4 years and 10 months of age before the trees were too high to shake the branches); pitfall traps: traps measuring 10 cm in diameter and 15 cm in height were placed at the center of each plot, filled with a solution (1%–2% detergent and 4% formaldehyde), and left for 72 h; adhesive cards: attractive yellow adhesive sheets (14 × 23 cm, ISCA brand) were positioned at the center of each plot, at the height of the treetops, and left for 72 h; soil collection: the samples (10 samples per plot, which are 10 cm in diameter and 5 cm in depth) were obtained, and the species were retrieved using the Berlese–Tüllgren funnel method (Brown, 1973); litter collection: samples (five samples per plot, 25 cm^2) were obtained, and the species were retrieved using the Winkler extractor method (Besuchet et al., 1987; Sabu et al., 2011).

Following the collection, the samples were preserved in a solution of 70% ethanol and 5% glycerin. They were then classified into different taxa by comparing them with reference collections or with the literature. The "Total" number of observed arthropods, "Richness" (defined as the number of observed species), and "Diversity" (defined as the inverse of the sum of the squares of the observed numbers of each species, divided by the total number of observed species) were analyzed using ANOVA with the agricolae package (version 1.3.5) in the R programming language (version 4.3.0). The significance level was set at alpha = 0.05. Statistical comparisons were carried out between the control group (FGN-K) and the eucalyptus event 1521K059. To control for the false discovery rate (FDR; type 1 statistical error), adjustments were made upward based on the magnitude of the F-test. Eta-squared effect sizes (η^2) were calculated for every

ANOVA using the *eta_squared*() function from the "effectsize v 0.8.6" R package (Ben-Shachar et al., 2020).

Organic material degradability assays

Screened nylon bags with a mesh size of 2 mm and dimensions of 20 × 20 cm were used to contain the litter samples. Each bag was filled with 35 g of biomass, comprising 5 g of branches and 30 g leaves collected right at the regulatory trial sites. At each of the four trial sites, five bags containing either the biomass from the event 1521K059 or commercial reference eucalyptus FGN-K were placed touching the ground in each plot. Altogether, there were five bags per sample type per time point at each location. On both days 0 and 180, the samples were analyzed following the methodology and calculations described by Santos and Whitford (1981). Initially, the samples were dried for approximately 1 h, at a temperature of 60°C–70°C, and the weight of the dry matter was recorded. Then, the samples were incinerated in a muffle furnace at 700°C to determine the content of ashes and organic matter. The loss of ashes and organic matter dry weight was calculated by comparing the results to those obtained on day 0. The average values were calculated across all sites for both the event 1521K059 and the commercial reference. Analysis of variance was conducted, and mean separations within the treatment and weight were determined by Tukey's test at a 5% error probability.

Results

Identification of the genomic insertion site

The insertion site of the T-DNA encoding the triplet pesticidal Cry proteins was identified by deep DNA sequencing and genome read mapping. Based on the publicly available eucalyptus genome, BRASUZ 2.0 (Myburg et al., 2014), it was established that event 1521K059 possesses a single heterozygous insertion site. This insertion site was identified on one of the two chromosomes #3 of the event 1521K059, located at approximately 66 Mbp, while no endogenous gene was affected by the insertion. Figure 1 illustrates a single inverted insertion detected in the genome. The complete sequencing of all four expression cassettes, starting from the T-DNA right border, identified the *Cry2Aa* gene controlled by the *cauliflower mosaic virus* 35S promoter, which was fused to the eucalyptus translation elongation factor, the EF-1 alpha gene (Eucgr J01112) intron, and to the *A. tumefaciens* nopaline synthase terminator (T-Nos). In addition, it identified the *nptII* selectable marker gene under the control of the CaMV 35S promoter and terminator, the *Cry1Bb* gene under the eucalyptus ribulose-bisphosphate carboxylase small subunit (Eucgr J01502) promoter and terminator, and, finally, the *Cry1Ab* gene controlled by the CaMV 35S promoter and terminated by T-Nos.

Event 1521K059 can effectively control the *T. arnobia* pest

Field and laboratory evaluations were conducted to assess the efficacy of the IR GM eucalyptus event 1521K059 against *T. arnobia*. Initially, we conducted tests to determine whether each individual pesticidal protein, expressed in eucalyptus, can effectively control the target pest on its own. Single pesticidal protein Cry1Ab or Cry1Bb, or Cry2Aa-expressing eucalyptus and WT FGN-K were employed to evaluate the activity of each pesticidal protein. The GM eucalyptus events expressing single pesticidal proteins effectively controlled second-instar *T. arnobia* caterpillars, resulting in 100% mortality within 7 days (Figure 2A).

Due to a low chance of a natural infestation, the efficacy of the event 1521K059 in the field was tested by intentional infestation in branch cages. The three field trials indicated that event 1521K059's branches conferred a mortality rate of 99.3%–100% for *T. arnobia* caterpillars (Figure 2B). In comparison, the mortality rate of the neonate caterpillars exposed to wt FGN-K branches was 20%–40% (Supplemental Figure S8), further substantiating the efficacy of event 1521K059 cuttings in combating *T. arnobia* second-instar infestation and preventing any damage within a laboratory cage environment.

When testing the impact of diluted leaf extracts from event 1521K059 (Figure 2C) in laboratory tests, even a 200-fold dilution of the extracts was sufficient to eliminate all the feeding neonate *T. arnobia* caterpillars. When exposed to a 400-fold dilution, some caterpillars survived but exhibited abnormal growth by failing to progress to the pupal stage, when reintroduced to their normal diet. Caterpillars exposed to event 1521K059's leaves diluted up to 1,200 times were able to enter the pupal phase but failed to emerge as adults. However, from a dilution of 1,600-fold and above, the caterpillars completed their development and successfully emerged as adults. *T. arnobia* caterpillars were fed undiluted wt leaves, as well as all dilutions of the diet, completed their entire life cycle, and developed into adults.

In silico allergenicity and toxicity analyses

In silico analyses performed to assess the potential allergenicity of Cry1Ab, Cry1Bb, and Cry2Aa, based on datasets as of 2022, found no significant matches (Table 1), indicating that these proteins, like other Bt Cry pesticidal proteins (Randhawa et al., 2011), are non-allergenic. The full-length alignments demonstrated no noteworthy similarity to any known allergen, and all results had E-values above 10e-4, suggesting a lack of meaningful biological resemblance between the sequences (Pearson, 1999). Furthermore, when employing a sliding window of 80 amino acids, no relevant alignments with an identity greater than 35% were observed for any of the tested proteins. Additionally, an analysis of eight amino acid peptides found no matches. The positive control profilin had more than 100 hits in all the three tests.

Searches conducted in the NCBI nr and UniProt databases using the Cry1Ab, Cry1Bb, and Cry2Aa protein sequences revealed alignments with other Cry pesticidal proteins, as well as proteins from the "δ-Endotoxins" and "Endotoxin_N domain-containing protein" groups. Some putative proteins showing a partial similarity to conserved domains found in Cry pesticidal proteins were also identified. However, apart from these, no relevant occurrences of the terms "toxic," "toxin," "anti-nutrition," "agglutinin," "trypsin inhibitor," and "protease inhibitor" were found in the BLASTP output files (Table 1). The positive control ricin had thousands of known similarities with toxins and is registered in the T3DB.

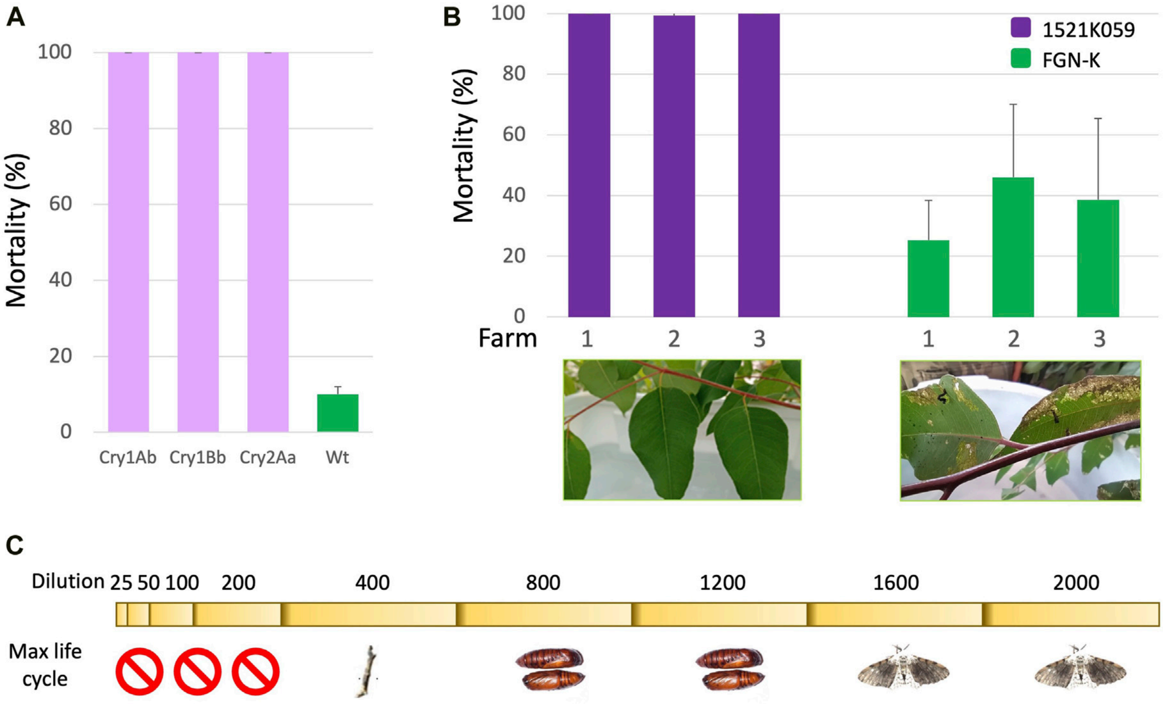

FIGURE 2
Analyses of transgenic eucalyptus expressing pesticidal proteins. **(A)** Eucalyptus events expressing Cry1Ab, Cry1Bb, or Cry2Aa effectively controlled second-instar *T. arnobia* caterpillars, resulting in 100% mortality within 7 days (Tukey's test at the 1% significance level). **(B)** In-field trials (two farms in São Paulo, SP-1 and SP-2, and one farm in Mato Grosso do Sul, MS) using branch cages; the event 1521K059 achieved 99.3%–100% mortality of *T. arnobia* neonate caterpillars, compared to the 20%–40% mortality in wild-type branch cages after 7 days of exposure. The representative leaf damage shown below in the graphs demonstrates the protection provided by the event 1521K059 against *T. arnobia* caterpillars (Tukey's test at the 1% significance level). **(C)** Dilutions of the event 1521K059 leaf extracts in the *T. arnobia* diet in laboratory assays. The 200-fold dilution was sufficient to eliminate all neonate caterpillars. The 400-fold dilution arrested the life cycle of the surviving caterpillars before the pupal stage. The 1,200-fold dilution still prevented adult emergence. Higher dilutions allowed caterpillars to complete the life cycle to adulthood.

TABLE 1 *In silico* allergenicity and toxicity analyses.

Protein	Size (aa)	Allergenicity			Toxicity				
		Full-length	80-mer	8-mer	NCBI nr		UniProt		T3DB
		Hit	Hit	Hit	Alignment	Toxic term	Alignment	Toxic term	Hit
Cry2Aa	633	0	0	0	1,781	0	115	0	0
Cry1Bb	655	0	0	0	2,094	0	150	0	0
Cry1Ab	622	0	0	0	2,073	0	135	0	0
Positive control									
Profilin	131	>100	139	138					
Ricin	576				8,102	>90%	>1,000	>90%	1

Simulated gastric fluid and simulated intestinal fluid digestibility

In the digestibility assays, Cry1Ab, Cry1Bb, and Cry2Aa exhibited similar behaviors to the control BSA in the SGF and SIF, i.e., rapidly digested (30 s–4 min) in the SGF but resistant to digestion (up to 48 h) in SIF (Table 2; Supplementary Figure S4; Supplementary Figure S5). Conversely, β-lactoglobulin showed high digestibility in the SIF but resisted digestion in the SGF.

Thermostability assessment

In thermostability studies, Cry1Ab and Cry2Aa were degraded as the temperatures were increased. The degradation was

TABLE 2 Digestibility results in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

Treatment	Protein	Maximum detection time
SGF	β-lac	32 min
	BSA	30 s
	Cry1Ab	4 min
	Cry1Bb	2 min
	Cry2Aa	30 s
SIF	β-lac	30 s
	BSA	48 h
	Cry1Ab	48 h
	Cry1Bb	48 h
	Cry2Aa	2 h

particularly pronounced at temperatures above 40°C (Table 3; Supplementary Figure S6). Cry1Bb underwent significant degradation, primarily at temperatures exceeding 60°C. The solubility of the pesticidal proteins gradually decreased as the temperature increased, as determined by densitometry. The immunoreactivity of the proteins, assessed by reduced binding to ELISA plates, also showed a gradual decrease with the increasing temperatures. At temperatures above 60°C, the antibodies in the ELISA plates failed to recognize the proteins and immunoreactivity reached 0%, indicating a substantial loss of conformation at higher temperatures (Table 3).

Maximum concentration levels

The biosafety tests and estimates of environmental exposure are based on the maximum recorded levels of the expression and concentration of pesticidal proteins in the evaluated event 1521K059. The highest concentration values across four farms and trees aged 6–24 months were used for the margin of exposure calculations. These were 53.76 μg/g for Cry1Ab, 8.33 μg/g for Cry1Bb, and 9.73 μg/g for Cry2Aa in leaf tissues. In pollen, the levels were 5.08 μg/g for Cry1Ab and 1.53 μg/g for Cry2Aa. The Cry1Bb expression is limited to green tissue by the Eucgr J01502 promoter and terminator (Figure 1), so no expression was detected in the stem, roots, and pollen. Therefore, the limit of quantification value of 1.24 μg/g for Cry1Bb was used as described in the methods.

Pesticidal protein margin of exposure for non-target indicator species

To evaluate the risk to non-target organisms upon their potential exposure to the pesticidal proteins in the eucalyptus event 1521K059, the worst-case scenario estimated EEC or EED of each protein was compared to the NOEC or NOED of the representative indicator species. The highest recorded Cry1Ab, Cry1Bb, and Cry2Aa concentrations (Table 4) were used to calculate the EEC or EED representing worst-case exposures. For earthworm and springtail, the EEC was the maximum leaf concentrations in their diet: 53.76 μg/g for Cry1Ab, 8.33 μg/g for Cry1Bb, and 9.73 μg/g for Cry2Aa. For *D. magna*, we utilized an estimate of the eucalyptus biomass from Ludvichak et al. (2022) as a highly conservative scenario. This approach aligns with the pond model based on maize (Carstens et al., 2012). The maximum leaf concentrations were converted to EECs in the test water based on the model: 0.68 mg/L for Cry1Ab, 0.1 mg/L for Cry1Bb, and 0.12 mg/L for Cry2Aa (detailed calculations in Supplementary Figure S1). For honey bees (detailed calculations in Supplementary Figure S4, S5), the pollen concentrations of 5.08 μg/g for Cry1Ab, 1.24 μg/g for Cry1Bb (LOQ as not detected), and 1.53 μg/g for Cry2Aa were converted to EEDs using pollen intake rates, resulting in 0.0104 μg/g for Cry1Ab, 0.025 μg/g for Cry1Bb, and 0.031 μg/g for Cry2Aa per larva development and 0.0218 μg/g for Cry1Ab, 0.0053 μg/g for Cry1Bb, and 0.0066 μg/g for Cry2Aa per day as per an adult worker (Table 5).

TABLE 3 Densitometric measurement (by SDS-PAGE) and immunoreactivity (by ELISA) of the effect of heat treatment on pesticidal proteins after 20 min of exposure.

Treatment (°C)	Densitometry (%)			Immunoreactivity (%)		
	Cry1Ab	Cry1Bb	Cry2Aa	Cry1Ab	Cry1Bb	Cry2Aa
0	100	100	100	100	100	100
20	100	100	100	100	100	99
40	100	100	100	100	95	81
60	54	100	83	5	93	37
80	53	38	0	0	0	0
90	33	29	0	0	0	0
100	5	23	0	0	0	0
110	1	12	0	0	0	0
120	1	1	0	0	0	0

TABLE 4 Maximum measured protein expression levels across farms and tree age (μg/g). The BOLD highlighted results were used for EED/D estimations.

Tissue	Age	Cry1Ab	Cry1Bb	Cry2Aa
Young leaves	6	51.75	5.8	**9.73**
	12	40.21	4.71	7.27
	24	28.96	3.22	9.38
Mature leaves	6	**53.76**	**8.33**	9.24
	12	30.15	3.07	6.44
	24	18.7	3.26	6.65
Stem	6	21.66	0	1.05
	12	13.72	0	0.51
	24	8.01	0	0.33
Roots	6	7.61	0	0.77
	12	4.59	0	0
	24	2.04	0	0
Pollen	During flowering	**5.08**	0	**1.53**

The highest concentration or dose of each pesticidal protein that showed no observable effects in the target indicator species was determined. These safe concentrations/doses were used as the NOEC or NOED to calculate the margin of exposure (MoE) for each species (see Table 5). In summary, 30 mg/L for Cry1Ab, 50 mg/L for Cry1Bb, and 20 mg/L for Cry2Aa, in the habitat of *D. magna* for 48 h (Supplementary Table S1), caused no harm and no immobilization in acute tests (OECD, 2004a) similar to the control. When added to the diet of *F. candida*, 2,600 μg/g for Cry1Ab, 750 μg/g for Cry1Bb, and 2,600 μg/g for Cry2Aa (Supplementary Table S2) had similar effects on survival, as did the control in chronic tests (OECD, 2016). Doses of 2,600 μg/g for Cry1Ab, 170 μg/g for Cry1Bb, and 2600 μg/g for Cry2Aa had similar effects on *E. fetida* survival, as did the control (Supplementary Table S3) in chronic tests (OECD, 2004b). Chronic larval toxicity studies with *A. mellifera* (OECD, 2021) showed that exposure to 4 μg Cry1Ab, 80 μg Cry1Bb, or 136 μg Cry2Aa during larval development was safe, with no significant differences in the survival or adult emergence compared to the control (Supplementary Table S4). Ten days of chronic oral exposure of adult bees (OECD, 2017) to 37 μg Cry1Ab, 14 μg Cry1Bb, or 18 μg Cry2Aa per bee per day (Supplementary Table S5) induced no mortality.

The margin of exposure (MoE) ranged from 20 to 43,573 times, indicating that the EEC or EED values were tens to thousands of times lower than the respective NOEC or NOED (Table 5).

Soil microbial community analysis

Event 1521K059 had no significant impact on the soil microbial community. Microbial assessments conducted 24 months after

TABLE 5 Calculated values for the no-observed-effect concentration/dose (NOEC/D), estimated environmental concentration/dose (EEC/D), and the margin of exposure (MoE).

Species	Pesticidal protein	NOED μg/larvae	EED μg/larvae	MoE (times)
Honey bee *Apis mellifera* larvae	Cry1Ab	4	0.0104	386
	Cry1Bb	80	0.0025	31,626
	Cry2Aa	136	0.0031	43,573
Species	**Pesticidal protein**	**NOED μg/day**	**EED μg/day**	**MoE (times)**
Honey bee *Apis mellifera* adults	Cry1Ab	37	0.0218	1,694
	Cry1Bb	14	0.0053	2,626
	Cry2Aa	18	0.0066	2,736
Species	**Pesticidal protein**	**NOEC μg/g of diet**	**EEC μg/g of diet**	**MoE (times)**
Earthworm *Eisenia fetida*	Cry1Ab	2,600	53.76	48
	Cry1Bb	170	8.33	20
	Cry2Aa	2,600	9.73	267
Springtail *Folsomia candida*	Cry1Ab	2,600	53.76	48
	Cry1Bb	750	8.33	90
	Cry2Aa	2,600	9.73	267
Species	**Pesticidal protein**	**NOEC mg/L**	**EEC mg/L**	**MoE (times)**
Daphnia magna	Cry1Ab	30	0.68	44
	Cry1Bb	50	0.1	500
	Cry2Aa	20	0.12	167

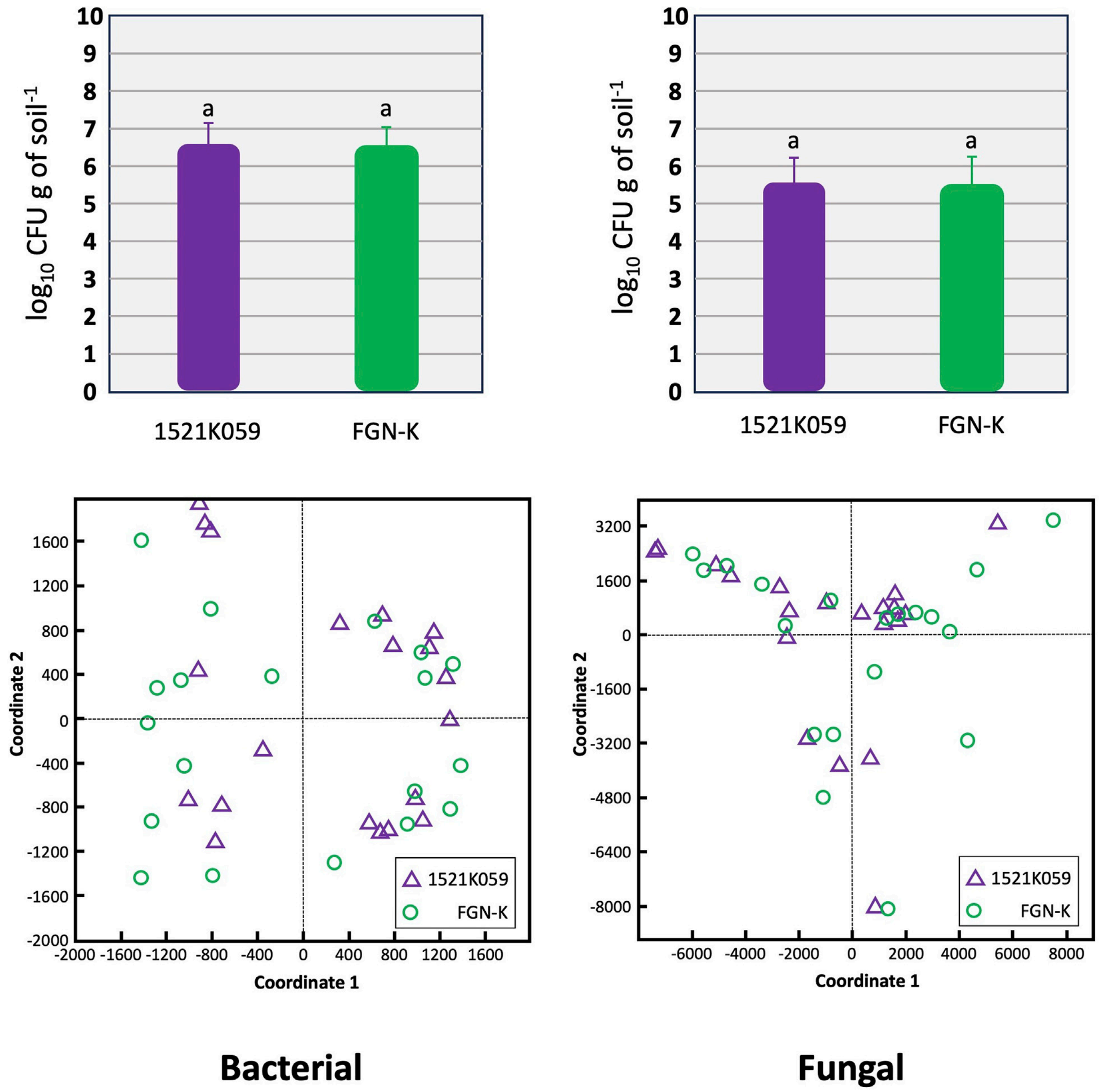

FIGURE 3
Comparison of the soil microbial composition in plots with the transgenic event 1521K059 versus plots with the wild-type clone FGN-K. Soil samples were collected from plots 24 months after either planting event 1521K059 or the corresponding wild-type clone FGN-K. To evaluate the microbial population diversity based on 16S rRNA sequencing, the principal coordinate analysis (PCoA) was performed using the QIIME alpha diversity pipeline (lower panel). Additionally, colony-forming unit (CFU) analyses were conducted on five biological replicates to assess the microbial densities (upper panel). Both analyses found no significant differences in the microbial diversity or CFUs between 1521K059 and wild-type FGN-K samples (Tukey's test at the 5% significance level).

planting found no notable difference in the composition and the density of bacteria and fungi between plots containing the event 1521K059 vs. those with the wt FGN-K clone (Figure 3 upper panels) (*p* >0.05). The CFU was quite similar across all four tested biomes in Brazil. Furthermore, the PCoA (Figure 3 lower panels) found no correlation between the soil microbial community and the cultivation of the GM event 1521K059 compared to the wt FGN-K clone.

Arthropod collection and analysis

The arthropod populations in areas cultivated with eucalyptus were compared between plots containing the eucalyptus event 1521K059 vs. those with the conventional FGN-K clone, in three different ecosystems across four experimental farms. Branches' samples from the Maranhão farm were not collected due to technical accessibility issues and were, therefore, not included in

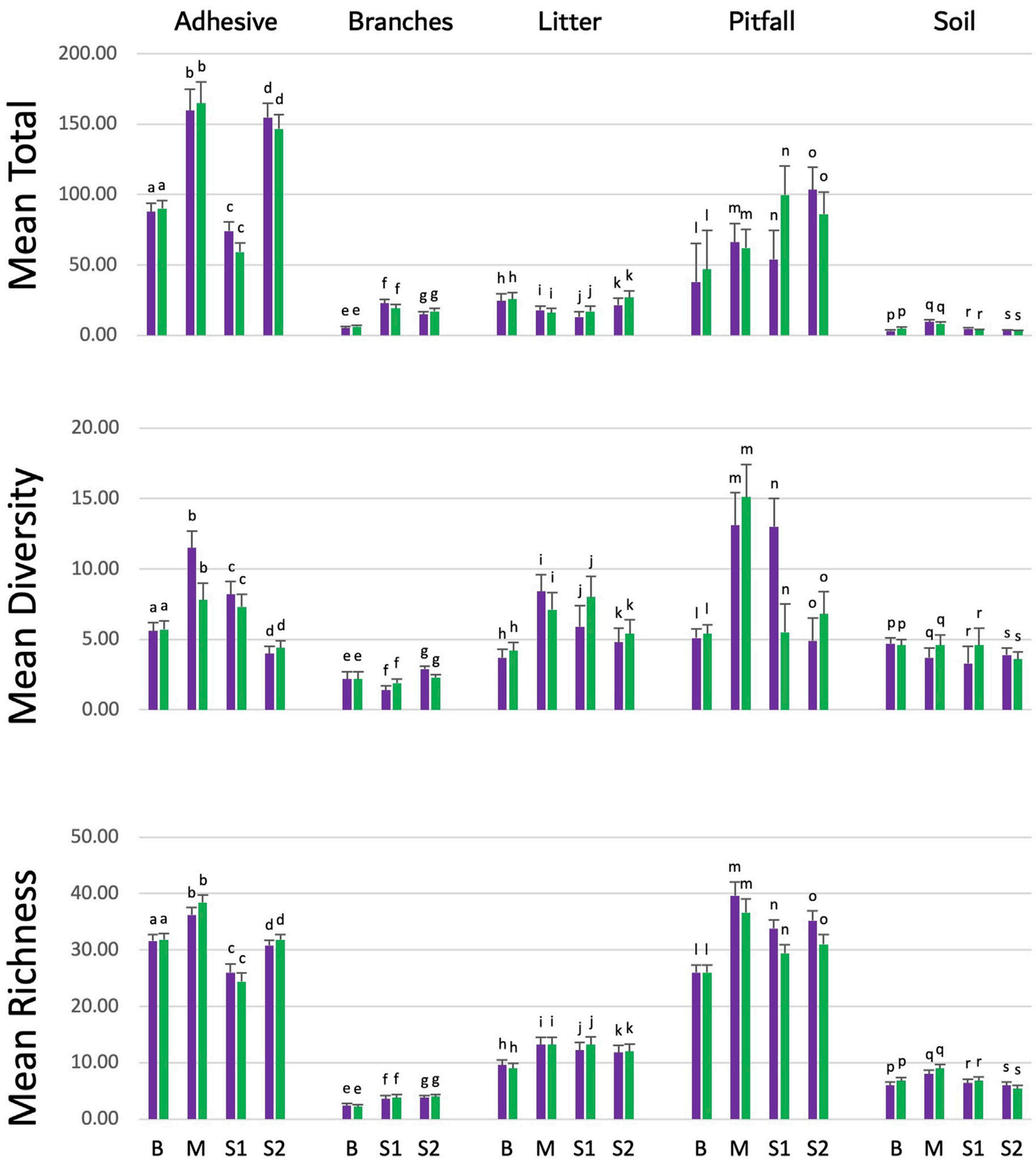

FIGURE 4
Arthropod richness and abundance in the soil from the transgenic event 1521K059 versus the wild-type clone FGN-K. Arthropod specimens were collected over 2 years from event 1521K059 and wild-type FGN-K plots using pitfall traps, adhesive traps, branch shaking, soil sampling, and litter sampling across four farms (B Bahia, M Maranhão, S1 São Paulo farm 1, and S2 São Paulo farm 2). Samples were analyzed in the laboratory, and the total number of arthropods, richness, and diversity was calculated. Variance analysis with Tukey's test at the 5% significance level was performed using the agricolae R package (version 1.3.5) in R (version 4.2.1). No significant differences in arthropod richness or abundance were found between 1521K059 and wild-type FGN-K plots across farms and collection methods.

the analysis. The results indicated that eucalyptus 1521K059 did not have a significant impact on the arthropod populations inhabiting these areas (Figure 4). For each combination of the collection method (adhesive, branches, litter pitfall, and soil) and parameter (diversity, richness, and the total), the calculated effect size (η^2) was consistently lower than 0.1, indicating a small effect size (Cohen, 1988; Olejnik and Algina, 2003). These findings were consistent across all five sampling methods (Supplemental Figure S3), indicating that the two eucalyptus varieties had similar effects on the different tested arthropod populations.

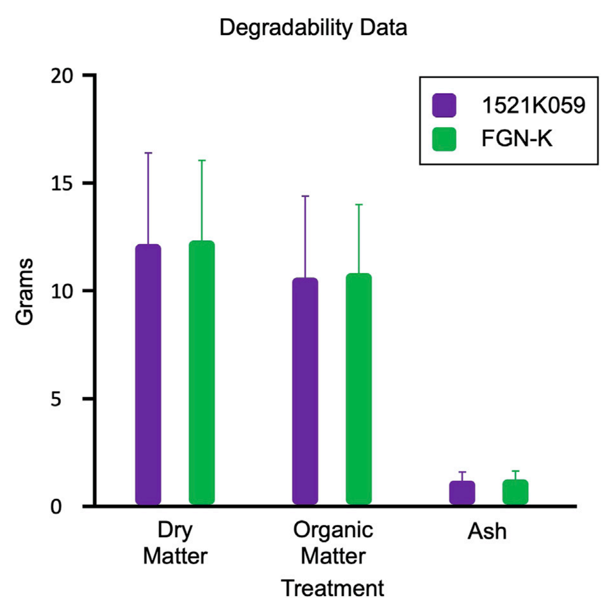

FIGURE 5
Comparable degradation rates of leaves and branches from the transgenic event 1521K059 and the wild-type FGN-K. The average dry matter, organic matter, and ash weight loss are presented for all four farms. No significant differences were found in the degradability of event 1521K059 compared to the wild-type FGN-K, as determined by Tukey's test at a 5% significance level.

Degradability of the branches and leaves in the field

Over a span of 180 days, the degradability assays found no significant differences in the degradation of the 1521K059 event vs. the FGN-K wt biomass (Figure 5). The relatively high standard deviations can be attributed to the exposure of the bags to field conditions in diverse biomes, where each farm possesses distinct characteristics, such as varying levels of precipitation, soil composition, humidity, and temperatures. These variations can influence the observed degradation rate on each farm (Ribeiro et al., 2018), and the site effect analysis can be seen in Supplemental Figure S7. At all sites, the transgenes and genetic modifications have no impact on the degradability of eucalyptus tissues in the soil.

Discussion

This study assessed the biosafety of the first commercially approved GM eucalyptus tree with lepidopteran pest resistance, labeled as event 1521K059. This pioneering Bt eucalyptus variant was engineered to express Cry1Ab, Cry1Bb, and Cry2Aa pesticidal proteins to control *T. arnobia*, one of the primary lepidopteran pests affecting eucalyptus. Each pesticidal protein can independently control the caterpillars, but utilizing the triplet stack enhances the product's durability and complements integrated pest management (IRM) programs. Detailed activity data for the event and information on the modes of action of these pesticidal proteins will be presented in an upcoming manuscript. The platform is a new tool contributing to the portfolio of biotechnological solutions aimed at assisting farmers in managing the growing threat of pest attacks, reducing the need for pesticides, and increasing the crop yield (Brookes and Barfoot, 2018). *Bt*, recognized as a safe biological pest control agent, along with its pesticidal proteins, had no adverse effects on the environment, human health, or animal welfare. Importantly, experimental data underscored the high specificity of these proteins, as evidenced in tests involving non-target organisms, encompassing both vertebrates and invertebrates (Shimada et al., 2006; Yu et al., 2011; Farias et al., 2015; Wang et al., 2018; Meissle et al., 2022).

Bt crop cultivation, adopted in 27 countries, has covered over 100 million hectares of agricultural land since 1995 (ISAAA, 2020; Tabashnik et al., 2023), earning safety approvals for food, feed, and environmental use (Mendelsohn et al., 2003; Koch et al., 2015). It represents the safest technology that is currently available to substitute for chemical pesticides, which pose direct negative consequences for farmers, consumers, non-target organisms, and ecosystems (Brookes, 2022; Yang et al., 2023). The defensive response of eucalyptus trees to feeding pests, which leads to the increased production of lignin and extractives, is known to negatively affect the wood quality (Khattab and Khattab, 2005; Zanuncio et al., 2020). The absence of caterpillar-induced damage is likely to directly augment the wood biomass, while the improved wood quality is projected to substantially increase pulp production (Zanuncio et al., 2020). Thus, insect-resistant Bt eucalyptus offers the potential to significantly enhance the yield and reduce the environmental impact of pest control measures.

Comprehensive biosafety assessments spanning 2 years positioned the insect-resistant Bt eucalyptus at a safety standard equivalent to other commercially established Bt crops, confirming its suitability for responsible farming practices. The *in silico* analysis (based on the datasets from 2022) indicated that the Cry1Ab, Cry1Bb, and Cry2Aa pesticidal proteins are not anticipated to present substantial risks to human health. Tests for protein digestibility and thermostability further corroborate to the safety of these proteins. Similar to other Cry pesticidal proteins that have been assessed and Cry1Ab that was tested before (Okunuki et al., 2002; de luis et al., 2009), Cry1Bb and Cry2Aa lost their immunoreactivity and potential activity at approximately 60°C (Wang et al., 2018) and were degraded when exposed to the SGF but resisted digestion in the SIF alone (Farias et al., 2015). Cry proteins are known to resist degradation in the SIF, but research has shown that if they first encounter gastric fluids, the resulting peptides are completely broken down when they arrive at the simulated intestinal environment (US EPA, 2010). This is crucial as it signifies that under the standard physiological conditions, where proteins are efficiently digested in the stomach first, Cry proteins would be reduced to amino acids or small peptides before reaching the intestines.

Arthropods and soil microbes play an important role in maintaining the ecological balance, including in agricultural fields and farms (Paoletti, 2012; Zhang et al., 2023). Due to their high sensitivity to alterations in crop growth and cultivation practices, they can act as "bioindicators" to identify impacts on specific field ecosystems. A three-year study on four farms investigated whether the genetically modified event 1521K059 impacted the arthropod and soil microbial populations in comparison to the non-modified wild-type clone FGN-K. No significant discrepancies were found between the plots of 1521K059 and FGN-K, suggesting that the introduction of the genetically modified event 1521K059 did not induce ecological alterations. This, along with similar degradation rates of the event 1521K059 and the FGN-K wild-type, suggests that the GM IR eucalyptus poses a low risk to the environment.

Given that eucalyptus products from Brazil are not employed for human or animal consumption, the assessment of the potential toxicity of the pesticidal proteins present in the genetically modified eucalyptus event 1521K059 was conducted in non-target organisms (NTOs), as required by the CTNBio, following the guidelines outlined in the OECD for assessing the potential impacts of chemicals on both human health and the environment (OECD, 2004a; OECD, 2004b; OECD, 2016; OECD, 2017; OECD, 2021). The evaluation encompassed honey bee *A. mellifera*, earthworm *E. fetida*, the springtail *F. candida*, and the aquatic invertebrate *D. magna*, which are the four well-established surrogate species, widely employed for safety analyses and as representatives of other NTOs in the environment. Eucalyptus is known to be attractive to bees (Cham et al., 2017), and exposure to the Bt protein primarily occurs through the consumption of pollen and nectar from eucalyptus flowers. Aquatic invertebrates, such as *D. magna*, may come into contact with the Bt protein through the ingestion of the solubilized protein in water or plant tissues from eucalyptus transported into the bodies of water. The 48-h acute toxicity tests for Bt pesticidal proteins are commonly conducted, as indicated by various studies (Federici and Siegel, 2008; Wolt et al., 2008; ILSI, 2011). Tests assessing the impact of these proteins on lepidopteran targets, which typically require 5–7 days to record 100% mortality, indicate a mortality rate of 20%–40% after 48 h (Babu et al., 2002). In contrast, mosquitoes demonstrate larval mortality ranging from 91.0% to 100% within 24 h (Derua et al., 2022). Following a thorough examination, which revealed no adverse effects, even at high doses of Cry1Ab, Cry1Bb, and Cry2Aa within the initial 48 h, there was no request to extend the *D. magna* tests to cover chronic reproduction (7–21 days). Edaphic fauna, comprising arthropods, like earthworms and springtails, can feed on eucalyptus leaves incorporated into the soil and on possible exudates or decomposed parts of plant roots. The Brazilian legislation does not specify the ecotoxicological studies to be conducted, leaving it to the applicant to determine which studies are most relevant to the environment where the GMO will be planted. The list of the selected indicator organisms was deemed sufficient by the CTNBio, and there is no requirement to conduct studies with predatory arthropods.

The MoE values (Table 5) exceeded the EEC and EED by more than a 10-fold margin and adhered to the NTO testing standards outlined by the U.S. EPA (U.S. EPA, 2007; U.S. EPA, 2010), suggesting the absence of the potential harm to any of these organisms. These MoE values are low-conservative estimate values suggesting worst-case scenarios, and the actual safety margins are potentially higher due to the lower pesticidal protein concentrations in the tree tissues and pollen and the extreme values used for the EEC/EED calculations.

The biosafety assessments of event 1521K059, including molecular characterization, toxicity and allergenicity, and the environmental impact, present a safety profile comparable to that of the conventional eucalyptus clone FGN-K. This supports the conclusion that this genetically modified insect resistant eucalyptus variety is safe for use in wood and fiber production and poses negligible risks to human or animal health or the environment.

The sequencing data on the event 1521K059 revealed a single insertion site in the genome, with no direct impact on any endogenous genes. This insertion can serve as a marker for tracking the events in the future planting and breeding activities. Trait introgression occurs at a slow pace in eucalyptus due to its inherent incompatibility with selfing and backcrossing (Hedrick et al., 2016). Consequently, facilitating the integration of the desired genes into eucalyptus breeding populations across different biomes requires more than a single GM event. Additional parental genetic backgrounds, carrying the genes, are essential for each biome's breeding population as the genetic background of 1521K059 is not universally suitable for all biomes in Brazil. Moreover, relying on a sole event may lead to linkage drag, connecting with an undesirable locus in the genome. Such outcomes might only become apparent in the years to come, owing to the yet unexplored genetic maps of eucalyptus (Bartholomé et al., 2015). Hence, in order to facilitate parallel breeding and promote sustainability in eucalyptus farms, it will be necessary to deregulate multiple GM events, each varying in their genetic background and/or T-DNA insertion sites. At the same time, an integrated resistance management program is being developed to ensure the durability of this caterpillar-resistant eucalyptus.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation. The T-DNA insertion site sequencing data is available at GenBank OR817761.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

DA: writing–original draft, formal analysis, funding acquisition, methodology and supervision. AM: data curation and writing–review and editing. AAS: conceptualization, methodology, and writing–review and editing. ACMP: data curation, investigation, software, and writing–review and editing. CSR: data curation, investigation, and writing–review and editing. EZ: formal analysis and writing–review and editing. ERG: formal analysis, funding acquisition, supervision, and writing–review and editing. ES: data curation, formal analysis, and writing–review and editing. JMWG: data curation, formal analysis, and writing–review and editing. LB: data curation, formal analysis, and writing–review and editing. MPG: data curation, project administration, and writing–review and editing. MMD: data curation and writing–review and editing. MCC: formal analysis, methodology, supervision, and writing–review and editing. RM: writing–review and editing. RNG: formal analysis, funding acquisition, methodology, supervision, and writing–review and editing. SA: data curation, formal analysis, and writing–review and editing. SL: formal analysis, investigation, methodology, and writing–review and editing. TBD: data curation, formal analysis, methodology, and writing–review and editing. TRD: data curation, formal analysis, methodology, and writing–review and editing. WS: formal analysis, software, and writing–review and editing. ACP: conceptualization, funding acquisition, project administration, supervision, and writing–review and editing.

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Conflict of interest

Authors DA, SA, and SL were employed by FuturaGene Israel Ltd. Authors AM, AAS, ACMP, CSR, ERG, JMWG, LB, MPG, RNG, TBD, TRD, and ACP were employed by Suzano S.A. (FuturaGene—Biotech Division). Author EZ, ES, MMD, and RM were employed by Suzano S.A. Author WJS was employed by W. J. Silva Agricultural Consulting Company.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2024.1322985/full#supplementary-material

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Paraguay's approach to biotechnology governance: a comprehensive guide

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This study analyzes Paraguay's biotechnology regulatory framework and its alignment with international standards amid biotechnological advancements. It also identifies areas of improvement for enhancing framework effectiveness. Through this work, we aim to provide a resource for policymakers, stakeholders, and researchers navigating Paraguay's biotechnology regulation.

KEYWORDS

Mercosur, genetically modified organisms, recombinant DNA, biologics, vaccines

1 Introduction

The regulation of biotechnology products is a complex process involving various institutions with different protection goals to ensure their safety and efficacy (Wolt and Wolf, 2018). Therefore, it is essential to have a sound regulatory framework to monitor and evaluate biotechnology developments and their applications (Xue and Shang, 2022).

This study presents a compilation of Paraguay's current regulatory framework for biotechnology focusing on recombinant DNA-derived products, vaccines, and biopharmaceuticals. It examines the country's efforts to create a regulatory environment that aligns with international standards while accommodating rapid biotechnological advancements. Even though any attempt to summarize and analyze regulations runs the risk of being incomplete and outdated, we are confident that it can serve as a resource for policymakers, researchers, and other stakeholders working to understand and navigate this complex regulatory landscape. We aim to provide an analysis of this regulatory framework, focusing on the roles and responsibilities of the various government bodies involved (Table 1). Finally, we aim to highlight potential areas for improvement and collaboration among stakeholders.

2 International treaties

Paraguay has signed several international treaties related to modern biotechnology which emphasize the conservation of biological diversity, the sustainable use of resources,

TABLE 1 Paraguayan government bodies involved in the regulation of biotechnology and their responsibilities relevant to the topic.

Institution	Responsibilities
Ministry of Agriculture and Livestock (MAG)	• Authorize regulated trials, pre-commercial release, commercial release, and other proposed uses of GMOs to be incorporated into agricultural and forestry production based on the opinion issued by the National Agricultural and Forest Biosafety Commission (CONBIO) (Presidencia de la República del Paraguay, 2012a).
National Service for Animal Quality and Health (SENACSA)	• Supervise biosafety conditions of the introduction of GMOs approved by MAG within the scope of its authority (Presidencia de la República del Paraguay, 2012a). • Evaluate GMO feed safety according to intended use (Presidencia de la República del Paraguay, 2012a). • Establish requirements for the registration, licensing, manufacturing conditions, commercialization, supervision and prohibition of biological, chemical, pharmacological and food products and supplies for veterinary use (República del Paraguay, 2004a, secs. 8h, 8n). • Establish animal health and quality requirements for the import and export of animals, genetic material, products, byproducts of animal origin, products and supplies for veterinary use (República del Paraguay, 2004a, secs. 8h, 8n).
National Service for Plant and Seed Quality and Health (SENAVE)	• Supervise biosafety conditions of the introduction of GMOs approved by MAG within the scope of its authority (Presidencia de la República del Paraguay, 2012a). • Ensure the identity and quality of seeds, and protect the right of creators of new cultivars (República del Paraguay, 2004b, secs. 6e, 9e). • Develop, and execute programs to improve the phytosanitary quality of products and byproducts of plant origin derived from the use of biotechnology (República del Paraguay, 2004b, secs. 6e, 9e).
National Forestry Institute (INFONA)	• Supervise biosafety conditions of the introduction of GMOs approved by MAG within the scope of its authority (Presidencia de la República del Paraguay, 2012a).
Paraguayan Institute of Agricultural Technology (IPTA)	• Advise the Ministry of Agriculture and Livestock and other institutions in the formulation and execution of agricultural policy in its sphere of action (República del Paraguay, 2010). • Cooperate technically with governmental and non-governmental institutions for research, dissemination and transfer of agricultural technology.
Ministry of Public Health and Social Welfare (MSPyBS)	• Regulate the manufacture, import, distribution, and sale of medicines, food, drugs, chemical products, biological and radioactive products, reagents, and all products for use and application in human medicine in accordance with current legislation (Presidencia de la República del Paraguay, 1998a). • Regulate, control, supervise and license activities potentially polluting the environment, in coordination with institutions with responsibility in the environmental sector (Presidencia de la República del Paraguay, 1998a).
National Institute of Food and Nutrition (INAN) of the Ministry of Public Health and Social Welfare	• Supervise the biosafety conditions of the introduction of GMOs approved by MAG within the scope of its authority (Presidencia de la República del Paraguay, 2012a). • Evaluate GMO food suitability according to the intended use (Presidencia de la República del Paraguay, 2012a).
National Directorate of Sanitary Surveillance (DINAVISA)	• Regulate, control and supervise health products such as drugs for human use, chemical products, reagents, and all other products for use and application in human medicine (República del Paraguay, 2021).
Ministry of Environment and Sustainable Development (MADES)	• Supervise biosafety conditions of the introduction of GMOs approved by MAG within the scope of its authority (República del Paraguay, 2018).

the equitable sharing of the benefits of genetic resources and the safe handling of genetically modified organisms (GMOs). They also address intellectual property rights and plant variety protection (Table 2).

In South America, the Southern Common Market (Mercosur) has been actively developing and implementing regulations for biotechnology products (Figure 1). Mercosur countries recognize the importance of cooperation and harmonization in biotechnology regulation which aim to establish consistent regulations across member states and facilitate trade, while maintaining safety and effectiveness standards (Dellepiane and Pagliusi, 2019). For each of the following sections, we have included Mercosur regulations that address these efforts.

Mercosur Common Market Group (GMC) Resolutions have the goal of setting standards for the harmonization of regulations within the group, namely, once a GMC Resolution is incorporated into a country's regulatory framework, said country must adjust its internal regulation to fit the standards set by that Resolution, and thus facilitate mutual recognition. To that end, GMC has also passed Resolutions on the procedures to elaborate, revise and revoke technical regulations (Mercosur GMC, 2017), and for mutual recognition of control systems (Mercosur GMC, 1998).

Mercosur's GMC has long been incorporating Codex Alimentarius guidelines for the harmonization of food safety standards (FAO, 1995; De F Toledo, 2014). However, as of the date of submission of this paper, no Codex guidelines referring

TABLE 2 International treaties relevant to modern biotechnology signed by Paraguay.

Treaty	Key objectives/Provisions	Incorporated to local regulatory framework through
United Nations Convention on Biological Diversity	Conservation of biological diversity; sustainable use of components; fair and equitable sharing of benefits from genetic resources; appropriate access to resources and transfer of technologies; management and control of risks associated with living modified organisms (LMOs)[a]	Law No. 253/1993 (República del Paraguay, 1993)
Paris Convention for the Protection of Industrial Property	Broad protection of industrial property, including agricultural and extractive industries and all manufactured or natural products	Law No. 300/1994 (República del Paraguay, 1994a)
Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS)	Exclusion of patentability for plants and animals other than microorganisms; protection of plant varieties either by patents or an effective sui generis system or a combination thereof	Law No. 444/1994 (República del Paraguay, 1994b)
International Convention for the Protection of New Varieties of Plants (1978 version)	Recognition and protection of the rights of breeders of new plant varieties or their successors; provision of protection through a special title or patent, with only one protection form allowed per botanical genus or species	Law No. 988/1996 (República del Paraguay, 1996)
Cartagena Protocol on Biosafety	Ensuring protection in the safe transfer, handling, and use of LMOs resulting from modern biotechnology; focus on transboundary movements and possible adverse effects on biological diversity and human health	Law No. 2309/2003 (República del Paraguay, 2003)
International Treaty on Plant Genetic Resources for Food and Agriculture	Conservation and sustainable use of plant genetic resources for food and agriculture; fair and equitable sharing of benefits arising from their use; sustainable agriculture and food security in harmony with the Convention on Biological Diversity	Law No. 3194/2007 (República del Paraguay, 2007a)

[a] Definitions of the Cartagena Protocol on Biosafety to the Convention on Biological Diversity (Secretariat of the Convention on Biological Diversity, 2000).
LMO: any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology.
Living organism: any biological entity capable of transferring or replicating genetic material, including sterile organisms, viruses and viroids.
Modern biotechnology: the application of *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection.
For the purposes of this study, the terms LMO, and GMO, will be considered equivalent and referred to as "GMO" in the main document, in accordance with the prevailing use in the literature.

specifically to products of biotechnology have been incorporated into the Mercosur framework, and thus the use of said guidelines is left up to each member state.

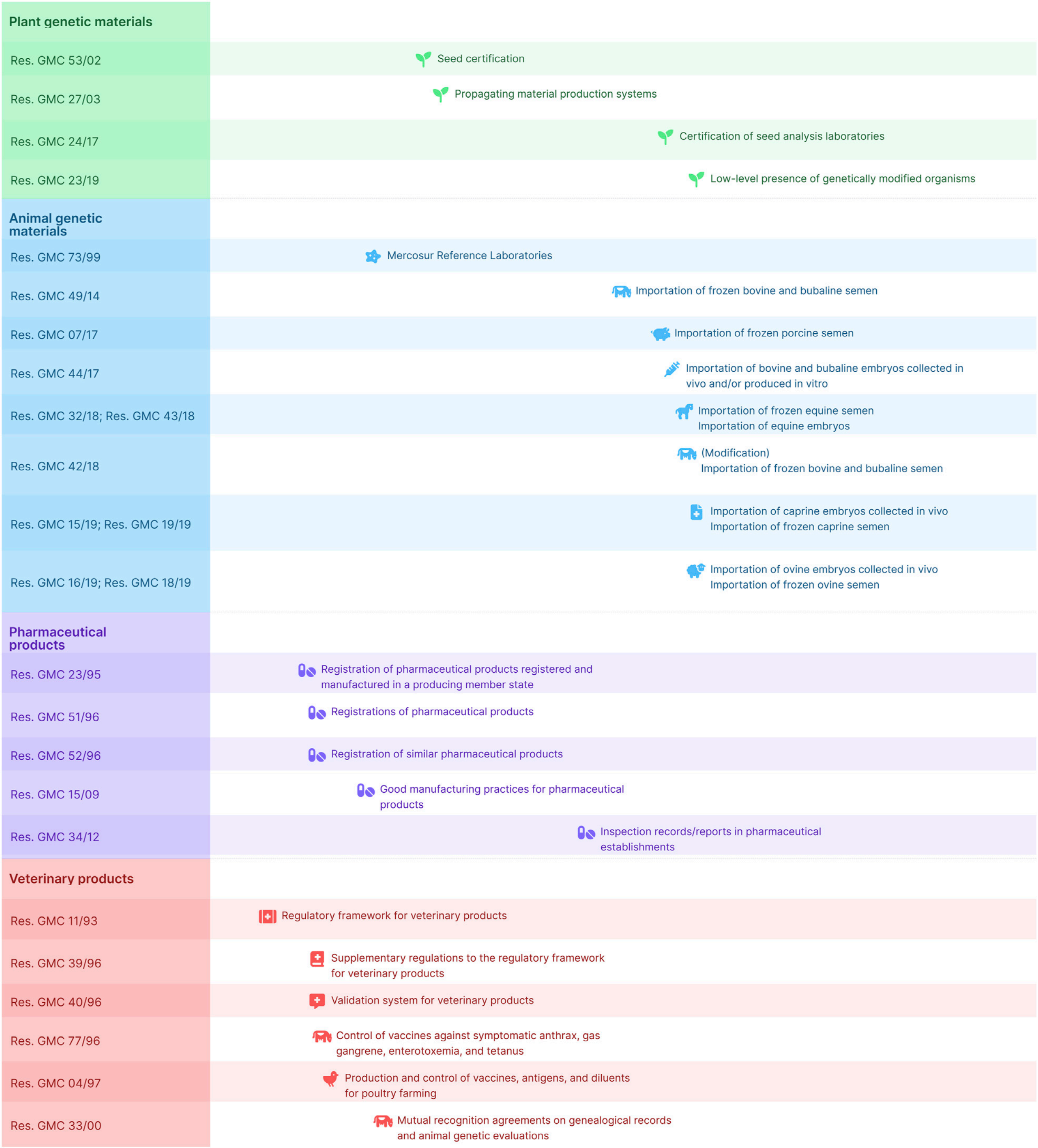
3 Crop biotechnology

MAG plays a central role in authorizing regulated trials, pre-commercial releases, commercial releases, and other proposed uses of genetically modified (GM) crops based on the opinion issued by CONBIO (Figure 2).

Submissions for approval of both regulated field trials and commercial release of plant GM events are received by MAG, and derived for analysis to CONBIO. MAG Resolution No. 27/2015 (MAG, 2015) contains both Form 1, for submission of regulated field trial applications; and Form 2, for submission of commercial release applications for GM crops (Fernandez Rios et al., 2018). Different treatments are established for events submitted for approval, based on their characteristics (Benitez Candia et al., 2020; Fernandez Rios et al., 2024).

In the case of the differentiated procedure established for events previously approved by sound and experienced regulatory systems (MAG, 2019a; MAG, 2019b), it is worth noting that Paraguay has not established a formal definition of an "experienced" regulatory system. However, the country recognizes that such a system employs scientifically sound methodologies for risk assessments to evaluate the safety of GMOs as food and feed and to assess their potential environmental impact. These systems are typically accompanied by several key features (McHughen, 2016), such as regulations that are commensurate with the level of risk and are based on a comparative risk analysis; the development of a rational risk hypothesis; the requirement of scientifically valid data of sufficient quality and quantity to inform on relative safety; and a clear distinction between scientific, political, ethical, and economic considerations.

The agri-food regulatory system is structured around a problem formulation approach, where protection goals are identified and plausible risk hypotheses are formulated. These hypotheses are tested with robust testing methodology. Such tests are conducted either in the laboratory or in confined field trials and follow a tiered-testing approach. For example, for non-target organisms (NTOs) the process starts with highly conservative early-tier tests in the laboratory and progresses to more complex, higher-tier experiments when necessary. Risk assessors must select data that can evaluate potential effects by translating policies and protection goals into risk assessment operational goals (Garcia-Alonso and Raybould, 2014; Wach et al., 2016). Experience has taught local regulators that most countries where GM crops have already been approved have protection goals that apply to a common set of valued ecological functions. One of the key elements that enabled Paraguay to develop a differentiated procedure for GM crops already approved in third countries was the utility of early tier tests using surrogate species for predicting field effects (Wach et al., 2016). The choice of appropriate surrogates for the assessment of NTOs is influenced by scientific factors, such as understanding the mode of action and spectrum of activity, and protection goals based on ecosystem services, including biological control, pollination, and decomposition of organic matter. Furthermore, the Paraguay agri-food regulatory system considers that the results from the use of



Plant genetic materials	
Res. GMC 53/02	Seed certification
Res. GMC 27/03	Propagating material production systems
Res. GMC 24/17	Certification of seed analysis laboratories
Res. GMC 23/19	Low-level presence of genetically modified organisms
Animal genetic materials	
Res. GMC 73/99	Mercosur Reference Laboratories
Res. GMC 49/14	Importation of frozen bovine and bubaline semen
Res. GMC 07/17	Importation of frozen porcine semen
Res. GMC 44/17	Importation of bovine and bubaline embryos collected in vivo and/or produced in vitro
Res. GMC 32/18; Res. GMC 43/18	Importation of frozen equine semen Importation of equine embryos
Res. GMC 42/18	(Modification) Importation of frozen bovine and bubaline semen
Res. GMC 15/19; Res. GMC 19/19	Importation of caprine embryos collected in vivo Importation of frozen caprine semen
Res. GMC 16/19; Res. GMC 18/19	Importation of ovine embryos collected in vivo Importation of frozen ovine semen
Pharmaceutical products	
Res. GMC 23/95	Registration of pharmaceutical products registered and manufactured in a producing member state
Res. GMC 51/96	Registrations of pharmaceutical products
Res. GMC 52/96	Registration of similar pharmaceutical products
Res. GMC 15/09	Good manufacturing practices for pharmaceutical products
Res. GMC 34/12	Inspection records/reports in pharmaceutical establishments
Veterinary products	
Res. GMC 11/93	Regulatory framework for veterinary products
Res. GMC 39/96	Supplementary regulations to the regulatory framework for veterinary products
Res. GMC 40/96	Validation system for veterinary products
Res. GMC 77/96	Control of vaccines against symptomatic anthrax, gas gangrene, enterotoxemia, and tetanus
Res. GMC 04/97	Production and control of vaccines, antigens, and diluents for poultry farming
Res. GMC 33/00	Mutual recognition agreements on genealogical records and animal genetic evaluations

FIGURE 1
Chronological synthesis of current Mercosur Resolutions. The figure depicts the timeframe and extent of resolutions adopted by the Mercosur Common Market Group concerning biotechnology, offering an overview of key regulatory achievements in the region.

surrogate species in tiered testing are transportable, as such testing can be replicated in other laboratories (Wach et al., 2016).

When Paraguay updated the regulatory framework for GM crops in 2015, it incorporated the concept of data transportability, which became a useful tool for avoiding redundant confined field trials (CFT). Through collaboration with the regulatory agency from Argentina, risk assessors learned that CFTs' site selection with a focus on the diversity of environments tested within the production zone of the crop of interest is a key element. At the same time, the appropriate methodology and agronomic management of the studies and the measured endpoints are relevant (Vesprini et al., 2020; Vesprini et al., 2022). If these conditions are met, and if through the application of problem formulation there were no risk hypotheses related to Paraguay's agro-climatic conditions, not only the data (as informative studies), but also the conclusions of

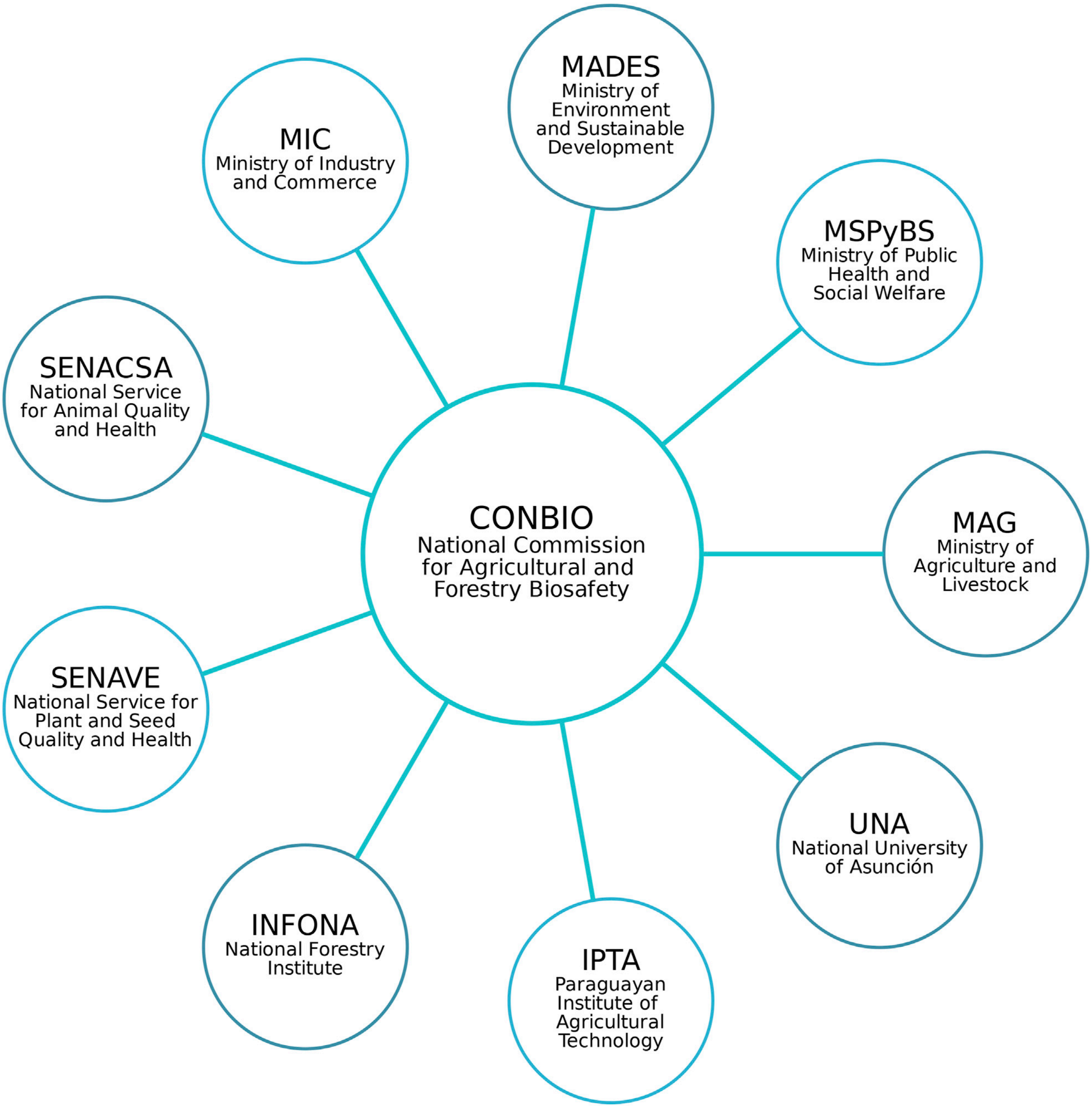


FIGURE 2
Composition of Paraguay's National Commission for Agricultural and Forestry Biosafety (Presidencia de la República del Paraguay, 2012a).

the CFT are transportable, and applicable to risk assessments for Paraguay, without the need to generate additional CFT data.

If a relevant risk hypothesis related to Paraguay's agro-climatic conditions is formulated, risk assessors scientifically assess the need (or not) for a new specific confined field trial that provides necessary unavailable data and answers that specific risk hypothesis. If a new confined field trial is required, risk assessors need to identify putative agro-climatic zones (Melnick et al., 2023) where this specific confined field trial could be conducted (e.g., where the production of that crop is common, and understanding the importance of any particular agro-climate for the production of a specific crop). This contributes to providing only relevant data that answer a specific risk hypothesis that persists even when all available data are analyzed.

Since 2014, SENAVE has maintained a registry of companies operating with regulated GMOs in the agricultural sector. To apply for registration, companies must fill Forms DBA-01 and DBA-02 (SENAVE, 2014b), whereby they declare relevant data about their legal representatives, technical advisors, and location where the companies operate. SENAVE has also approved a procedure for the risk management of activities with regulated GMOs in the agricultural sector, through Resolution SENAVE No. 283/2014 (SENAVE, 2014a) (Supplementary Table 1). Meanwhile, a Mercosur GMC Resolution has been incorporated into

Paraguay's regulatory framework which approves a mechanism to reduce the low-level presence of unapproved GMOs (Mercosur GMC, 2019; Presidencia de la República del Paraguay, 2021).

4 Genetically modified microorganisms

The regulation of genetically modified microorganisms (GMMs) in the agri-food system is governed by the same norm as crop biotechnology and subject to analysis by CONBIO, which includes conducting case-by-case risk assessments of activities involving GMMs and identifying potential environmental risks or safety concerns to human and/or animal health resulting from the use of GMMs and their byproducts (Presidencia de la República del Paraguay, 2012a). However, the differentiated mechanism for events previously assessed in third countries is not currently applicable. Paraguay has approved the use of several GM yeasts for ethanol production (OECD, 2023). Because yeast-derived products and distiller's dried grains with solubles (DDGS) can be used as animal feed, a CONBIO safety assessment is required.

5 Animal products of biotechnology

GM animals have a wide range of applications, from laboratory research to agriculture and public health. The regulation varies depending on these applications. In the context of the agri-food system, an assessment by CONBIO is necessary, and genetically modified animals are regulated according to the same standards as crop biotechnology.

Paraguay has recently granted the first commercial release of a GM insect, *Spodoptera frugiperda*, containing a self-limiting gene which allows for the production of male-only insects (MAG, 2024). Once released into the environment, these modified males will seek out and mate with wild females. The self-limiting gene will be transmitted to offspring, preventing female offspring from reaching maturity and reproducing. By continuously releasing GM males in a specific area, there will be a decrease in the number of wild females and consequently a reduction in the overall population of these insects (Reavey et al., 2022).

For applications related to public health, an evaluation by the MSPyBS may be required in some cases. However, there are currently no established guidelines outlining the assessment process in the public health context.

One particular case is that of synthetic beef (and plant-based meat substitutes). The use of the word "meat" (carne) is regulated by Law No. 6916/2022 (República del Paraguay, 2022), and is reserved for the edible muscular part of animals slaughtered and declared fit for human consumption by the official veterinary inspection, consisting of the soft tissues surrounding the skeleton, including their fat covering, tendons, vessels, nerves, aponeurosis, the skin of swine and poultry (except that of the order Struthioniformes) and all those tissues not separated during the slaughter operation. The diaphragm is also considered meat. At the time of submission of this paper, a draft bill is being studied in Congress to ban synthetic meat altogether (Franco Alfaro, 2023). These laws were promoted by the agribusiness sector as a way to combat market competition for conventional meat, claiming that by calling alternatives 'meat', competitors are fooling consumers (La Nacion, 2022). France has a similar policy against using meat-related terms for plant-based products (Carreno, 2022). However, research suggests that labeling these products as "meat" does not inherently cause confusion (Gleckel, 2020; Profeta et al., 2021; Tosun et al., 2021).

Several Mercosur GMC Resolutions have been integrated into the Paraguayan regulatory framework, focusing on animal health requirements for importing various types of animal genetic materials. This includes frozen semen and embryos from bovines, bubalines, porcines, equines, caprines, and ovines (Supplementary Table 2).

6 Biologics for human use

The registry of biologics for human use[1] is made by DINAVISA, following public health policies from MSPyBS and is regulated by the Presidential Decree No. 6611/2016 (Presidencia de la República del Paraguay, 2016b; Presidencia de la República del Paraguay, 2016a). DINAVISA's webpage makes available all forms required for the registry of biologics (DINAVISA, 2023).

Decree No. 6611/2016 categorizes requirements into two segments: general requirements (Table 3); and additional requirements (Table 4) for the registry of vaccines, hemoderivatives, innovative biologics, biosimilars, biologics for orphan diseases, and biologics obtained through recombinant DNA technology. Paraguay does not have specific regulation for biotechnological pharmaceutical products; their regulation is established within that of biologics.

The registration of all products requires preclinical and clinical trial information, and in the case of biosimilars, said studies must be done in a comparative manner. Paraguay does not require the performance of local clinical trials. The biosimilarity study seeks to rationally predict the same safety and clinical efficiency between the innovator product and the biosimilar. The process must define the quality and safety attributes necessary for comparison and must include preclinical and clinical studies. The scope of this study shall be defined based on the characteristics of the product and the differences in the production and purification mechanisms, and the results obtained may determine the need for additional studies for biosimilarity testing. The World Health Organization (WHO) has recently issued a new guideline on biosimilars (Kurki et al., 2022; WHO, 2022), thus we can expect local regulation instruments to be updated soon.

Vaccines present different situations. In the case of novel vaccines, preclinical and clinical studies are required; while for conventional and

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[1] Defined in Decree No. 6611/2016 as: "substances composed of or derived from proteins, nucleic acids, sugars or a complex combination of the above or living entities such as cells or tissues, obtained from living organisms or their tissues. They include viruses, therapeutic serums, toxins, antitoxins, vaccines, blood, blood components or derivatives, allergenic products, hormones, colony-stimulating factors, cytokines, antibodies, heparins, among others. The sources and methods of production include, but are not limited to cell culture, microorganisms, extraction from tissues or biological fluids, recombinant DNA techniques, transgenesis, hybridoma techniques, propagation of microorganisms in embryos or animals, etc. They are products used for the prevention, treatment, or *in vivo* diagnosis of certain diseases."

TABLE 3 Paraguay's general requirements for the sanitary registry of biologics for human use according to Decree No. 6611/2016 (Presidencia de la República del Paraguay, 2016a).

No.	Document	Description
1	Application forms	Approved by regent and legal representative.
2	RUE	Authenticated copy of valid unique company registration (RUE), granted by DINAVISA.
3	Free Sale Certificate	Authenticated copy of the certificate of free sale or documentation that accredits the commercialization of the drug issued by the Health Authority of the country of origin or provenance (as appropriate).
4	Manufacturer Approval	Authenticated copy of the approval of the manufacturer of the active ingredient, of the final product, and of the conditioner, issued by the competent health authority of the country of origin, in case it is not included in the Certificate of Good Manufacturing and Control Practices or in the Good Storage and Warehousing Practices.
5	Good Practices Certification	An authenticated copy of the Certificate of Good Manufacturing and Control Practices of the manufacturer of the active ingredient, of the manufacturer of the final product and of the conditioner, and of the storage issued by the sanitary authority of the country of origin or provenance (as applicable).
6	Production Description	A copy of the production process of the active ingredient up to the obtaining the final product with the description of said process and the complete set of data describing the manufacturing and control process, up to the final storage.
7	Storage Practices Certificate	An authenticated copy of the certificate of Good Storage Practices of the applicant and of the company in charge of the storage of the drug, issued by DINAVISA.
8	Third-Party Relations	An authenticated copy of the statement of the relationship with third parties involved in the processes to obtain the final product, from the production process to the final product, if applicable.
9	Contractual Documents	An authenticated copy of the contract of representation, or alternatively the contract of distribution, or contract of manufacturing, as applicable.
10	Risk Management Plan	Information requirements set on Annex III of Decree No. 6611/2016.
11	Technical-Scientific Data	Preclinical and clinical studies and any other information related to the development, quality, efficacy and safety of the product. In the case of biosimilar products, comparability studies with the innovator product. Also a stability study must be performed.
12	Foreign Document Authentication	All documents of foreign origin must be duly authenticated, consularized, or apostilled and legalized, and if written in a different language, accompanied by a translation into Spanish by a translator registered in the Supreme Court of Justice. Likewise, all documents must be valid on the date of the application.

combined vaccines developed by new manufacturers, the demonstration of non-inferiority with marketed vaccines of proven efficacy and safety is required. When new vaccination schedules or new indications are proposed, they must be accompanied by the corresponding clinical trials.

Good Manufacturing and Control Practices are required in all cases. The guidelines for the certification of compliance of Good Manufacturing and Control Practices were approved through MSPyBS Resolution No. 020/2015 (MSPyBS, 2015a; MSPyBS, 2015b).

Sanitary registries are valid for 5 years, and can be renewed for similar periods. The renewal of sanitary registries for biologics necessitates compliance with the requirements specified in article 6 of the Decree, ensuring that there have been no changes in the production process from the active ingredient to the final product, modifications of the therapeutic indications, change of manufacturer, and other changes that DINAVISA considers essential to maintain the quality, safety, efficacy and immunogenicity of the biologic[2]. If there have been changes in the production process from the active ingredient to the final product, modifications of the therapeutic indications, change of manufacturer and other changes that DINAVISA considers essential to maintain the quality, safety, efficacy and immunogenicity of the biologic, a new application for sanitary registry must be submitted.

Regarding pharmaceutical products in Mercosur, several important resolutions have been incorporated into the Paraguayan regulatory framework. These include standards for registering pharmaceutical products across member states (Mercosur GMC, 1995; Presidencia de la República del Paraguay, 1997), criteria for company registration (Mercosur GMC, 1996c; Presidencia de la República del Paraguay, 1997), and detailed requirements for documentation and information necessary for registration (Mercosur GMC, 1996d; Presidencia de la República del Paraguay, 1997). In addition, standards for good manufacturing practices have been established to ensure product quality and safety (Mercosur GMC, 2009; Presidencia de la República del Paraguay, 2012b).

7 Biologics for veterinary use

Paraguay's regulation of veterinary products, supervised by SENACSA, is characterized by a multilayered approach aimed at ensuring the safety and efficacy of veterinary biologics. The foundational legal framework, established by Law No. 667/1995 (República del Paraguay, 1995) and amended by Law No. 2426/2004 (República del Paraguay, 2004a), mandates the registration of all veterinary products and entities dealing with such products.

Meanwhile, Decree No. 6991/2017 (Presidencia de la República del Paraguay, 2017) entrusts SENACSA with exclusive jurisdiction over the authorization, operation, and supervision of entities dealing

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[2] Except for products specified in article 3, paragraph b.1 of the Decree, which must follow requirements established in article 12, specified in the section for biologics obtained through recombinant DNA technology (Table 4).

TABLE 4 Paraguay's specific additional requirements for the registry of vaccines, hemoderivatives, innovative biologics, biosimilars, biologics for orphan diseases, and biologics obtained through recombinant DNA technology.

Type of biologic	Specific requirements	Regulated by
Vaccines[a]	• Information about the active principle and the final product (requisites further explained in the regulation). • Nonclinical study reports, which for *novel vaccines* encompass information on pharmacology, pharmacokinetics, toxicology (requisites further explained in the regulation), and an assessment of the possible shedding of the microorganism (for attenuated vaccines); while for *conventional vaccines*, they include bibliographic information supporting the pharmacodynamic or safety information for the formulation subject of the application for registration. • Clinical study reports (Phases I, II and III). The commercial release of each batch of imported vaccine must be requested separately through the application form approved by Resolution DINAVISA No. 64/2022.	Annex I of Decree No. 6611/2016 (Presidencia de la República del Paraguay, 2016b)
Hemoderivatives	• Applicants must submit to DINAVISA a "Plasma Master File": documentation separate from the marketing authorization dossier containing detailed information on the characteristics of all human plasma used as starting/raw material for manufacturing subfractions, fractions, excipient components, and active ingredients. Each human plasma fractionation/processing center or establishment is responsible for preparing and keeping this information up-to-date within their Plasma Master File. The applicant or holder of the registry assumes responsibility for the drug. • The Plasma Master File must: include information such as the origin, quality, and safety of the plasma; outline the system of collaboration between manufacturers or processing entities and collection/testing centers; specify the conditions of their interaction and specifications agreed among them, and list the medicinal products for which the file is valid. • When submitting a complete dossier for the evaluation and certification of medicinal products not yet authorized, it is required to include a Plasma Master File. Applicants must provide all available preclinical and clinical information to demonstrate the safety and efficacy of the drug under evaluation compared to the reference biologic. Alternatively, if the drug is already registered with one of the specified Regulatory Health Agencies mentioned in article 4 of Decree No. 6611/2016, this information can also be provided. The Plasma Master File will undergo a scientific and technical evaluation by DINAVISA. A positive evaluation will result in the issuance of a certificate of compliance for the Plasma Master File along with an accompanying report. The file must be updated and recertified annually.	Annex I of Decree No. 6611/2016 (Presidencia de la República del Paraguay, 2016b)
Innovative biologics	• Quality studies: comprehensive information regarding the physicochemical, biological and immunological properties of both the active ingredient and the final product. • Efficacy, safety and immunogenicity studies: specific criteria for demonstrating effectiveness, safety and immune response will vary depending on the type of biological drug and will be determined on a case-by-case basis as specified in the annexes of the Decree following international guidelines set by WHO or ICH. The reports required include preclinical pharmacokinetics, pharmacodynamics, toxicity, immunogenicity, and interaction studies. For clinical evaluation, the reports must cover phase I trials, phase II trials, phase III studies, phase IV studies (if any), immunogenicity studies, and interaction studies.	Decree No. 6611/2016, article 7 (Presidencia de la República del Paraguay, 2016a)
Biosimilar drugs	• These drugs must demonstrate the biosimilarity of the biological drug in terms of quality, through complete physicochemical and biological characterization by side-by-side comparison with the reference drug. • Preclinical and clinical studies needed to demonstrate biosimilarity regarding efficacy, safety and immunogenicity will follow specific requirements determined on a case-by-case basis, following WHO or ICH international guidelines. These aspects must align with guidelines for innovative biologics, laid out in article 7, paragraph B of Decree No. 6611/2016.	Decree No. 6611/2016, article 8 (Presidencia de la República del Paraguay, 2016a)

(Continued on following page)

TABLE 4 (*Continued*) Paraguay's specific additional requirements for the registry of vaccines, hemoderivatives, innovative biologics, biosimilars, biologics for orphan diseases, and biologics obtained through recombinant DNA technology.

Type of biologic	Specific requirements	Regulated by
	• The preclinical and clinical studies for the comparability exercise must be conducted with the same product applying for registration, in the same pharmaceutical form, concentration, dosage and route of administration as the reference drug; and must be multicenter, randomized, with a statistically significant number of patients as defined in the approved protocol, and in the same indications approved for the reference drug; conducted in centers authorized by the competent Health Authority where the studies are conducted. • The reference drug shall be the innovator drug, except for proteins obtained through first-generation recombinant DNA technology (specified in article 3, paragraph b.1 of Decree No. 6611/2016) and pegylated proteins. • The characterization of the product to be registered must be performed with appropriate techniques for the determination of physicochemical properties, biological activity, immunochemical properties and impurities. These criteria must be considered as key elements when planning the comparability exercise, taking into account the complexity of the molecular entity involved; and depending on the physicochemical properties of the molecule, the battery of tests must be extended. • Comparative preclinical and clinical studies must be performed. The extent of this study will depend on the difference obtained with the innovator during the analysis. The requirements for conducting preclinical and clinical studies, their depth, and breadth will be determined by the nature and structural complexity of the active substance, information on the clinical behavior of the biological drug (including immunogenicity), and impurities profile. • The biosimilar drug must be registered or approved with one of the Regulatory Agencies of the countries referred to in article 4 of Decree No. 6611/2016, paragraphs a, c, and d.	
	• Biologics obtained through manufacturing processes clearly different from the ones used for the reference drug will be evaluated on a case-by-case basis according to the nature of the drug.	Decree No. 6611/2016, article 9 (Presidencia de la República del Paraguay, 2016a)
	For monoclonal antibodies, in addition to the requirements previously mentioned for biosimilars, detailed information must be presented on: • Starting materials (cell lines). • Production. • Active ingredient. • Preclinical and clinical trials to an extent consistent with the nature of the product and therapeutic indication, and evidence of similarity found during the comparability exercise in the characterization phases of physicochemical properties, biological activity and impurities. Depending on the information provided, it will be determined whether additional preclinical, clinical, and quality information is required. The extrapolation of indications will be evaluated case by case.	Decree No. 6611/2016, article 10 (Presidencia de la República del Paraguay, 2016a)
Biologics for orphan diseases	• Application for importation, signed by the treating physician, stating the patient's name, specifying the orphan disease and the quantity of medicines to be imported, with the period of time in which they will be used. • Original prescription and its copy, stating the patient's name, name of the drug and active ingredients, total amount of the drug for a treatment that cannot exceed 60 days. • Informed consent signed by the patient and the treating physician. • Medical diagnosis and summary of medical history. • All the aforementioned documents must be signed and stamped by the treating physician and they will have the nature of a sworn statement, the treating physician being exclusively responsible for the use of the biological drug on the patient. • Copy of the patient's identification document. • Copy of the treating physician's identification document and current professional registration.	Annex VI of Decree No. 6611/2016 (Presidencia de la República del Paraguay, 2016b)

(Continued on following page)

TABLE 4 (*Continued*) Paraguay's specific additional requirements for the registry of vaccines, hemoderivatives, innovative biologics, biosimilars, biologics for orphan diseases, and biologics obtained through recombinant DNA technology.

Type of biologic	Specific requirements	Regulated by
	• Copy of the prospectus or quali-quantitative formula of the active ingredients of the product to be imported or reliable scientific documentation that supports the use of the medicinal specialty. • Copy of the remittance or invoice of origin, where the batch and expiration date of the biological medicine is stated once it has entered the country.	
Biologics obtained through recombinant DNA technology	• Information regarding the quality of the drug.	Scope defined: Decree No. 6611/2016, article 3, paragraph b.1 (Presidencia de la República del Paraguay, 2016a)
	• Certificate of free sale.	Specific requirements: Decree No. 6611/2016, article 12 (Presidencia de la República del Paraguay, 2016a)

[a]Conventional vaccines are those that already meet the requirements of the WHO, or monographs in international pharmacopoeias, or are part of the immunization programs included in internationally recommended schedules; while novel vaccines are those for which there is no history of safety and efficacy, either because there is no known vaccine against the microorganism to be prevented, or because they contain a new combination of antigens, a new pharmaceutical form, a new route of administration, new adjuvants, or new preservatives (Presidencia de la República del Paraguay, 2016a).

with veterinary products. The registration of all activities related to veterinary products is managed through SENACSA's SIGOR (Regional Office Management Information System) online platform (SENACSA, 2021b; SENACSA, 2021c).

Resolution SENACSA No. 2803/2011 (SENACSA, 2011) outlines the criteria for the registration and licensing of importers. In addition, Resolution SENACSA No. 199/2012 (SENACSA, 2012a; SENACSA, 2018a) sets operational standards for vaccine-vending houses and distribution centers, while Resolution SENACSA No. 785/2012 (SENACSA, 2012b) sets comprehensive guidelines covering a wide array of operational aspects of laboratories including personnel management, facility layout, and quality control processes.

Biosafety information requirements for the registration of veterinary biologics include biological and chemical composition, specifications and methods of control for components of the formula and for culture media, substrates and other biological materials used, methodology of product manufacturing, method of control of the finished product, and evidence of safety and efficacy (literature review and clinical trials, when applicable) (SENACSA, 2020; SENACSA, 2021b).

The registration process for subunit immunogens produced via biotechnological processes mandates comprehensive control of the components and final product, ensuring their safety, quality, and efficacy. This encompasses chemical and biological characterization, manufacturing processes, and quality control protocols. The registration process focuses specifically on managing biological risks, emphasizing the prevention of public, animal, and environmental health hazards during production (SENACSA, 2021b; SENACSA, 2021a).

Disease control is another critical aspect addressed. Resolutions SENACSA No. 687/2017, No. 1641/2017, and No. 124/2018 focus on foot-and-mouth disease vaccine production standards, while Resolution SENACSA No. 690/2017 outlines standards for *Brucella abortus* vaccine production. These standards include infrastructure requirements for the manufacturers, dosage and specific strains to be used in production, and quality control (SENACSA, 2017b; SENACSA, 2017c; SENACSA, 2017a; SENACSA, 2018b).

Mercosur GMC Resolutions scaffold the harmonization of regional regulatory frameworks for veterinary products. In particular, vaccines against diseases such as symptomatic anthrax and gas gangrene are addressed, along with vaccine production in poultry. The adoption of Mercosur resolutions into Paraguay's regulatory framework indicates an effort to align veterinary health standards and promote a unified strategy for animal healthcare (Table 5).

8 Intellectual property of products of biotechnology

Patents are regulated in Paraguay through Law No. 1630/2000 (República del Paraguay, 2000), which establishes the requirements for the obtention of a patent, types of patent, matters excluded from patent protection, duration of the patent, and other relevant regulations. In particular, article 5 states that plants and animals (except microorganisms), and processes that are essentially biological for the production of plants or animals, are excluded from patent protection.

In addition, article 16 determines that during the application for a patent, when the invention refers to a product or procedure related to some biological material that is not available to the public and cannot be described in such a way that the invention can be implemented by a person skilled in the matter, the description shall be complemented by the deposit of said material in a deposit institution recognized by the General Directorate of Industrial Property. Such deposit shall not be required if it has already been made in any state member of the World Trade Organization or if the examination of novelty has already been carried out by the authority of any such country. The executing body of intellectual property policy is the National Directorate of Intellectual Property (República del Paraguay, 2012).

In compliance with article 27 section 3.b of Law No. 444/1994 (incorporation of TRIPS agreement) (República del Paraguay, 1994b), Law No. 385/1994 (República del Paraguay, 1994c) establishes several instruments for the protection of plant varieties, which apply to GM crops. Its implementing authority is SENAVE since the passing of Law No. 2459/2004 (República del Paraguay, 2004b). Further specifications on the use of these instruments can be found in Decree No. 7797/2000 (Presidencia de la República del Paraguay, 2000).

TABLE 5 Resolutions dealing with veterinary products approved by the Mercosur Common Market Group and incorporated into the Paraguayan regulatory framework.

Resolution GMC no.	Title	Incorporated to paraguayan regulatory framework through
11/93 (Mercosur GMC, 1993)	Regulatory framework for veterinary products	Presidential Decree No. 891/98 (Presidencia de la República del Paraguay, 1998b)
39/96 (Mercosur GMC, 1996a)	Supplementary regulations to the regulatory framework for veterinary products	Presidential Decree No. 891/98 (Presidencia de la República del Paraguay, 1998b)
40/96 (Mercosur GMC, 1996b)	Regulation of the validation system for veterinary products	Presidential Decree No. 891/98 (Presidencia de la República del Paraguay, 1998b)
77/96 (Mercosur GMC, 1996e)	Technical regulation for control of vaccines against symptomatic anthrax, gas gangrene, enterotoxemia, and tetanus	Presidential Decree No. 891/98 (Presidencia de la República del Paraguay, 1998b)
4/97 (Mercosur GMC, 1997)	Technical regulations for the production and control of vaccines, antigens, and diluents for poultry farming	Presidential Decree No. 891/98 (Presidencia de la República del Paraguay, 1998b)
33/00 (Mercosur GMC, 2000)	Mutual recognition agreements on genealogical records and animal genetic evaluations	Incorporation instrument unavailable/not found

9 Difficulties with data collection

One of the primary difficulties in Paraguay's regulatory framework for biotechnology is the absence of a centralized database of regulations. Currently, these regulations are scattered across various platforms, which often leads to accessibility issues, and some are entirely unavailable online, meaning interested parties must make a written request of a physical copy at the respective government office, resulting in extended waiting periods.

This decentralization impedes the ability to monitor the development of regulations, thereby complicating the process of determining whether a norm is currently in effect, or has been repealed, replaced, or modified. Paraguay has a unified online portal of public information (Portal Paraguay - Acceso a la Información Pública, 2023) where such requests can also be made, but they are not always answered in a timely manner. Additionally, the absence of official signatures or letterheads on digitally available documents necessitates additional verification steps, which further diminishes the efficiency of the system. We gathered the regulations analyzed for this work and deposited them in a repository to ensure their availability to our readers (in Spanish) (Benitez Candia et al., 2024).

Mercosur GMC Resolutions present a specific challenge. We were unable to find a local incorporation instrument for Resolution GMC No. 33/00 (Table 5), which results in uncertainty about its domestic status.

10 Considerations on the situation of the regulatory framework for biotechnology in Paraguay

The biotechnology framework in Paraguay is closely aligned with Mercosur, yet the regulatory agencies of the member states exhibit distinct characteristics that may result in variations in their approaches (Magnuson et al., 2013; Mukherjee et al., 2022). Cooperation within the region is primarily sustained by the exchange of information and a certain level of harmonization of legal and regulatory requirements. However, effective harmonization necessitates the acceptance of common values and objectives, shared interests and challenges, mutual economic and other advantages, avoidance of disputes, collaboration on other concerns, and streamlining of procedures (McLean et al., 2002). Unfortunately, achieving this level of harmonization is a daunting task.

Moreover, regulatory systems have often been implemented on a "piece-by-piece" basis (McLean et al., 2002) in response to the urgent needs of the moment, and are more reactive than preventive systems. An inventory and evaluation of priorities, policies, existing regulatory regimes, and scientific and technical means is ideally a prerequisite to the development and implementation of policies and regulations (McLean et al., 2002; Schoemaker et al., 2020). However, building such a system and making it operational is complicated by the fact that there is no single best approach nor standard that reflects cultural, political, financial, and scientific heterogeneity. When establishing a regulatory framework, considerable attention must be paid to factors such as regulatory triggers, transparency, public involvement in policy-making and regulatory decision-making processes, and proportionate methods for assessing and managing risk.

While Paraguay has made efforts to improve its regulatory framework, the triggers for regulatory review are not adequately defined in several current norms. The country has recently implemented science-based approaches to assessing and managing risks in relation to GMOs. However, there is a deficiency in terms of public consultations and participation. Current processes for public engagement are lacking, resulting in a disconnect between regulatory bodies and the broader community. Establishing an effective public consultation process would not only enhance regulatory decision-making, but also promote a more transparent approach to biotechnology governance. It is essential to involve stakeholders and the public in discussions about biotechnology to ensure informed policymaking and foster trust.

Several regulations have proven particularly challenging in their interpretation. An example is article 10 of Law No. 3283/2007 (República del Paraguay, 2007b), which addresses the validation of evaluations for sanitary registration from specific countries. The wording of the law presents a challenge to understanding whether the procedure involves automatic acceptance from third-country assessments, and the extent of this provision. At the very least, interpretations are manifold. In addition, articles 4 and 5 of Decree No. 6611/2016 determine that for biologics with a sanitary registry from the regulatory agencies specified in the aforementioned law, therapeutic indications "can be recognized and expanded", which makes interpretation of both regulations together even more challenging.

These difficulties have significant ramifications for a range of stakeholders, including researchers, industry experts, and policymakers; and can impede the development, authorization, and commercialization of biotechnology products, consequently affecting scientific progress. At minimum, the process and criteria for risk assessment and risk management must be widely published to instill trust in the system as credible and predictable among developers, stakeholders, and the public (Crow et al., 2016; Wolt and Wolf, 2018).

We suggest the evaluation of current scientific and technical capacity in Paraguay in order to facilitate the development of a more fit-for-purpose system. A sound regulatory system necessitates continuous updates on the latest scientific advancements; without such updates, the regulator's knowledge base will have a limited lifespan. We hope that this initial assessment of current legislation will be the starting point for determining and implementing appropriate, scientifically sound regulations.

Author contributions

NB: Writing–review and editing, Writing–original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal Analysis, Data curation. MU: Writing–original draft, Resources, Investigation. PS: Writing–review and editing, Validation, Resources, Investigation, Formal Analysis. EN: Writing–review and editing, Validation, Resources, Investigation, Formal Analysis. AA: Writing–review and editing, Writing–original draft, Validation, Supervision, Resources, Project administration, Formal Analysis, Conceptualization. DF: Writing–review and editing, Writing–original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal Analysis, Data curation, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2024.1373473/full#supplementary-material

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Applying knowledge and experience from potato (*Solanum tuberosum*) to update genetic stability data requirements in the risk assessment for vegetatively propagated biotech crops

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Regulatory agencies require data on genetic stability as part of the safety assessment for biotech crops, even though the genetic stability of a plant is not necessarily an environmental, human or animal health safety concern. While sexual reproduction has the potential to introduce genomic variation in conventionally bred and biotech crops, vegetative propagation is genetically stable. In vegetatively propagated crops, meiosis does not occur thus limiting the number of homologous recombination events that could lead to chromosomal rearrangements in progeny plants. Genetic stability data is often, but should not be, an automatic requirement for the safety assessment of vegetatively propagated biotech crops. Genetic stability data from biotech potato events has demonstrated that vegetative propagation of potato tubers does not affect the stability of introduced DNA sequences or lead to loss of trait efficacy. The knowledge and experience gained from over 30 years of assessing the safety of biotech crops can be used by regulatory authorities to eliminate data requirements that do not address environmental, food or feed safety concerns. As a first step, regulators should consider removing requirements for genetic stability as part of the safety review for vegetatively propagated biotech crops.

KEYWORDS

genetic stability, vegetative propagation, regulation, risk assessment, potato

1 Introduction

Plants naturally evolve, and genetic heterozygosity in plants is due to mutations, transposable elements, homologous recombination, gene silencing, and even whole genome duplication resulting in polyploidy (Flint-Garcia, 2013; Soltis and Soltis, 2021). The ability of plants to evolve has enabled the improvement of domesticated crops using conventional breeding techniques (i.e., crossing between sexually compatible species) (Flint-Garcia, 2013). Biotechnology has been used for almost 30 years to improve crops through the introduction of new genes (i.e., transgenes), and more recently crops are being improved using gene editing (e.g., CRISPR/Cas9, etc.) (Chen et al., 2019). Unlike conventionally bred crops, plants developed using genetic modification have been required to undergo risk assessments prior to environmental release or commercialization for food and feed use (Brune et al., 2021).

Risk assessment is used to evaluate the impact of biotech crops on the environment, as well as human and animal safety when these products are used for food or feed (Waters et al., 2021). Risk assessments utilize established problem formulation criteria to identify and evaluate the likelihood of potential risks based on hypotheses of hazard and exposure (Devos et al., 2019). Rather than using the risk assessment process as a catch-all to characterize every and all possible adverse effect, risk assessment can apply experience and knowledge gained from breeding and 30 years of development, regulatory review, and use of biotech crops to become more efficient without undermining risk management decisions (Anderson et al., 2021; Brune et al., 2021). Improvements to the risk review process will benefit the entire agricultural industry by lowering costs associated with product development and promoting technology adoption by farmers, food processors, and even consumers, without impacting on the safety of new crops. Our experience has identified genetic stability as an area where risk analysis could be more efficient, primarily when applied to vegetatively propagated plants.

As part of the risk assessment for biotech crops, regulatory agencies require an extensive characterization of the product, including molecular details of inserted DNA and its location in the genome (EFSA, 2011). In addition, regulatory agencies also require an evaluation of genetic stability to ensure that introduced traits are stably inherited in progeny plants. The regulatory requirement to include genetic stability data in the risk assessment review for biotech crops comes from the CODEX Alimentarius guideline for foods derived from modern biotechnology, which states that molecular characterization of inserts in genetically modified plants should demonstrate that "…all expressed traits are expressed and inherited in a manner that is stable through several generations" (FAO/WHO, 2009). As most national food safety agencies align with CODEX, the requirement for genetic stability data is widely adopted for biosafety reviews of new biotech food crops.

Genetic stability as stated in CODEX includes 1) expression of the trait, and 2) heritability of the trait. In this review, we focus primarily on heritability of the trait as an indication of stable transformation of introduced DNA. Whether defined as expression or heritability, genetic stability does not inform on environmental impact or food/feed safety of a product (Anderson et al., 2021; Brune et al., 2021). Evaluation of genetic stability is a quality control measure for developers to ensure that commercialized products have the traits they claim.

Here we document over 20 years' experience working with transgenic potato varieties to further substantiate that vegetative propagation of potato plants is genetically stable. Applying the experience and knowledge gained from this work would improve the efficiency of the regulatory review process. We recommend that automatic requirements for genetic stability data be removed from the risk assessment for vegetatively propagated biotech crops.

2 Vegetatively propagated crops are genetically stable

Conventional breeding practices that rely on sexual reproduction contribute to the maintenance of genetic heterozygosity within crop populations where meiosis and gamete fertilization have the potential to alter the chromosomal makeup of the cell during segregation and recombination. However, even for row crops developed using biotechnology and propagated by seed, newly inserted genes have been shown to be inherited in a stable and consistent manner similar to endogenous genes, across multiple generations (Privalle et al., 2020).

Crops such as banana, citrus, cassava, potato, and strawberry are vegetatively propagated for commercial production in order to fix desirable genotypes within cultivated varieties. Vegetative propagation circumvents challenges in the breeding process, such as self-incompatibility and inbreeding depression that have the potential to cause the loss of desirable traits (McKey et al., 2010). Vegetative propagation is considered an advantage for food production where desirable characteristics are maintained by avoiding meiosis, segregation, and homologous recombination that would introduce genetic variation in progeny plants. Vegetative propagation thus conserves the quality of planting material through multiple years of propagation (McKey et al., 2010). Examples include, the Russet Burbank potato variety, which is widely grown in the United States and has been continuously propagated for over one hundred years while maintaining genetic integrity and trait quality (Brown, 2015); and citrus trees, which have been vegetatively propagated, to maintain desirable traits, as clones or apomictic seed for several hundred years (Wu et al., 2018). For vegetatively propagated crops, detectable polymorphisms or epigenetic changes resulting in unwanted traits are eliminated from commercial production fields in order to maintain integrity of desirable genotypes (McKey et al., 2010).

Applying genetic stability data requirements [i.e., "inherited in a manner that is stable through several generations" (FAO/WHO, 2009)] to the safety assessment of vegetatively propagated crops raises questions and presents challenges for data collection and interpretation. For example, how does one define "inheritance" or "generation" in vegetative propagation? Any attempt to delineate a generation in a vegetatively propagated crop leads to an arbitrary classification. For example, tubers, which are the vegetative propagule of potatoes, are given a field year designation such as field year 1 (FY1). Designations are not universal and vary by geography, but are used to track vegetative propagations and are different from seed crop generations, which are the result of crosses between parent plants or self-pollination. Prior to field release, disease-free potato plantlets from tissue culture are used to produce small tubers (mini-tubers) that are designated as FY0 (Figure 1). The FY0 tubers are planted in the field and the resulting plants and tubers are designated FY1. FY1 to FY3 tubers are used primarily for commercial tuber propagation, while FY4 to FY6 tubers are sold to potato farmers for commercial crop production (Bohl and Johnson, 2010). The potato propagation pipeline is constantly replenished from tissue-culture, disease-free mother plants (Bohl and Johnson, 2010).

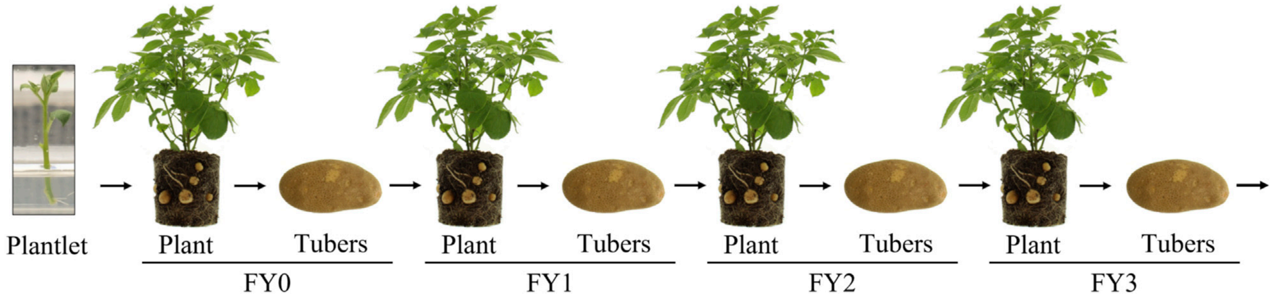

FIGURE 1
Vegetative propagation of potatoes. Tissue culture plantlets are transferred to soil or grown in hydroponic systems to produce mini-tubers, designated FY0. FY0 tubers are planted to produce FY1 plants and tubers. FY1 tubers are planted to produce FY2 plants and tubers, and so on. FY1, FY2, and FY3 tubers are typically replanted for tuber seed production. FY4, FY5 and FY6 tubers are typically sold commercially. Genetic stability data from 16 biotech potato events were collected on tubers from the vegetative propagation designated FY2.

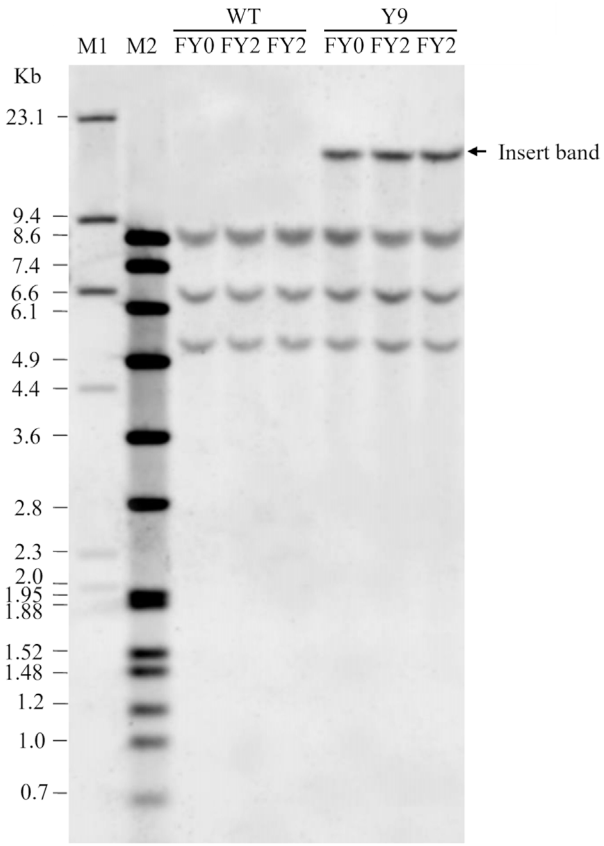

FIGURE 2
Genetic stability data. Figure adapted from (16). Southern blot result showing the stability of the inserted DNA in the Y9 potato event following three vegetative propagations. FY0 is propagation 1, FY1 (not shown) is propagation 2, and FY2 is propagation 3. The insert band is visible in all Y9 event samples demonstrating the genetic stability of vegetative propagation. The WT sample is a negative control and does not have the insert band. M1 and M2 are DNA molecular weight markers. Kb is size in kilobases.

Published results showing stability in transgenic, vegetatively propagated crops include only a limited number of examples [i.e., sugarcane (Caffall et al., 2017; Yao et al., 2017), apple (Borejsza-Wysocka et al., 2010), pear (Lebedev, 2019), and apomictic rice (Liu et al., 2023)]. The scarcity of published results showing genetic stability in vegetatively propagated crops is presumably because researchers consider these crops genetically stable.

To address the requirements of various global regulatory agencies, data were collected to demonstrate the genetic stability of transgenes from sixteen potato (*Solanum tuberosum*) varieties (Clark and Collinge, 2013; Clark et al., 2014; Spence et al., 2015; Pence et al., 2016) (example shown in Figure 2). In total, the data have been reviewed by eighty, independent scientific reviewers (Table 1). These reviewers assessed the data for environmental, food, and feed safety, and all concluded that transgenes in vegetatively propagated potatoes are stable and not a safety concern. Based on these results of genetic stability in vegetatively propagated potatoes, some regulatory agencies have begun to reconsider making genetic stability data an automatic requirement for vegetatively propagated crops (Burzaco, 2019).

3 Improvements to the risk assessment of vegetatively propagated crops

Data on genetic stability do not necessarily inform on the safety of the transformed event. In almost all crops, whether conventionally bred or developed using biotechnology, if a new trait is not genetically stable the variety would not be commercialized.

Plants developed using biotechnology are not necessarily less stable than plants developed through conventional breeding, or even than wild relatives (Privalle et al., 2020). While molecular characterization of the inserted DNA is important for food safety assessments, genetic stability data should only be required when there is an identified pathway to harm. One possible pathway to harm that requires knowledge of genetic stability data is a loss-of-function trait that if unstable may reintroduce a health risk—for example, if genetic instability were observed in the silenced expression of solanidine glucosyltransferase in the high glycoalkaloid-containing "Lenape" potato variety (McCue et al., 2003). Genetic stability data for traits that pose this type of risk could be requested by regulators as a condition of approval for purposes of risk management.

TABLE 1 Genetic stability data from 16 potato events submitted for regulatory approvals.

Event	Data type	Regulatory agency[a]	Reference
E12	Southern blot	USDA, FDA, FSANZ, HC, CFIA, MHLW, MAFF, DOB, COFEPRIS, BPI, SFA	Clark and Collinge (2013)
E24	Southern blot	USDA, FDA	Clark and Collinge (2013)
F10	Southern blot	USDA, FDA, FSANZ, HC, CFIA, COFEPRIS	Clark and Collinge (2013)
F37	Southern blot	USDA, FDA	Clark and Collinge (2013)
J3	Southern blot PCR	USDA, FDA, FSANZ, HC, CFIA, COFEPRIS	Clark and Collinge (2013)
J55	Southern blot PCR	USDA, FDA, HC, CFIA	Clark and Collinge (2013)
J78	Southern blot PCR	USDA, FDA	Clark and Collinge (2013)
G11	Southern blot	USDA, FDA	Clark and Collinge (2013)
H37	Southern blot	USDA, FDA	Clark and Collinge (2013)
H50	Southern blot	USDA, FDA	Clark and Collinge (2013)
V11	Southern blot	USDA, FDA, FSANZ, HC, CFIA	Spence et al. (2015)
W3	Southern blot	USDA	Clark et al. (2014)
W8	Southern blot	USDA, FDA, EPA, FSANZ, HC, CFIA	Clark et al. (2014)
X17	Southern blot	USDA, FDA, EPA, FSANZ, HC, CFIA, MHLW, MAFF, DOB, COFEPRIS, BPI, SFA	Pence et al. (2016)
Y9	Southern blot	USDA, FDA, EPA, FSANZ, HC, CFIA, MHLW, MAFF, DOB, COFEPRIS, BPI, SFA	Pence et al. (2016)
Z6	Southern blot	EPA, FSANZ, HC, MHLW, MAFF	Z6 Genetic Stability Report (J. R. Simplot Company; unpublished)

[a]USDA, United States Department of Agriculture; FDA, United States Food and Drug Administration; EPA, United States Environmental Protection Agency; FSANZ, Food Standards Australia/New Zealand; HC, Health Canada; CFIA, Canadian Food Inspection Agency; MHLW, Ministry of Health, Labour and Welfare (Japan); MAFF, Ministry of Agriculture, Forestry and Fisheries (Japan); DOB, Department of Biosafety (Malaysia); COFEPRIS, Federal Commission for Protection Against Sanitary Risks (Mexico); BPI, Bureau of Plant Industry (Philippines); SFA, Singapore Food Agency.

The International Union for the Protection of New Varieties of Plants (UPOV) maintains a system for plant variety protection with certain data requirements to show that new plant varieties are distinct, uniform, and stable (DUS) (UPOV, 2002). However, UPOV does not require stability data for potato variety registration when progeny plants are uniform. The UPOV DUS guidelines state that the level of variation within self-pollinated and vegetatively propagated varieties is relatively low (UPOV, 2002), and that when a variety has been shown to be uniform, it can also be considered stable (UPOV, 2011). Building on this understanding of uniformity and stability, the UPOV *S. tuberosum* Testing Guideline (TG/23/6) notes that when a potato variety has been shown to be uniform, it can be considered stable and no tests for stability need to be performed (UPOV, 2004). Uniformity is an integral part of the line selection process for new potato varieties.

The experience and knowledge gained working for the past 20 years with transgenic potatoes has demonstrated the stable presence of inserted DNA and consistent performance of introduced traits following vegetative propagation, as expected. The conclusion that vegetative propagation is genetically stable applies to all vegetative crops whether developed by breeding, biotechnology, or gene editing. Requiring data that does not address safety questions for regulatory approval of vegetatively propagated crops adds unnecessary burden to an already long list of data required by regulatory agencies for risk assessment review. By removing requirements for unnecessary data, such as genetic stability data for vegetatively propagated crops, the efficiency of obtaining biotech approvals can be improved and regulatory costs reduced.

4 Discussion and actionable recommendations

Updates to regulatory guidance and policies is needed as new crops are improved, new technologies developed, and experience in assessing biotech crops grows. If guidance is not kept current, data requirements can result in increased regulatory burden for both developers and regulatory agencies. As an example, the rapid adoption of new gene editing technologies has left many agencies struggling to update their regulatory policies and guidance to keep pace with the development of new traits. This results in regulatory backlogs, which delay the launch of new products and prevent access to beneficial technologies for farmers, processors, consumers, and the environment.

Regulatory requirements for genetic stability data for vegetatively propagated biotech crops are not supported by science and should not be a requirement for risk assessment unless a plausible pathway to harm is identified. After 30 years of experience evaluating biotech crops, it is appropriate for regulatory authorities to eliminate data requirements that do not address environmental, food, or feed safety concerns.

By applying knowledge gained from the review of biotech products over the past three decades, regulatory agencies can reduce the regulatory burden of future biotech products without reducing the robustness of the safety review. Agencies can make an informed decision to remove this requirement based on known genetic stability of vegetatively propagated crops. As an initial step, regulators should consider removing requirements for genetic stability for vegetatively propagated biotechnology crops as part of the safety review. Regulatory authorities are encouraged to work with CODEX Alimentarius to clarify that stability assessments recommended in CAC/GL 45-2008 are not necessary for vegetatively propagated plants.

Author contributions

MP: Investigation, Writing–original draft, Writing–review and editing. MK: Writing–original draft, Writing–review and editing. JD: Writing–review and editing. GR: Supervision, Writing–review and editing.

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Conflict of interest

Authors MP, MK, JD, and GR were employed by J. R. Simplot Company.

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Modernizing and harmonizing regulatory data requirements for genetically modified crops—perspectives from a workshop

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Genetically modified (GM) crops that have been engineered to express transgenes have been in commercial use since 1995 and are annually grown on 200 million hectares globally. These crops have provided documented benefits to food security, rural economies, and the environment, with no substantiated case of food, feed, or environmental harm attributable to cultivation or consumption. Despite this extensive history of advantages and safety, the level of regulatory scrutiny has continually increased, placing undue burdens on regulators, developers, and society, while reinforcing consumer distrust of the technology. CropLife International held a workshop at the 16th International Society of Biosafety Research (ISBR) Symposium to examine the scientific basis for modernizing global regulatory frameworks for GM crops. Participants represented a spectrum of global stakeholders, including academic researchers, GM crop developers, regulatory consultants, and regulators. Concurrently examining the considerations of food and feed safety, along with environmental safety, for GM crops, the workshop presented recommendations for a core set of data that should always be considered, and supplementary (i.e., conditional) data that would be warranted only on a case-by-case basis to address specific plausible hypotheses of harm. Then, using a case-study involving a hypothetical GM maize event expressing two familiar traits (insect protection and herbicide tolerance), participants were asked to consider these recommendations and discuss if any additional data might be warranted to support a science-based risk assessment or for regulatory decision-making. The discussions during the workshop highlighted that the set of data to address the food, feed, and environmental safety of the hypothetical GM maize, in relation to a conventional comparator, could be modernized compared to current global regulatory requirements. If these scientific approaches to modernize data packages for GM crop regulation were adopted globally, GM crops could be commercialized in a more timely manner, thereby enabling development of more diverse GM traits to benefit growers, consumers, and the environment.

KEYWORDS

genetically modified (GM), regulation, food and feed, safety assessment, environmental risk assessment (ERA), problem formulation, cultivation, data requirements

1 Introduction

Genetically modified (GM) crops that have been engineered to express transgenes have been commercially cultivated since 1995 and are annually grown on 200 million hectares globally. These crops have delivered important societal benefits, such as increased crop yields, resilience to adverse growing conditions, reduced tillage leading to improved soil health, reduction in the need for crop protection inputs, preservation of natural resources, and improved rural economies (Klümper and Qaim, 2014; Dively et al., 2018; Zilberman et al., 2018; Smyth, 2020; Ala-Kokko et al., 2021; Macall et al., 2021; Peshin et al., 2021; Brookes, 2022a; Brookes, 2022b; Brookes, 2022c). These benefits have led to rapid adoption of GM technology for agricultural production, including 80% of global cotton and 73% of global soybean. One-third of global maize production includes GM traits for herbicide tolerance, insect protection, or both (AgbioInvestor, 2023). GM traits have been introduced in other row crops such as oilseed rape, sugar beet, and alfalfa and, at a smaller scale, in specialty crops such as apples, eggplant, squash and potatoes (ISAAA, 2020). Hundreds of studies have been conducted to assess the safety of GM crops, and there have been no substantiated cases of resulting harm to people or livestock that consume GM crops or to the environment in which they are grown (European Commission, 2010; Snell et al., 2012; Van Eenennaam and Young, 2014; NASEM, 2016).

Despite this track record of safety and benefits, regulatory data requirements for approval and commercialization of GM crops have continued to grow globally. GM technology is primarily limited to major global crops, like maize and soybean, and to major input traits, such as insect protection and herbicide tolerance. While there are many efforts underway to use GM technology for other traits and to improve minor crops, especially for small holders in the developing world (David, 2009; Shelton, 2021; Woodruff, 2024), securing the regulatory approvals to enable cultivation and avoid potential trade disruptions can present often insurmountable challenges to commercialization. Only a few large multinational developers can afford the US$115 million cost and also persist for the 16 years that it currently takes, on average, to bring a new trait to the global market. More than one-third of those costs, and more than one-half of that time, are taken by the regulatory process (AgbioInvestor, 2022). These extensive and complex regulatory systems also mean that governments must invest significant resources in developing and maintaining regulatory bodies staffed with sufficient people and expertise, creating a burden on taxpayers and society. Countries that cannot afford such an investment are missing out on the benefits of GM crops.

CropLife International and its member companies that develop GM crops (BASF, Bayer Crop Science, Corteva™ Agriscience, and Syngenta) have proposed a modernized regulatory framework and streamlining of data requirements for GM crops that is based on scientific rationale and builds on the 25 years of experience with the technology, and the history of its safe use (Mathesius et al., 2020; Anderson et al., 2021; Bachman et al., 2021; Brune et al., 2021; Goodwin et al., 2021; McClain et al., 2021; Roper et al., 2021; Waters et al., 2021). The development of the proposed framework was motivated and guided by considering four key questions. 1) Are today's regulations for GM crop approvals risk-proportionate? 2) Do today's data requirements act as an unnecessary barrier to beneficial innovation? 3) How can knowledge and experience accumulated over the last 25 years inform modernization of regulations? 4) Can data requirements be streamlined and harmonized across countries and authorities? These questions were used to guide the determination of the types of data that are necessary to ensure GM crops are developed and deployed without increased risks for food and feed safety or the environment compared to conventional crops. Under this framework, core data, which are important for the problem formulation step of the risk assessment of the GM crop, were identified. The core data are used for problem formulation to identify plausible cause-and-effect hypotheses of harm from the GM crop. Depending upon the outcome of the problem formulation for a specific crop by trait combination, additional supplementary (i.e., conditional) studies may be needed, on a case-by-case basis, to analyze any plausible risk identified. Figure 1A outlines proposed core and supplemental studies for a Food and Feed Safety Assessment; Figure 1B outlines proposed core and supplemental studies for an Environmental Risk Assessment. CropLife International took an approach that is consistent with principles of risk assessment such that the proposed data requirements can fully inform decision-making by a regulatory agency, without the extraneous data present in many current regulatory submissions that does not meaningfully contribute to the risk assessment of the GM crop.

To further examine whether CropLife International's proposed modernized data requirements are sufficient for food and feed safety assessments and for environmental risk assessments, a workshop was held at the 16th International Society of Biosafety Research (ISBR) Symposium (St. Louis, USA) in 2023. Using a case study of a hypothetical GM maize event containing two familiar transgenic traits (herbicide resistance and insect protection). The workshop participants were charged with considering whether the proposed data in the case study are scientifically both necessary and sufficient to determine the food, feed and environmental safety of the hypothetical GM crop.: CropLife International member representatives that served as moderators during the workshop authored this publication to report the outcomes and summarize the discussions that took place among the participants. The participants varied in their backgrounds and prior experience with risk assessment and included individuals from regulatory

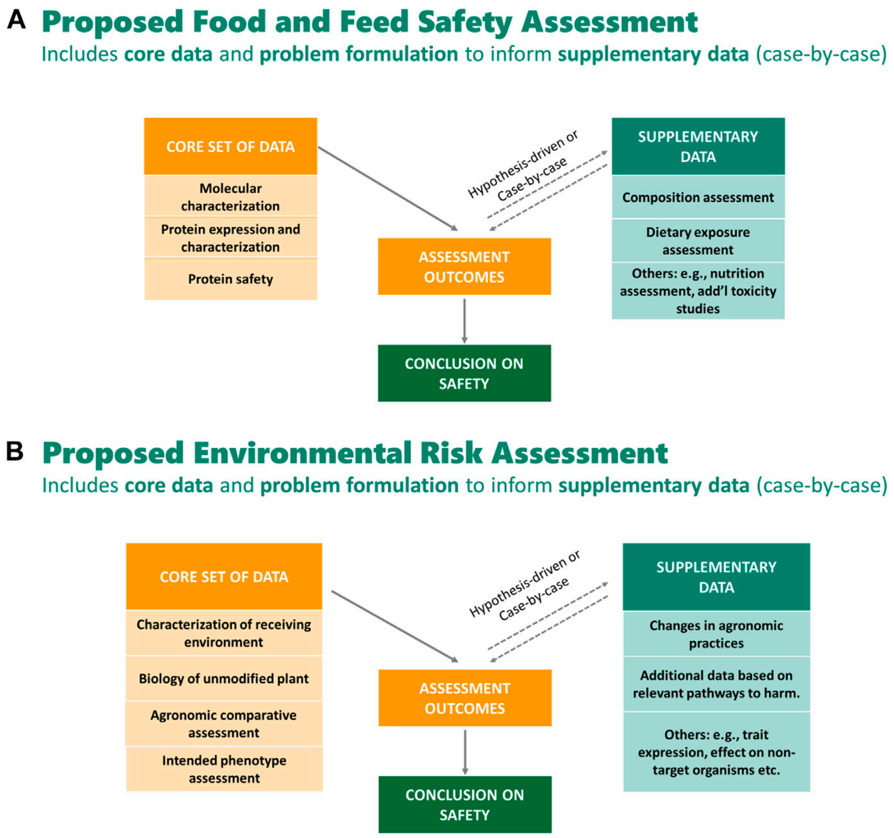

FIGURE 1
(A) proposes a set of data recommended for a science-based food and feed safety assessment for a typical GM crop and considers as core studies: basic molecular characterization, protein characterization and expression, and protein safety (i.e., history of safe use of the protein and source organism and bioinformatics to identify potential toxins and allergens). The outcomes of these core data are used to inform the problem formulation step and decide, on a case-by-case basis which, if any, supplementary studies are needed to make a conclusion on safety (Brune et al., 2021; Waters et al., 2021). **(A)** is adapted from Brune et al., 2021 and Waters et al., 2021. **(B)** proposes a set of data recommended for a science-based environmental risk assessment for a typical GM crop and considers as data: understanding the receiving environment and the basic biology of the unmodified plant; assessing the agronomic similarity of the GM crop to its conventional counterparts (i.e., agronomic comparative assessment); and understanding the intended trait of the GM plant and assessment of how the intended trait may lead to environmental harm. The core data should be used first to inform the problem formulation. If a conclusion cannot be made about the pathway to harm using the core data, additional case-by-case hypothesis-driven supplementary studies should be considered (Anderson et al., 2021).

agencies, technology developers, consultant groups, and academia. A wide range of geographical areas were represented.

2 Case study description

For the case study, a hypothetical GM maize event was presented to the workshop participants for evaluation. The hypothetical event was intentionally simple for this exercise (i.e., a familiar crop with traits that are similar to many transgenic events that have already been reviewed and approved by regulatory agencies globally, with several in commercial production for many years), which enabled the participants to analyze in greater depth the need for data that is routinely submitted but may not contribute to the safety assessment. More specifically, a maize (*Zea mays*) event containing a single insertion encoding for two proteins from a single T-DNA introduced using standard disarmed Agrobacterium tumefaciens-based transformation was described. The two hypothetical traits provide protection against lepidopteran pests and tolerance to treatment with glyphosate herbicide, using a hypothetical Cry1 protein from *Bacillus thuringiensis* (*Bt*) and a hypothetical EPSPS protein variant isolated from maize, respectively. The workshop participants were asked to separately consider a food and feed safety assessment or an environmental risk assessment for this same hypothetical GM maize event. Additional distinctions between the presentation of the case study for the different assessments are outlined below.

2.1 Food and feed safety assessment

For the Food and Feed Safety Assessment, the results from hypothetical evaluations of core data on the characterization and safety assessment of the event were provided (summarized in Table 1). Throughout this paper, the term ‘data’ refers to both the results of experiments or studies as well as information gathered from literature reviews, consensus documents and other similar sources. As described in Waters et al. (2021), the core data for a food

TABLE 1 Summary of food and feed safety assessment core data of the hypothetical GM maize.

Molecular characterization	
Number of insertion loci and inserts per locus	Insertion of one T-DNA from plasmid at a single locus. Based on sequencing of genomic DNA.
Presence or absence of unintended sequences (e.g., plasmid backbone)	Confirmed absence of backbone sequences from the transformation plasmid
Sequence of the inserted DNA and flanking borders	No changes in protein coding sequences. Small changes detected at junctions with genomic DNA.
Stability of the inserted DNA across multiple generations	Single T-DNA insertion is stably inherited over three breeding generations
Protein characterization and expression	
Identity of newly expressed proteins confirmed	EPSPS protein isolated from GM maize consistent with the theoretical molecular weight/amino acid sequence and the protein displayed expected enzyme activity
	Cry1 protein isolated from GM maize consistent with the theoretical molecular weight/amino acid sequence and the protein demonstrated expected insecticidal activity towards target insect pests
Protein expression as intended	EPSPS protein: Constitutive expression driven by ubiquitin gene promoter from *Zea mays*
	Cry1 protein: Constitutive expression driven by 35S promoter from Cauliflower mosaic virus
Protein safety	
History of safe use	EPSPS protein: History of safe use of source organism (maize) and similar EPSPS proteins
	Cry1 protein: History of safe use of source organism (*Bt*) and similar Cry1 proteins
Toxicity	Neither protein is related to any proteins of toxicological concern by bioinformatics search
Allergenicity	Neither protein is related to allergens in qualified allergen database by bioinformatics search

and feed safety assessment are: 1) molecular characterization, 2) protein characterization, and 3) protein safety (allergenicity and toxicity). The results of the molecular characterization demonstrated that there was an insertion of a single T-DNA sequence into the maize genomic DNA without any vector backbone sequences. There were no changes in the intended protein coding sequence and constitutive expression of both proteins were driven by familiar promoter elements (35S from cauliflower mosaic virus and ubiquitin promoter from *Zea mays*, respectively). Finally, the inserted DNA and the traits were indicated as being stable over three generations. The protein characterization data given to participants indicated that the molecular weight and amino acid sequence were as expected for both proteins. The function of the hypothetical Cry1 protein was established as having activity limited to target lepidopteran pest species, with no activity against other insect orders. Field tolerance to glyphosate from the hypothetical EPSPS protein variant was also as expected. The protein safety data indicated that both proteins are similar to proteins that have a history of safe use for food and feed; neither EPSPS proteins nor Cry proteins have any known toxicity or allergenicity concerns. Bioinformatics analysis comparing the amino acid sequences of both hypothetical proteins to a protein database also demonstrated that neither protein is related to any protein of toxicological concern nor related to any allergens in the qualified allergen database.

A familiar crop with familiar traits and minimal genetic disruptions was used for the workshop to promote discussion of what data is really needed to establish the food and feed safety of a GM crop event. It was also noted to workshop participants that extensive protein expression data in the plant was not obtained, nor was detailed proximate or nutrient composition data included. Further, while it was established that bioinformatics confirmed no homology to known allergens or toxins, no exposure assessments, no animal feeding studies, or other more direct assessments of potential for harm from the hypothetical event were included. As presented, the case study stated that considering 1) the assessment from the core data, 2) the familiarity of the crop and traits, and 3) the lack of direct interaction with other metabolic pathways of the plant, there was no hypothesis of food and/or feed safety risks for the new GM maize crop, and therefore additional supplementary data are not warranted to establish food and feed safety, in accordance with the approach established in Brune et al. (2021), McClain et al. (2021) and Roper et al. (2021).

2.2 Environmental risk assessment

For Environmental Risk Assessment (ERA), the intention of the case study was to model how problem formulation and core data should be leveraged to inform ERA of a GM crop for cultivation safety. Problem formulation is a process used in the ERA to develop plausible pathways to harm resulting from cultivation of the GM crop. Problem formulation first considers core data, then considers other data on a case-by-case basis if it is deemed necessary to inform the risk assessment. For ERA, core data includes information related to the receiving environment, description of basic biology of the unmodified plant, assessment of the agronomic similarity of the GM crop to its conventional counterparts, and characterization of the intended traits of the GM crop (summarized in Table 2). For the purpose of the case study, the protection goal was broadly stated as protection of biodiversity, specifically protection of beneficial or charismatic species. For the purposes of the workshop, the core characteristics of the event as described for the food and feed

TABLE 2 Summary of environmental risk assessment core data.

Characterization of the receiving environment	
Receiving environment	Agroecosystem where Zea mays (maize) will be cultivated
Presence of wild relatives	There are no wild relatives of maize present in the targeted cultivation country
Changes in agronomic practices	There are no changes to the standard agronomic practices for hypothetical GM maize, relative to nonmodified maize
Description of the biology of unmodified *Zea mays*	
Survival	Maize requires human intervention for propagation and survival (OECD, 2003)
Weediness	Maize does not have weedy characteristics. While volunteers can occur the following season, maize is frost intolerant, the seeds have limited dispersal ability and they are not dormant (OECD, 2003)
Reproduction and gene flow	Maize propagates through seed and is wind-pollinated (OECD, 2003)
Agronomic similarity of the GM crop	
Multi-location field trial	A field study was planted during the 2022 growing season at 10 sites in the United States and Canada, which were selected to represent North American growing regions for commercial maize. Standard agronomic endpoints were assessed for hypothetical GM maize and nonmodified varieties. Results from this study demonstrate that hypothetical GM maize is agronomically similar to non-modified maize
Characterization of the intended phenotype	
Protein function	Hypothetical Cry1 protein - provides protection against lepidopteran insect pests: European corn borer (ECB), Asian corn borer, southwestern corn borer (SWCB), corn earworm (CEW), and fall armyworm (FAW)
	Hypothetical EPSPS protein- Functions in the chloroplast as a step in the biosynthesis of aromatic amino acids. EPSPS catalyzes the reversible reaction of shikimate-3-phosphate and phosphoenolpyruvate to produce 5-enolpyruvylshikimate-3-phosphate and phosphate. The EPSPS enzyme also serves as a selectable marker for plant transformation
Mode of action	Hypothetical Cry1 protein - ingestion of Cry1 is followed by receptor-binding in the insect mid-gut, which results in pore formation in the mid-gut of sensitive insects. The mode of action of Cry proteins in GM crops is well-documented (OECD, 2007)
	Hypothetical EPSPS - not inhibited by glyphosate and retains the standard EPSPS enzymatic function in the presence of glyphosate
History of safe use	Hypothetical Cry1 protein - History of safe use: Multiple crops have been globally assessed and approved as products that express Cry1 proteins (ISAAA, 2023)
	Hypothetical EPSPS - Multiple crops have been globally assessed and approved as products that express glyphosate-tolerant versions of EPSPS proteins from different sources (ISAAA, 2023)

assessment were considered the same (e.g., molecular features), with additional information focused on agronomic and environmental aspects provided to guide the ERA discussion.

The participants were presented with the following set of core data (summarized in Table 2) and were asked to consider if a plausible pathway to harm could be developed related to weediness, invasiveness, gene flow to wild relatives or hazard to non-target organisms: 1) assessment of the receiving environment indicating no wild relatives of maize present in the cultivation country and no changes to the standard agronomic practices relative to non-modified maize; 2) assessment of the basic biology of maize, using consensus documents, demonstrating non-modified maize has no weediness characteristics and requires human intervention for propagation and survival; 3) multilocation field trial data demonstrating hypothetical maize was agronomically similar to non-modified maize; and 4) assessment of the intended phenotype (i.e., insect protection and herbicide tolerant traits are not intended to increase fitness or survival in the environment).

Based on the core data assessed, the case study proposed that there are no plausible hypotheses for how cultivation of the hypothetical maize could result in environmental harm related to weediness, invasiveness, and gene flow to wild relatives. Thus, additional data will not further contribute to meaningful assessment of environmental safety. However, the case study proposed that a plausible pathway to harm to non-target organisms could be developed based on the intended insect protection phenotype. The hypothetical Cry1 protein was presented as providing protection against specific lepidopteran insect pests (European corn borer, Asian corn borer, Southwestern corn borer, corn earworm, and fall armyworm).

The mode of action of Cry proteins in GM crops is well-documented (Bravo et al., 2007; OECD, 2007). In this case study, additional supplemental protein expression data and non-target organism hazard data were provided to the participants, and they were asked to consider if additional plausible pathways to harm could be developed. The set of supplemental data (summarized in Table 3) was as follows: 1) multilocation field trial data measuring the concentration of the hypothetical Cry1 protein in several plant tissues to inform exposure assessment; 2) an exposure assessment for different non-target organisms to consider the likelihood and magnitude of exposure to the hypothetical

TABLE 3 Summary of environmental risk assessment supplementary data.

Expression of the hypothetical Cry1 protein in GM maize	
Multi-location field trial	• A field study was planted during the 2022 growing season at 10 sites in the United States and Canada, which were selected to represent North American growing regions for commercial maize. Hypothetical Cry1 protein expression was analyzed from representative plants at 6 sites • Hypothetical Cry1 protein expression in GM maize was measured in several plant tissues, including pollen (R1), leaf, root, and whole plant (several vegetative and reproductive growth stages) • Results from this study: the hypothetical Cry1 protein in GM maize is below the limit of detection (LOD) of the analytical assay in pollen and root. The hypothetical Cry1 protein in GM maize was detectable in leaf and whole plant, with the highest concentration detected in R1 leaf (mean = 30 ng/mg; maximum = 45 ng/mg)
Specificity	
Specificity of the Cry1 protein	• Cry1 protein activity is well-documented to be limited to the order Lepidoptera (Van Frankenhuyzen, 2009; Anderson et al., 2021) • The hypothetical Cry1 protein provides protection against lepidopteran insect pests, including European corn borer (ECB), Asian corn borer, southwestern corn borer (SWCB), corn earworm (CEW), and fall armyworm (FAW)
Exposure assessment—non-target organisms	
Ladybird beetle	• May consume pollen, plant tissues, or prey that have previously consumed plant tissues • For the purposes of this case study, a worst-case scenario would assume a ladybird beetle consumes GM maize leaf tissue
Soil dwelling organism	• Detritivores may consume roots or plant tissues that have fallen to the ground • There is no exposure to detritivores via roots (root hypothetical Cry1 protein expression is below LOD) • For the purposes of this case study, a worst-case scenario would assume a detritivore consumes GM maize leaf tissue
Aquatic organism	Although aquatic habitats may be located near agricultural areas, exposure of aquatic organisms to biotech crops is limited temporally and spatially (Bachman et al., 2021) and aquatic exposure to Bt corn is extremely small (US-EPA, 2010)
Non-target predator	A non-target predator may consume prey that has previously consumed the hypothetical GM maize plant tissues. For the purposes of this case study, a worst-case scenario would assume there is no degradation of the hypothetical Cry1 protein in the prey; however, previously it has been shown prey contains lower concentrations of Cry protein relative to the Cry protein concentration in planta (Raybould et al., 2007)
Non-target honey bee	There is no exposure to honeybees (pollen hypothetical Cry1protein expression is below LOD). Non-target lepidopteran–non-target Lepidoptera do not consume maize pollen directly, but they may ingest maize pollen that has been deposited on host plants growing within or closely adjacent to maize fields. There is no exposure to pollen-feeding non-target lepidopterans (expression in pollen is below LOD)
Hazard assessment—non-target organisms	
Ladybird beetle	Tier I study was conducted; diet contained hypothetical Cry1 protein at approximately 10x the environmentally relevant exposure. The no observable adverse effect concentration (NOEC) was >10X the environmentally relevant exposure, resulting in a margin of exposure >10
Soil dwelling organism	Tier I study was conducted; diet contained hypothetical Cry1protein at approximately 10x the environmentally relevant exposure. The no observable adverse effect concentration (NOEC) was >10X the environmentally relevant exposure, resulting in a margin of exposure >10
Aquatic organism	Tier I study was not conducted because aquatic exposure to Bt corn is extremely small (US-EPA, 2010)
Non-target predator	Tier I study was conducted; diet contained hypothetical Cry1protein at approximately 10x the environmentally relevant exposure. The no observable adverse effect concentration (NOEC) was >10X the environmentally relevant exposure, resulting in a margin of exposure >10
Non-target honeybee	Tier I study was not conducted because there is no exposure to honeybee (expression in pollen is below LOD)
Non-target lepidopteran	Tier I study was not conducted because there is no exposure to pollen-feeding non-target lepidopterans (pollen hypothetical Cry1 protein expression is below LOD). Lepidoptera that consume maize leaf tissue or grain are considered maize pests
Fate of the hypothetical Cry1 protein in the environment	
Soil Fate	There is a large body of evidence that Bt Cry proteins do not accumulate or persist in soil (Clark et al., 2005; Stotzky, 2005; Icoz and Stotzky, 2008)

Cry1 protein; and 3) results of non-target organism Tier I hazard studies for several surrogate species representing different taxonomic orders (e.g., ladybird beetle, a soil dwelling organism, and a non-target predator) conducted with the Cry1 protein in the diet.

The multilocation field trial data showed that the Cry1 protein was only detectable (above the limit of detection) in the leaf and whole plant, with the highest concentration found in R1 leaf. The protein was below the limit of detection of the analytical assay in pollen and root. Based on the tissue expression, the exposure assessment concluded that since there is no expression of the Cry1 protein in pollen, there would be no route of exposure to non-target pollen feeding organisms (e.g., honeybee). Finally, the Tier I hazard studies indicated that no hazard was observed at concentrations that exceeded >10x the expected environmental concentration.

Usually, the assessment of adverse effects in non-target organisms follows a tiered approach that starts with laboratory studies at levels that exceed worst-case exposure conditions (Romeis et al., 2011). Tier I laboratory studies with non-target organisms are typically conducted using at least 10X the worst-case expected environmental concentration. In this case, the results of the hypothetical Tier I dietary studies indicated no hazard (i.e., adverse effects) at concentrations that exceeded 10x the worst-case expected environmental concentration, and thus a conclusion that evidence is sufficient without conducting additional hazard testing was indicated. Based on data from the exposure assessment and non-target hazard assessment studies, the case study proposed that there were no plausible pathways to harm to non-target organisms due to lack of exposure and/or lack of risk because there were no adverse effects at concentrations that exceeded 10X the worst-case expected environmental concentration. Participants were asked to consider whether they agreed with the conclusions proposed by the case study based on core data and additional supplementary data related to protein expression, non-target organism exposure, and non-target organism hazard.

Additional information such as molecular data to confirm that the insert is an intact single copy, stable across generations, and that there is no insertion of DNA from the plasmid backbone were not provided in the ERA case study. These additional data for product characterization have historically been submitted to regulators as part of cultivation applications, but they are not directly relevant to ERA (Anderson et al., 2021).

3 Learnings from breakout group discussions

After participants attended the introductory presentation session of the workshop, they were distributed into smaller discussion groups of approximately 10 people, with CropLife International member representatives serving as moderators. Each participant had the opportunity to choose either the Food and Feed Safety Assessment or the Environmental Risk Assessment, depending on their respective areas of interest.

The goal of the smaller group discussion sessions was to allow participants to go into deeper conversations about the proposed modernized paradigm for a risk assessment of a GM crop. Discussions were aided by a distribution of a printed booklet that included a description of the hypothetical GM maize event and the data collected, and that outlined the key concepts of using the core data for a Food and Feed Safety Assessment and Environmental Risk Assessment. Moderators provided some time for the participants to review the information and then introduced the case study by giving a brief overview of the information provided in each data section of the case study. Participants were encouraged to provide feedback and to bring up questions and/or comments about topics/elements of the case study that they considered not sufficiently covered by the data provided. They were also asked to complete a worksheet allowing for comments on the specific steps of the assessment process.

Discussions during this small group session were productive and highly informative. Overall, the participants were engaged, willing to discuss, and mostly supportive of the general assessment framework of primarily using core data and only using further assessments on a case-by-case basis.

A summary of key points from the breakout group discussions is shared below. This section is not intended to be a complete summary of the discussion, rather the authors have captured points of interest with an emphasis on points that are worth considering for future workshops and discussions on this topic.

3.1 Food and feed safety assessment

In the small group session, participants were asked to consider 1) the assessment from the core studies (see Table 1), 2) the familiarity of the crop and traits, and 3) the lack of direct interaction with other metabolic pathways of the plant, and then decide whether there was a hypothesis of food and/or feed safety risks for the new GM maize crop. Because of these considerations, the position for the case-study was that, for the hypothetical event, additional supplemental studies are not warranted to establish food and feed safety, and the participants discussed whether they agreed with this position.

Below are some key feedback and questions captured during the workshop regarding the proposed approach for the assessment of Food and Feed Safety of the hypothetical GM maize event.

3.1.1 Molecular characterization (transformation method, transformation construct, DNA insert characterization)

Overall, the participants agreed that the proposed molecular characterization core data is aligned with what is currently provided and that the information was sufficient to inform a food and feed safety assessment. One potential exception to the core data package that was discussed is data demonstrating that the insert is stable over at least three generations. The participants suggested that this study could be considered as supplemental, and not necessarily required as part of the core data package, if the insert is demonstrated to be inserted into the chromosome and is not interrupting endogenous genes or regulatory elements, and there is no other reason to expect that the insert might be unstable (e.g., insertion site near a transposon). There was some discussion that three generations of data may not be considered enough by all regulatory agencies and that additional generations could be required for polyploid crop species. Additionally, participants raised questions about Agrobacterium transformation not being targeted and discussed providing data on whether any internal genes were modified. It was also noted by workshop participants that the use of Next-Generation Sequencing (NGS) to characterize the insert is not yet accepted by all regulatory agencies, but also there was recognition of the utility of NGS to provide a more comprehensive characterization of the insert and the insertion site compared to traditional methods (e.g., Southern blots).

3.1.2 Protein characterization (molecular weight, protein sequence confirmation, protein function)

Participants agreed that the protein characterization information was sufficient to inform the food and feed safety assessment, with some discussions around whether a registrant would always be able to provide what is required, as some proteins may be more challenging to characterize (e.g., difficulties in isolating the proteins in an active form, generating specific antibodies, or generating SDS-PAGE and Western blot data). A question was also raised on maize codon optimization and if the protein would still be considered the same as the native version. Future workshops can reinforce that maize codon optimization of the GM trait gene does not alter the trait protein sequence. Thus, it should not change the safety profile of the protein if there is no change to the amino acid sequence. Discussion also occurred regarding familiarity with promoters and the relationship to expression levels. The participants discussed if there might be a need to better understand the protein expression levels for unfamiliar promoters and also if increased expression levels might raise a concern of potentially increased allergenicity risk.

3.1.3 Protein safety/toxicology/allergenicity (background, source, history of safe use, bioinformatics)

Participants agreed that the EPSPS protein information for safety was sufficient to inform the food and feed risk assessments, but questions were raised about Cry proteins around digestibility and heat stability. There was also discussion regarding how similar a protein would need to be to a known protein to be considered familiar. Additionally, concerns were raised in the small group discussion on the limited protein expression data provided in the case study as it related to an exposure assessment. In response, the moderators noted that an exposure assessment is not necessary, because no hazard was identified from the proteins. However, when a hazard is identified, then protein expression levels are needed to enable assessment of potential exposure (Brune et al., 2021).

3.1.4 Additional information needed to determine event safety

It was stated by one participant that if there was a disruption of a native gene, then composition data could be requested. Discussion also occurred regarding the concept of History of Safe Use (HOSU), and the amount of data, time and similarity (e.g., consideration of minor protein sequence differences) needed to establish something as having sufficient familiarity to be considered safe without additional data. One participant suggested that protein sequence data would be needed to demonstrate a HOSU and could be useful in determining the activity of the protein.

3.1.5 General feedback for food and feed safety assessment

Although participants generally agreed that the case study with a familiar crop and familiar traits is a good starting point for the discussions, several suggestions were made for further discussions to also provide a case study on an unfamiliar event or protein, to lay out how each study informs the safety assessment, to provide more on the problem formulation process, and to provide more graphics and to use examples. Discussion also occurred around the challenges of communicating and making changes to the currently provided data in regulatory applications. On this topic, proposals from participants included suggestions to emphasize more the end goal of getting needed products on the market sooner with less regulatory burden for all stakeholders and to publish more data prior to submission of the application in the scientific literature, and to be ready to provide additional data upon request.

3.2 Environmental risk assessment

After introducing the case study, the CropLife International moderator described a list (provided with the case study) of the specific potential pathways to harm that are relevant to the cultivation of the hypothetical maize event. Additionally, an explanation for how the core data can be used to sufficiently assess environmental risk was provided. For plausible pathways to harm that may not be sufficiently addressed by the core data (i.e., potential harm to non-target organisms), another list of potential pathways to harm that are specific to non-target organism (NTO) exposure was also presented.

Below are some key feedback and questions captured during the workshop regarding the proposed approach for the Environmental Risk Assessment of the hypothetical GM maize event.

3.2.1 Weediness potential

There was an overall consensus among the workshop groups that weediness can be adequately assessed using only core data. Participants agreed that there is not a plausible pathway to harm in the case study since maize is highly domesticated and volunteers will not survive without human intervention and management. One group discussed questions around the potential for dormancy, which may be a weediness trait, and whether it can be assessed in the core data (multilocation field trial; Table 2). It was concluded within the groups that the similarity in agronomic characteristics between the GM maize event and the non-GM maize in the case study core data is sufficient to show that there is a highly unlikely risk of weediness potential. This follows the principle of placing risk in the context of current practice (i.e., that the modified maize will have no greater risk than that of cultivation of the non-modified maize) (Raybould and MacDonald, 2018). However, one workshop group had unresolved discussions on whether a difference in agronomic performance between different geographical regions may result in differences in the risk assessment and what specific agronomic elements are the most relevant to consider. Some participants in this group proposed scenarios in which the agronomic data generated in field trials performed outside of the cultivation country may not sufficiently represent the agronomic outcomes of field trials performed within the cultivation country.

3.2.2 Gene flow potential to wild relatives

There was general consensus that there is no environmental safety concern of gene flow in the case study based on the core data because there were no wild relatives present in the hypothetical cultivating environment. There was some interest from participants in further exploring how the risk assessment and data requirements will change if the cultivation environment did contain wild relatives. Also, there was some discussion on the threshold of relatedness between the GM maize and a wild relative species that constitutes a safety concern in terms of gene flow. Ultimately, there was additional consensus that product registrants should demonstrate that there are no wild relative species that are reproductively compatible with GM maize (regardless of species relatedness) to position that there is no gene flow concern. Alternatively, if there are wild relative species in the area of cultivation an assessment of the likelihood and consequences of trait introgression into the wild relative population may be warranted based on a problem formulation approach (Anderson et al., 2021). Participants generally stressed the importance of citing published literature (e.g., accepted consensus references on crop-specific biology) as part of the core data to support the environmental risk assessment. Although it was acknowledged that gene flow will not likely occur between GM maize and wild relatives in the case study example, there was some discussion around whether gene flow may occur between the GM maize and adjacent local non-GM maize varieties and negatively impact crop integrity and biodiversity. The case study focused on assessing plausible pathways to harm related to gene flow between GM maize and sexually compatible weedy relatives. Future workshops can address concerns that were raised about coexistence of GM and non-GM cropping systems. Such a workshop may have to distinguish between environmental risks and market or socio-political concerns. For example, countries that have landrace populations for which the genetic make-up *per se* is a protection goal may have societal concerns about coexistence (for example, there could be changes the genetic identity of the landrace).

3.2.3 Plausible pathways to harm for non-target organisms (NTO)

All groups aligned that the only plausible pathway to harm from the case study that could not be sufficiently addressed with core data alone was the potential for harm to NTOs from potential exposure to the hypothetical Cry1 protein (Table 2). Participants discussed the plausible pathways to harm that are specific to NTOs. There was general agreement that no additional data was needed to assess the potential for the EPSPS protein conferring the herbicide tolerance trait to cause harm to NTOs. However, participants acknowledged that public perception of herbicide tolerance traits could influence regulatory decisions and may need to be considered when determining the registrability of a GM crop. Such perceptions are not reflective of an actual risk, and the additional data generated do not inform the science-based risk assessment. For other pathways to harm, there was consensus that if there was either no hazard or no detectable exposure, then there is low risk to NTOs. For example, honeybees that may directly consume maize pollen and NTO lepidopterans that may indirectly consume maize pollen that drifts onto their host plants should have low risk in the ERA case study since the GM maize event has expression less than the limit of detection (LOD) of the insecticidal protein in pollen tissue (Table 2). It was generally accepted by workshop participants that if expression of the insecticidal protein is <LOD in tissues that might be consumed by an NTO, further toxicity testing to determine hazard is not warranted.

Participants were also mostly aligned that aquatic environments generally experience minimal exposure to GM crop tissue and so additional toxicity testing is not needed for aquatic NTO species in most situations. However, some participants expressed uncertainty on whether this may be an issue if GM crops are cultivated very close to aquatic environments, which may affect exposure levels to NTO aquatic species. For NTO species where there is a plausible pathway to harm, all groups agreed that further data (exposure assessment or NTO Tier I laboratory testing) might be needed. Some discussions among participants regarding appropriate surrogate species to use for NTO testing and to what extent test species need to match those found in the cultivation regions were not resolved in the workshop. There was some additional discussion around the large body of scientific literature describing the surrogate species concept for testing Cry proteins and other types of plant incorporated protectants (e.g., Romeis, et al., 2011; Romeis et al., 2013; Bachman et al., 2021). While the terms “focal species” and “indicator species” were not discussed directly as part of the workshop, understanding protection goals and selecting appropriate surrogate species or indicator species to inform the science-based assessment of risk is an important consideration (Rose, 2007; Roberts et al., 2020). Despite the lack of consensus on species selection, there was clear alignment among participants that NTO species representatives should only be tested if there is a valid hypothesis that there is a plausible pathway to harm for that specific organism type. For this reason, NTO studies should only be conducted when hypothesis-driven (Figure 1B).

3.2.4 General feedback and future considerations for ERA

Although participants agreed that a generic ERA case study is a good starting place, participants indicated that future workshops using a modified case study tailored for specific geographical regions will be even more helpful. As different countries have different sets of questions and concerns from local regulatory agencies, using more country-specific scenarios and less familiar pest-control traits in a case study may be more directly relevant in that region.

Related to gene flow, there was not a consensus about potential for harm in small team discussions. Future workshops would benefit from guided discussion to help develop problem formulation for gene flow. For example, it could be established as a baseline that for gene flow to occur naturally in the environment, and when assessing the potential for harm from gene flow between GM maize and local maize varieties, it should be compared to potential for harm from gene flow of non-GM maize and local maize varieties (OECD, 2023). Furthermore, future workshops can reinforce that if gene flow to local maize varieties is a relevant concern for a specific cultivation country, then there is a large body of literature to leverage to assess if additional data is needed to inform the risk assessment (See OECD, 2023 Annex B for recent review) such a workshop would need to distinguish between the true environmental impact and concerns related to trade or economic issues.

Also, there were productive discussions on the topic of data transportability. Participants generally accepted the concept of transportability for lab study data. However, due to a lack of time for discussion, some unresolved questions remained regarding the transportability of field study data. Future workshops will benefit from guided discussion to help explain the principle of data transportability. An underlying principle of data transportability is that if no biologically relevant differences between a GM crop and its conventional counterparts are observed in one country or region, data from these studies can be used to inform the risk assessment in another country, regardless of agroclimatic zone (Bachman et al., 2021). Following the recommendations for modernizing global regulatory frameworks for GM crops, additional agronomic data should only be collected in the local environment if there are plausible pathways for harm that cannot be fully informed by the core data.

Furthermore, there was some interest from participants in discussing how the proposed risk assessment paradigm might apply to combined GM products (i.e., breeding stacks), yield and stress traits (e.g., drought resistance), and streamlining of import registrations.

One topic that generated discussion across groups was the value of product characterization data in an environmental risk assessment. In the proposed modernized regulatory framework (Anderson et al., 2021), underlying characterization data for the GM event are not regarded as core to the regulatory assessments (such as molecular data to confirm that the insert is an intact single copy, stable across generations, and that there is no plasmid backbone DNA). Although these data do not directly inform the ERA (Anderson et al., 2021), it was discussed that an understanding of the characteristics of the GM product provides foundational information that enables the regulatory assessments to focus on the intended introduced trait during the problem formulation stage. Therefore, it was proposed to consider including, as part of the modernized ERA framework, a set of foundational information and data from the characterization of the GM event that confirms that (1) the intended gene sequence was inserted and functions as intended, as well as the number of such insertions; (2) the plants produce the intended newly expressed protein (NEP); (3) the intended phenotype is achieved.

4 Key considerations and takeaways from the workshop

The case study for the workshop considered a single event, albeit one that contained genetic material encoding for two proteins leading to two distinct traits (herbicide tolerance and insect protection). However, the majority of commercialized products contain multiple GM events that are combined through conventional breeding (also known as stacked trait products). The typical regulatory process first assesses all single events, before applying regulatory processes, if any, to the stacked trait products. In this sense, the case study used for the workshop reflected a realistic scenario in which regulators assess a single event regardless of whether the event will be commercialized as a single event or as a stacked trait product.

Regulatory processes for stacked trait products vary globally, with many countries recognizing the long, safe history of conventional breeding and not requiring additional assessment once all the single events are approved. It is the position of CropLife International that additional safety assessment of a stacked trait product produced by conventional breeding should not be required unless there is a plausible and testable hypothesis for interaction of the traits (Goodwin et al., 2021). This case study did not address stacked trait products however, further iterations could include consideration of stacked trait products and how to evaluate possible interaction of traits.

The workshop was convened to explore the proposed modernized data requirements for regulatory assessments of GM crops (Anderson et al., 2021; Waters et al., 2021). The participants were charged with considering whether currently implemented regulations for GM crops are risk-proportionate or whether they create an unwarranted barrier to the introduction of new traits. The organizers presented a position that knowledge and experience from 25 years of research and development could inform regulatory modernization and that streamlined data requirements could advance harmonization across countries and authorities.

Overall, considering the case study discussed, the participants at the workshop found the proposed modernized data requirements generally to be necessary and sufficient for decision making to support the safe commercial introduction of a new GM crop. There was a clear consensus that some of the current data requirements are no longer routinely warranted for familiar traits such as that discussed in the case study, given the track record of GM crops not presenting unexpected or unintended effects on food or feed safety or environmental risk relative to their conventional counterparts. Participants appreciated the benefit of harmonized hypothesis-based risk assessments to enable future deployment of GM crops that can address emerging agricultural challenges associated with increasing demand for affordable healthy food and changing agricultural environments. The points discussed in this publication will be used to further clarify recommendations for supplementary case-by-case data and guide the development of future, more targeted workshops and related discussions. In particular, applying the proposed framework to traits and crops with which there is less familiarity and established HOSU than those used in the case study may be associated with greater uncertainty in the foundational information of the GM event. Additional case studies involving less familiar traits and different crops should be used to further test the robustness of the modernized regulatory framework.

The workshop focused on what data was scientifically necessary and sufficient to make a conclusion on the food, feed and environmental safety of the GM crop. However, several participants noted that certain data not included in the case study was either required in their jurisdiction or routinely submitted by applicants. While it was beyond the scope of this workshop, future targeted workshops or symposia could address the extent to which regulatory authorities have the flexibility to decide, on a case-by-case basis, what data is necessary to make a conclusion on safety. In some jurisdictions the recommendations of the modernization project could be implemented by applicants by including a scientific rationale in their submission for why a specific study is not necessary. In other cases, changes to laws, regulations, or written guidance would be needed to implement these recommendations.

The case study for the first workshop, as described in this publication, was a valuable tool to foster discussion about science-based data requirements for the assessment of GM crops. If these scientific approaches to modernize data packages for GM crop regulation were adopted globally, delays to the commercialization of GM crops could be reduced, thereby allowing farmers access to new GM traits that will benefit not just growers, but consumers and the environment as well. For more information on the case study used in the workshop, or if there is interest in hosting a similar workshop, please contact the corresponding author.

Author contributions

NS: Conceptualization, Writing–original draft, Writing–review and editing. AS: Conceptualization, Writing–original draft, Writing–review and editing. JS: Writing–original draft, Writing–review and editing. JA: Writing–original draft, Writing–review and editing. MH: Writing–original draft, Writing–review and editing. DM: Writing–original draft, Writing–review and editing. CM: Writing–original draft, Writing–review and editing. MS: Writing–original draft, Writing–review and editing. SS: Writing–original draft, Writing–review and editing. EU-W: Writing–original draft, Writing–review and editing.

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Conflict of interest

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Rare but diverse off-target and somatic mutations found in field and greenhouse grown trees expressing CRISPR/Cas9

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Introduction: CRISPR gene editing, while highly efficient in creating desired mutations, also has the potential to cause off-target mutations. This risk is especially high in clonally propagated plants, where editing reagents may remain in the genome for long periods of time or in perpetuity. We studied a diverse population of *Populus* and *Eucalyptus* trees that had CRISPR/Cas9-containing transgenes that targeted one or two types of floral development genes, homologs of *LEAFY* and *AGAMOUS*.

Methods: Using a targeted sequence approach, we studied approximately 20,000 genomic sites with degenerate sequence homology of up to five base pairs relative to guide RNA (gRNA) target sites. We analyzed those sites in 96 individual tree samples that represented 37 independent insertion events containing one or multiples of six unique gRNAs.

Results: We found low rates of off-target mutations, with rates of 1.2×10^{-9} in poplar and 3.1×10^{-10} in eucalypts, respectively, comparable to that expected due to sexual reproduction. The rates of mutation were highly idiosyncratic among sites and not predicted by sequence similarity to the target sites; a subset of two gRNAs showed off-target editing of four unique genomic sites with up to five mismatches relative to the true target sites, reaching fixation in some gene insertion events and clonal ramets. The location of off-target mutations relative to the PAM site were essentially identical to that seen with on-target CRISPR mutations.

Discussion: The low rates observed support many other studies in plants that suggest that the rates of off-target mutagenesis from CRISPR/Cas9 transgenes are negligible; our study extends this conclusion to trees and other long-lived plants where CRISPR/Cas9 transgenes were present in the genome for approximately four years.

KEYWORDS

CRISPR, gene editing, off-target editing, *Populus*, *Eucalyptus*, tree biotechnology

1 Introduction

Gene editing technologies using site-specific nucleases (SSNs) such as CRISPR/Cas9 has been a transformative method for scientific research and biotechnology (Adli, 2018). Gene editing using wild type CRISPR/Cas systems, most commonly SpCas9 from *Streptococcus pyogenes*, has been widely employed throughout angiosperm plants, most commonly through *Agrobacterium*-mediated stable integration of a Cas/gRNA-containing transgene (Goralogia et al., 2021; Cardi et al., 2023). For most plants, removal of stably integrated transgenes via segregation is the common approach, after which null segregants containing the desired mutations absent transgenes are the starting points for scientific research or biotechnology. Current regulations in the United States permit null segregants with simple edits that are theoretically obtainable through normal breeding methods to be exempt from regulation by USDA-APHIS, facilitating field research and commercial applications (Hoffman et al., 2022).

For clonally propagated plants, there are few reliable methods to efficiently remove stably integrated gene-editing transgenes without compromising clonal integrity (Goralogia et al., 2021). The most applicable approaches, recombinase-mediated transgene excision, DNA-free protoplast- or biolistic-mediated transformation via Cas-RNP complexes, and transient viral delivery systems, have been achieved in several species but remain difficult to apply at scale due to widespread recalcitrance to transformation and/or regeneration among species and genotypes (Fossi et al., 2019; Dalla Costa et al., 2020; Pompili et al., 2020; Liu et al., 2023). Due to the presence of an excision "footprint," the editing-transgene/excision method will currently trigger regulatory scrutiny as a GMO everywhere in the world—an undesirable outcome where field research or commercial development are important goals. An exception includes footprint-free transposases like piggyBac; however, they have some technical challenges, and have only been demonstrated in rice (Nishizawa-Yokoi and Toki, 2021). Although an avenue for deregulation of simple edits in clonal crops remains open through USDA-SECURE by trait-mechanism of action (MOA) approval of stably integrated Cas and gRNA genes, to our knowledge no such applications have been successfully approved.

Because clonally propagated plants by their nature do not require the production of sexual propagules, one option is to introduce sexual sterility traits by genome editing and simply leave the editing transgenes permanently in the genome. This would be permissible if the rate of continued off-target mutation is very low, and if the risks of residual sexual spread or vegetative propagation in the environment, especially to wild or crop relatives, is acceptable. This is an attractive option for fiber crops such as forest trees, where sexual reproduction is not important to their commercial products (Fritsche et al., 2018). This approach would greatly limit or prevent the flow of editing transgenes into sexually compatible species (a potential public acceptance and regulatory concern, especially for a forest tree species with wild relatives), and mitigate or completely prevent the risks of gene drives that could occur over long time periods through outcrossing. Of course, though edited, such transgenic plants would not obtain exemptions; they would be regulated and subject to normal reviews by the relevant agencies in the United States and abroad (Goralogia et al., 2021; Hoffman et al., 2022).

One potential effect of leaving editing transgenes in the genome for long periods of time is a heightened potential for off-target mutations. Off-target mutations are those that occur due to CRISPR/Cas activity but are located at unintended loci. Due to the nature of gRNA binding and Cas complex formation, these are most likely to occur at sites similar to but divergent from (mismatched) the true target sites (Pattanayak et al., 2013; Bae et al., 2014). This contrasts with the much more random nature of somatic mutations that occur in clonally propagated plants due to factors such replication errors and exposure to radiation and UV light. Fixed somatic mutations, though rare, are often important for breeding in clonal crops, and many cultivars in tree fruits come from so called "bud sports" which differ from the rest of the tree but whose characteristics persist through long-term vegetative propagation (Ban and Jung, 2023). In animals, the occurrence of off-target mutations due to CRISPR/Cas appear to be higher than in plants, though a highly cited study discovering such mutations was retracted after other reports had contrary observations and employed superior controls (Anderson et al., 2018; Iyer et al., 2018; Schaefer et al., 2018). Concerns over off-target editing have also led to the development and wide use of Cas-nickase or high-fidelity systems, which have much lower off-target rates due to an absence of DNA double-strand-breaks (Kleinstiver et al., 2016; Gao et al., 2017). Although there have been many studies of off-target mutation in plants, including in Arabidopsis, maize, rice, and grape, these studies involve very short timeframes from transformation to sequencing, analysis only of null-segregants of T0s, or involve *in vitro* DNA-CRISPR/Cas interactions (Tang et al., 2018; Xu et al., 2019; Young et al., 2019; Wang et al., 2021; Sturme et al., 2022). In addition, many studies look at a narrow band of potential off-target sites (e.g., one or 2 bp divergence to target sequence), or use whole-genome sequencing but with lower overall coverage than is desirable to detect low-frequency mutations. To estimate the types and rates of rare off-target mutations, we used a targeted-sequencing approach that delivered high sequence depth, queried a very large number of potential off-target sites, studied plants where CRISPR/Cas had been present for more than 2 years, and examined a large number of insertion events. We report very low off-target and somatic mutation rates, where mutated sites had no obvious relationship to target site sequences.

2 Materials and methods

2.1 Plant materials and timeline

In a previous 2018 study, we produced a population of clonally propagated, CRISPR/Cas9 edited poplar trees in two diploid hybrid genotypes (Elorriaga et al., 2018). They had been produced with the intent to induce sterility by editing the *LEAFY (LFY)* and *AGAMOUS (AG)* loci to cause frameshifts and large deletions. These are genes believed to be required for inflorescence and floral organ specification, respectively, and have highly conserved functions in most angiosperms. These transgenic trees, to our knowledge, were the first edited trees approved for field trial in the United States. We also produced *Eucalyptus* trees in a previous 2021 study, targeting the *LFY* locus, with the same goal (Elorriaga et al., 2021). In brief, the

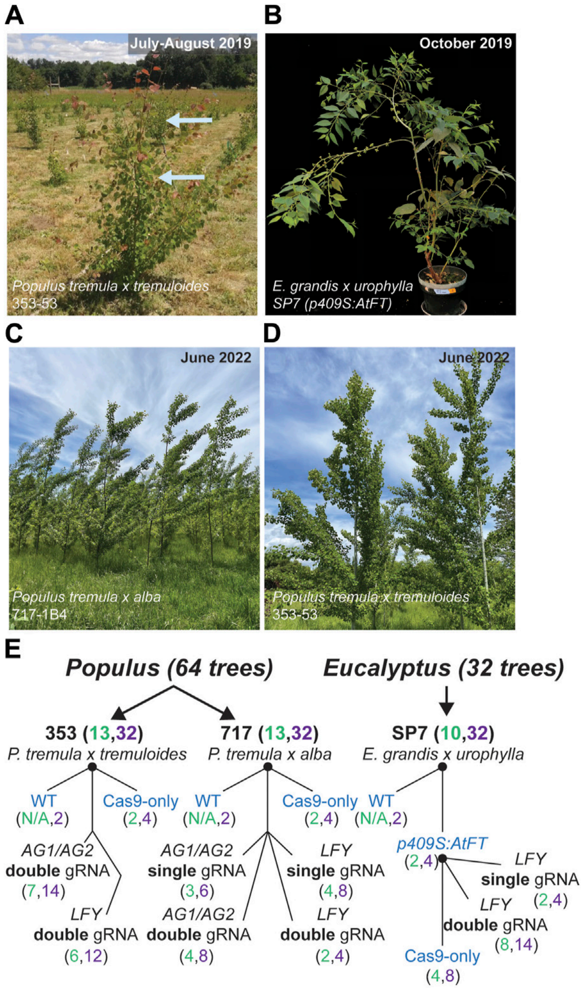


FIGURE 1
Tree species, study design and transgenic constructs used to study target, off-target and somatic mutations after transformation with CRISPR/Cas9. **(A–D)** Representative images of poplar genotypes in the field as of summer 2022, and for early flowering eucalypts in the greenhouse. **(A)** Newly forming shoots are rust-colored in this clone. Tissues sampled in the field from poplars in summer 2019. **(E)** Construct structure and gRNA targets within surveyed poplar and eucalypt genotypes. Control constructs given in blue text. Black nodes represent clonal parental material. Numbers of events and ramets of each event in each construct are given in the following format (Event #, Ramet #). Overall numbers of unique CRISPR/Cas9 events and ramet numbers are shown in parentheses after genotype ID.

editing constructs contained a human codon-optimized Cas9 gene driven by a double enhancer 35S promoter; it also contained a nos terminator fragment, gRNAs driven by AtU6 small nuclear RNA promoters, and a kanamycin or hygromycin antibiotic resistance gene driven and terminated by nos transcriptional elements.

For poplars, the two genotypes employed were *Populus tremula x alba* 717-1B4 (female, hereafter abbreviated ‘717’) and *P. tremula x tremuloides* 353-53 (male, hereafter abbreviated ‘353’), both a product of research at INRA, France. For *Eucalyptus*, we used one genotype, a *Eucalyptus grandis x urophylla* hybrid called “SP7” that was provided by Futuragene/Suzano (Figures 1A–D). We included six representative CRISPR/Cas9 editing constructs in the study (Figure 1E). In *Eucalyptus*, the editing constructs were transformed into early flowering transgenic backgrounds (two independent events, construct *p409S:AtFT*) which was developed in prior work (Klocko et al., 2016a). Together, these constructs targeted the poplar *PtaLFY* or *PtaAG1/PtaAG2* loci or eucalypt *EgLFY* genes, in either a single or double gRNA configuration (genotype 353 had only been transformed with double gRNA constructs). As controls, included for each genotype set were transgenic plants expressing Cas9 but without gRNAs (hereafter abbreviated ‘Cas9-only’), wild type (non-transgenic) trees, and transgenic *p409S:AtFT* parent events (*i.e.*, into which the editing constructs were transformed for eucalypts (Figure 1E).

The study timeline, which covered 46–59 months, is detailed in Supplementary Figures S1, S2. Cloning of constructs began in 2014 for poplars and in 2015 for eucalypts, and transformation began the subsequent year for each set. Poplar transgenic events in clones 717 and 353 were planted in the Fall of 2017 at a field site near Corvallis, Oregon, and eucalypt events (in clone SP7) were planted for study in the greenhouse starting Fall of 2018 on the Corvallis campus of Oregon State University. Samples for DNA analysis shown in this study were taken in summer and fall of 2019, and thus represent two full growing seasons in the field for poplars, after significant time also spent under *in vitro* culture during micropropagation (approximately two additional years). Representative images of trees in the field (approximately 3 years after sampling) are shown in Figures 1C, D, and at the time of sampling in Figures 1A, B. The transgenic tissues had been growing approximately 4 years since transformation, and those in 717 were growing for nearly 5 years.

2.2 Tissue collection, DNA purification, and preparation for sequencing

Our goal was to survey as large a number of constructs and events, with high confidence sequencing data, at as many potential off-target sites as feasible within our budget and available plant material. Due to technical constraints, we selected a 96-tree sample size, divided into thirds to fit our plant materials; there were 32 trees of poplar clone 717, 32 trees of poplar clone 353, and 32 trees of eucalypt clone SP7. Two clonal ramets of each insertion event were selected at random for sampling.

Poplar samples were collected in July-August 2019 at a field site near Corvallis, Oregon. *Eucalyptus* samples were collected in September-December 2019 from plants grown in greenhouses at the Oregon State University campus. A total of ten leaves were harvested from the first fully expanded leaf on the main stem (~3 leaves from the apical bud). Leaf tissue was ground in a mortar and pestle chilled with liquid nitrogen, and samples were aliquoted into 1.5 mL tubes with 500ul volume of powdered tissue placed into each tube. Nucleic acid purification was performed on both poplars and eucalypts using the CTAB method (Barbier et al., 2019). DNA quality was analyzed by nanodrop (Thermo-Fisher) and by Qubit fluorometric analysis. The samples were then frozen at −20°C until shipment. Final DNA preparation for sequencing was performed at Arbor Biosciences (Ann Arbor, Michigan) and DNA was sonicated to approximately 500bp prior to target-bait capture.

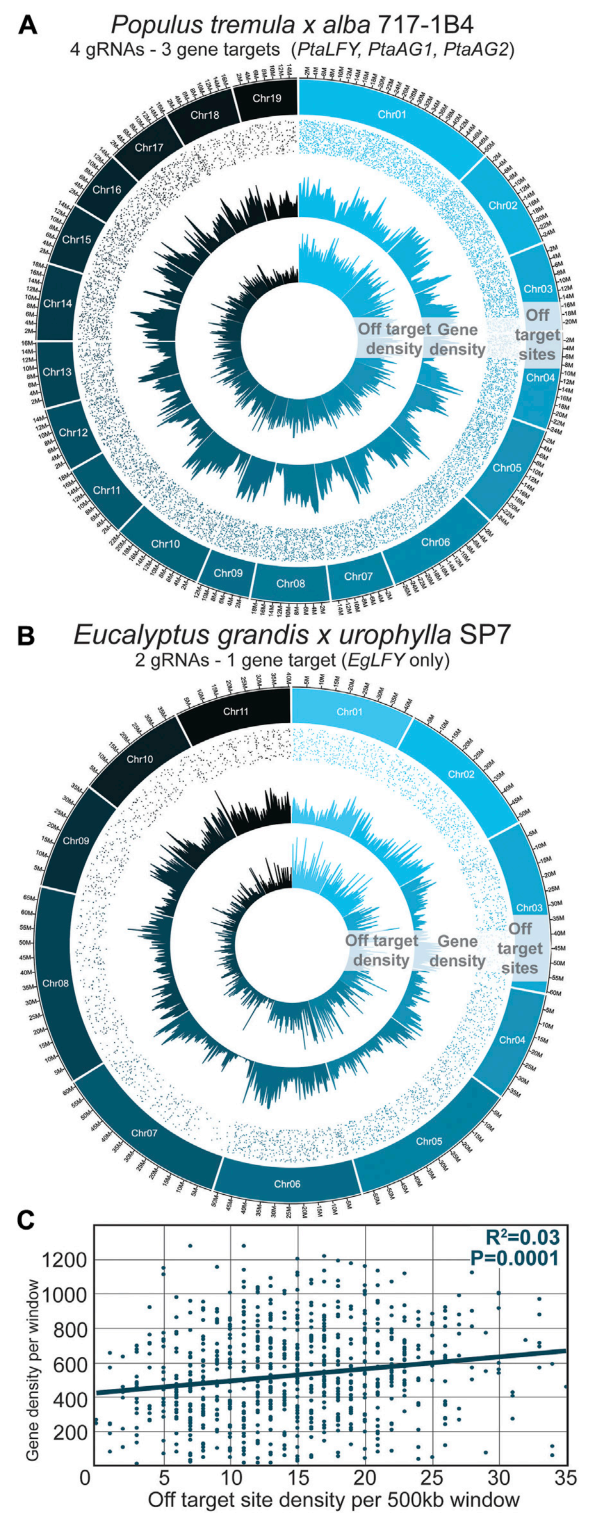


FIGURE 2
A targeted sequencing approach using bait-capture effectively covered regions with potential off-target sites. **(A)** 717-1B4 P. tremula x alba reference genome used to query off-target sites within 353 and 717 transformed poplars. CasOFFinder program was used to find sites with up to 5bp of DNA mismatch to the four target gRNA sequences. Off-target site density and gene density were computed over 500 kb windows. Off-target site locations are shown as dots in the outer ring of each plot, with each dot representing a unique site. **(B)** SP7 *Eucalyptus grandis x urophylla* genome used similarly for off-

(Continued)

FIGURE 2 (Continued)
target site analysis. CasOFFinder program was also used to find sites with up to 5bp of DNA mismatch to the two designed gRNA sequences, using the *Eucalyptus grandis* v2 genome. **(C)** Corresponding gene density and off-target site density in the poplar 717-1B4 genome. Best fit linear regression is shown by the solid blue line.

2.3 Probe design and construction

Potential off-target sites were determined using the CRISPR RGEN tool CAS-OFFinder using the *P. tremula x alba* 717-1B4v2 reference sequence for both 717 and 353 poplar clones (https://www.aspendb.org/downloads), and the *E. grandis* v2 genome for the genotype SP7 eucalypt (https://phytozome-next.jgi.doe.gov/info/Egrandis_v2_0) (Bae et al., 2014; Myburg et al., 2014; Mader et al., 2016). No genome sequence is currently available for the 353 poplar clone but given the ability of the 80 bp baits to bind slightly divergent regions and the presence of a *P. tremula* parental genome in 353s pedigree, we were confident (and supported by our results) that 717-designed baits would be adequate for the majority of target loci. Sites were analyzed with up to five base pairs of mismatch to the target sequence, or up to four bases of mismatch with a DNA or RNA bulge of 1bp. Both the canonical NGG Protospacer Adjacent Motif (PAM) as well as the NRG PAM were permitted. Sites located on unassembled scaffolds were accepted. 17,774 probes of 80bp length were designed that were centered to the potential off-target site (Hill et al., 2019). Sites with poor synthesis scores (3% of total) had alternative baits designed in the flanking region.

2.4 Capture and sequencing

Bait synthesis, hybridization, capture, and sequencing was performed at Arbor Biosciences. For efficiency in sample processing, each sample was subjected to the entire bait library (i.e., the two poplar genotype and eucalypt baits were applied to their own DNA samples as well as to those from the other genotypes). Sequence capture was accomplished with streptavidin-binding magnetic beads (Invitrogen). Captured sample libraries were prepared, then sequenced on an Illumina NovaSeq 6,000 platform with S4 flow cells to yield 150bp PE reads.

2.5 Bioinformatic processing

An overview of the bioinformatics pipeline for this study is shown in Supplementary Figure S3. Sequence quality of the samples was initially assessed using FastQC. Alignment of the raw sequence reads to the respective reference genomes used the Burrows-Wheeler Aligner (bwa https://bio-bwa.sourceforge.net/). The resulting .bam files were processed to be analyzed by the Mutect2 program (part of GATK tool suite) (Benjamin et al., 2019). This included using two steps in Picard and samtools (https://broadinstitute.github.io/picard/, https://www.htslib.org/) first the AddOrReplaceReadGroups

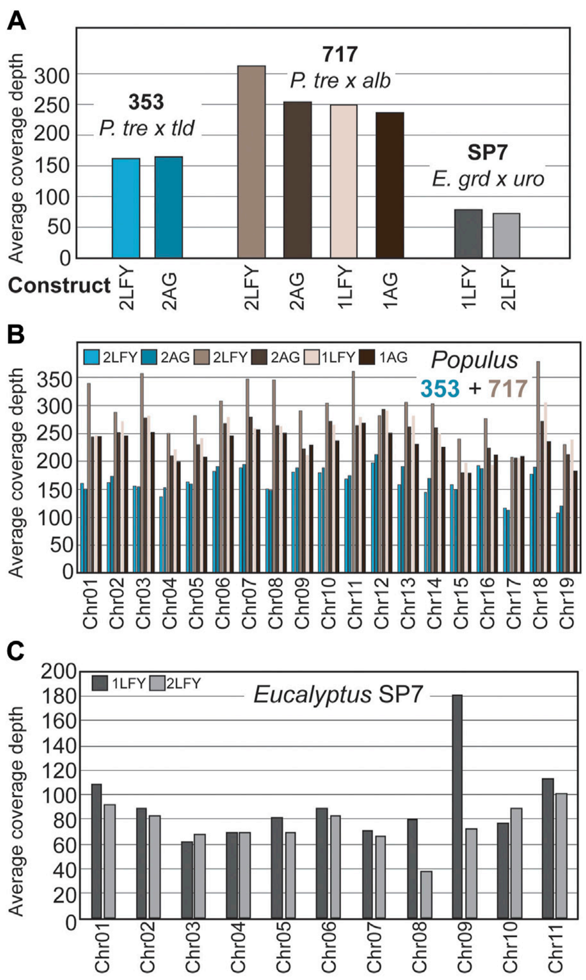


FIGURE 3
Sequencing depth for off-target sites in genomes. **(A)** Coverage within 80bp target bait sites, averaged over all sites within a given construct. **(B)** Coverage within target bait sites, averaged over chromosomes within a construct in the two poplar genotypes. **(C)** Coverage within target bait sites, averaged over chromosomes within a construct in eucalypt genotype SP7.

command was used to assign sample numbers in the header of bam aligned reads, and the resulting. bam files were sorted using the SortSam command to set SORT_ORDER = coordinate. Detection of off-target and somatic mutations was performed using the Mutect2 program. Mutations were assessed proximal to the mismatch sites using the intervals input. Intervals were set by aligning the 80bp bait .fasta files to the respective genomes using bwa, then the resulting .bam files were converted into bed format using bedtools (https://bedtools.readthedocs.io/en/latest/). Sites with flanking baits were assessed in 80bp windows about their genomic coordinates using the same method .bed files were converted into a GATK intervals file using Picard BedToIntervalList command, and a sequence dictionary file was made using the CreateSequenceDictionary command. For final analysis using Mutect2, all wild type and Cas9 only controls were pooled for each respective genotype as "normal" samples, and all transgenic events and ramets for a given construct were pooled as "tumor" samples for analysis. Default settings were used for Mutect2. For quality control of identified sites, the program FilterMutectCalls was used (https://gatk.broadinstitute.org/hc/en-us/articles/360036856831-FilterMutectCalls). Analyses of parameter inputs for FilterMutectCalls analysis are shown in Supplementary Figure S4. Final analysis included the following input parameters: -max-events-in-region 20, --f-score-beta 1. Resulting sites which passed filtering were assessed by manual examination of sequence alignments.

2.6 Potential off-target site chromosome plots and coverage analysis

We constructed circular genome maps using the program Circa (https://omgenomics.gumroad.com/l/circa), using .gff gene models (https://www.aspendb.org/downloads) or potential off-target sites over 500 kb windows (number of potential off-target sites/500 kb) in the *P. tremula x alba* 717-1B4_v2 genome or the *E. grandis x urophylla* SP7 genome.

To assess coverage over 80bp bait windows, coverage depth (DP) values at pre-filtered Mutect2 output sites were used as proxies for coverage. Coverage depth values were averaged in entire samples over the whole genome or by chromosome within sample, then treated as individual measurements to assess coverage over the population. Haplotype-phased sites in *Eucalyptus* were merged for analysis.

2.7 Assessment of off-target and somatic mutations

For manual scoring of individual sites for mutations, a series of criteria were assessed by visualizing events and ramets against wild type controls at specific sites in an IGV browser (https://www.igv.org/) (Robinson et al., 2011). A logic-tree for assessment of sites is shown in Supplementary Figure S5. Briefly, sites were excluded if the alternative allele was not supported by more than five reads. Sites with likely alignment errors were also excluded (examples shown in Supplementary Figure S6). Sites were assessed for evidence of the same exact SNP or indel in the wild type sequence and excluded if the wild type had similar allele frequencies to the flagged site. Sites within 20 bp of the 5′ or 3’ borders of mismatched gRNA site were binned as potential off-target sites, and those beyond that distance were binned as somatic mutations. We fully evaluated off-target sites which exceeded 10% allele frequency in at least two ramets. A haplotype-phased high quality genome sequence for eucalypt clone SP7 became available during data analysis and was used to assess off-target and somatic mutations in that clone (https://www.futuragene.com/wp-content/uploads/2023/06/Eucalyptus-genome-Press-Release.pdf).

To calculate off-target site mutation allele frequencies, reads were manually counted in an IGV browser and called mutant if alternative bases (SNPs or indels) were present in a read between the +2 and −5 sites relative to the mismatched PAM site. 150-200 reads were counted in this manner, or until the total reads in the sample were assessed.

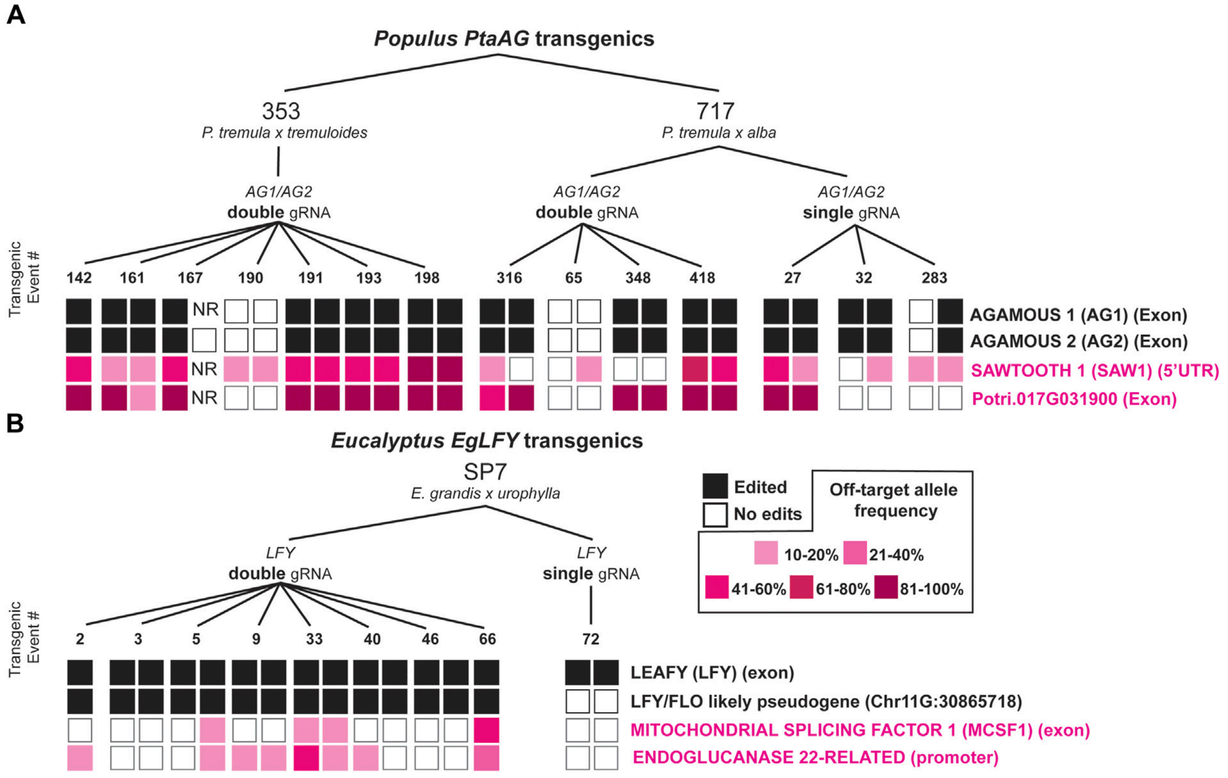


FIGURE 4
Target and off-target editing outcomes with four constructs in *Populus* and *Eucalyptus* CRISPR/Cas9 transgenics. **(A)** 353 and 717 poplars transformed with single and double gRNA constructs directed to the *PtaAG1* and *PtaAG2* genes (Elorriaga et al., 2018). Target editing outcomes are shown with filled black squares, and off-target editing (with greater than 10% allele frequency) are shown with filled pink squares, with increasing intensity for higher allele frequencies as per the key in the center. "NR" labels mean no reads were sequenced at the locus to determine edits. Biallelic edits, heterozygous edits, and transgenic but unmutated transgenic events at the target loci were included for analysis. **(B)** SP7 eucalypts transformed with single and double gRNA constructs directed to the *EgLFY* locus. Target editing outcomes are also shown with filled black squares, and off target editing are shown with filled pink squares.

2.8 Validation of off-target mutations

To verify the mutations observed by targeted sequencing, we amplified the two poplar off-target sites identified at the Potri.007G032700 and Potri.017G091300 loci, using the primers (7G: F:5′-ATTCCGTAGAGTGCGTTGGT-3′, R:5′-TTTGTTGCTCTTTGCAGCAC-3′, 17G:F: 5′-CACGAAGTAGGAGATGATGGCGATT-3′, R: 5′-CAGAGGCTTCTCAATGTGTGGATGG-3′). DNA was isolated from 3-5 dormant buds prior to bud break in April of 2022, at lower accessible branches due to tree height. Regions were amplified using Q5 DNA polymerase (New England Biolabs) PCR products were excised from agarose gels and purified using a column purification kit (Zymo) and submitted for long-read sequencing by Oxford Nanopore method (Plasmidsaurus: https://www.plasmidsaurus.com/).

3 Results

3.1 Off-target site genome distribution

The distribution of off-target sites in poplar and eucalypts is shown in Figures 2A, B, respectively. The number of sites investigated was different between poplars and eucalypts given the lower number of construct/gRNA pairs investigated in eucalypts, with 5,557 sites surveyed vs 12,217 in poplars. When viewed in relation to gene density over each chromosome, there was not a visually obvious correlation between gene density and potential off-target site density (Figures 2A, B). When the association was analyzed using 500 kb genome windows, a highly significant and positive, but very weak, correlation was found; gene density explained only 3% of the variance in off-target site density (Figure 2C; r = 0.17, $p < 0.0001$).

3.2 Targeted sequencing depth

To estimate the coverage depth obtained by the bait-capture targeted sequencing approach at off-target sites, we computed the depth of coverage at pre-filtered variant sites identified by Mutect2 (Figure 3A). We found highly variable recovery per site, and different coverage depth between genotypes, with 717 having the best coverage (mean = 242, SD = 200, CoV = 0.82), followed by 353 (mean = 163, SD = 131, CoV = 0.80) and then SP7 (mean = 72, SD = 96, CoV = 1.32). The highest average

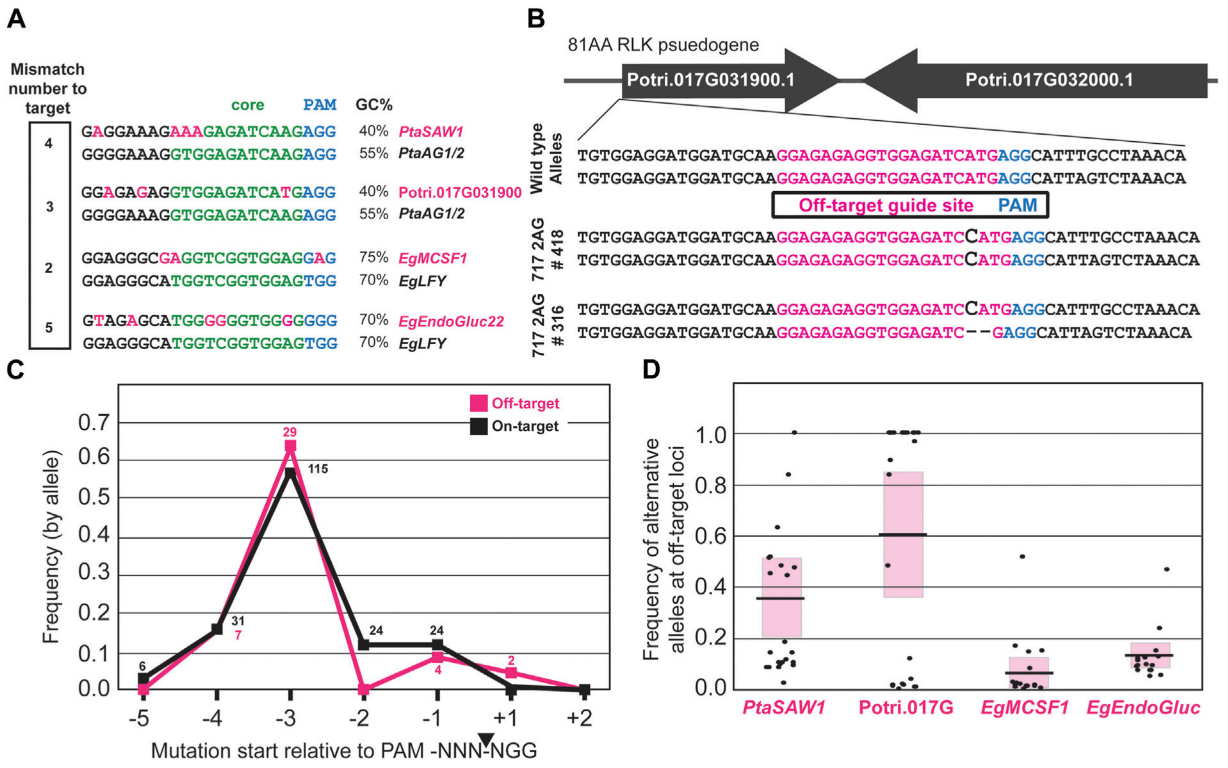


FIGURE 5
CRISPR-mutated off-target site features. **(A)** Off-target sites where CRISPR-mediated mutation occurred in poplars and eucalypts (top row), relative to the target site (bottom row). Variant nucleotides are highlighted in pink. Green nucleotides show the "core" consensus region of Cas9-gRNA affinity. **(B)** Examples of off-target edits at a locus on chromosome 17 in two transgenic events targeting the *PtaAG1* and *PtaAG2* loci. **(C)** Frequency of mutations relative to the PAM site of off-target sites (pink), vs target sites (black). Allele counts observed for each PAM position are shown above each point. **(D)** Frequency of alternative alleles in all poplar and eucalypt events and clonal ramets. Each point represents the mutant allele frequency (in a +2 to −5 window relative to PAM, averaged across both copies in haplotype-phased SP7 genome) in an individual tree. Pink blocks represent a single standard deviation about the mean (black bars).

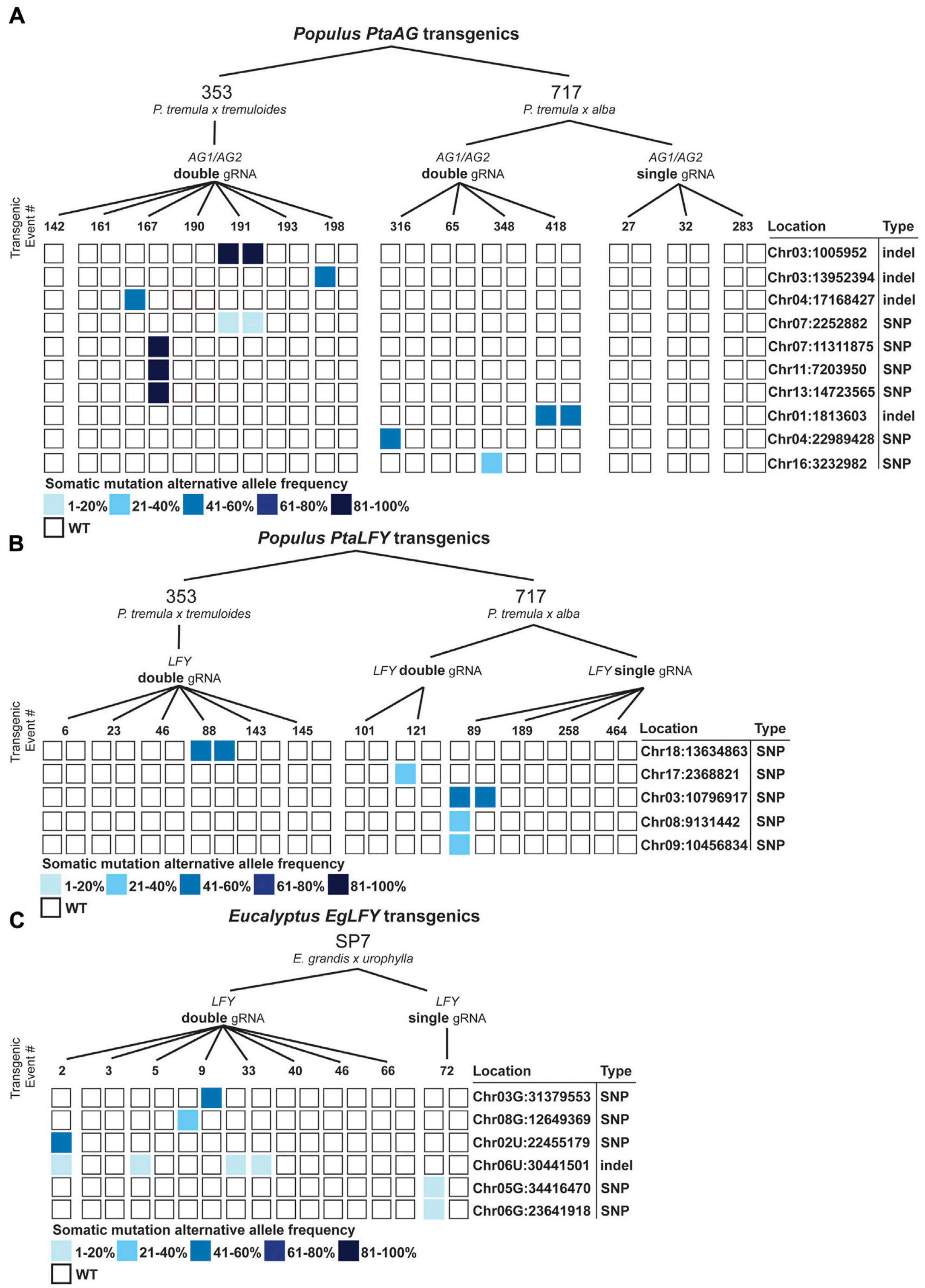
coverage depth was over 300 reads per site for the single gRNA construct *PtaLFY* in 717, and the lowest was 60 reads per site for the single gRNA construct *EgLFY* in SP7. For poplar, the same trends in coverage depth were obtained when the data were examined by chromosome (Figure 3B).

3.3 Mutect2 detection of variant sites

We initially compared our control samples to each other (wild type vs Cas9 only constructs) and found two somatic mutations in 717 and one in 353. We also detected four novel somatic mutations in eucalypts between the different early flowering parental backgrounds. We also identified that one of the Cas9 only control events in the 717 clone was mislabeled after tissue culture as both ramets in this event had mutations indicative of a *PtaAG* double-gRNA transformant, thus it was excluded from future study. Events had expected on-target edits with the exception of 717 single gRNA *PtaAG*-targeting event 283-1, which was likely mislabeled in tissue culture, but was retained for analysis of off-target mutations. Subsequent analysis comparing controls "normal" to transgenics "tumor" within each construct was completed independently in each clonal background (353, 717, SP7).

3.4 Off-target mutation analysis

In poplar and *Eucalyptus*, we found four total sites in the genome which had been unintentionally mutated by CRISPR/Cas9 (Figure 4). In poplar, this included the Potri.017G091300 gene (an 81-amino acid encoding RLK-like gene with no RNA-seq support for the gene model and a mutation site located in an exon), and the Potri.007G032700 gene with whose nearest orthologue in Arabidopsis *SAWTOOTH 1 (SAW1)* encodes a BEL1-like homeodomain transcription factor (Kumar et al., 2007). The mutation site in *PtaSAW1* is located in the 5′UTR. In eucalypts, this included Eucgr. E01328, whose nearest orthologue in Arabidopsis is *MITOCHONDRIAL CAF-LIKE SPLICING FACTOR 1 (MCSF1)*, with the mutation site in an exon, and a second gene Eucgr. I01325 is a predicted glycosidase ENDOGLUCANASE 22-related (hereafter abbreviated '*EgEndoGluc22*'), whose nearest Arabidopsis orthologue is *GLYCOSYL HYDROLASE 9B18 (GHB9B18).* For each of the sites we found many events and ramets with allele frequencies exceeding 10%, with the greatest being *PtaSAW1* (78% ramet mutation rate exceeding 10% AF), and the least being *EgEndoGluc22* (25% ramet editing rate) (Figures 4A, B). Though *PtaSAW1* saw the highest ramet-level editing rate



Location	Type
Chr03:1005952	indel
Chr03:13952394	indel
Chr04:17168427	indel
Chr07:2252882	SNP
Chr07:11311875	SNP
Chr11:7203950	SNP
Chr13:14723565	SNP
Chr01:1813603	indel
Chr04:22989428	SNP
Chr16:3232982	SNP

Location	Type
Chr18:13634863	SNP
Chr17:2368821	SNP
Chr03:10796917	SNP
Chr08:9131442	SNP
Chr09:10456834	SNP

Location	Type
Chr03G:31379553	SNP
Chr08G:12649369	SNP
Chr02U:22455179	SNP
Chr06U:30441501	indel
Chr05G:34416470	SNP
Chr06G:23641918	SNP

FIGURE 6
Accumulated somatic mutations in *Populus* and *Eucalyptus* CRISPR/Cas9 transgenics. **(A)** Somatic mutations in 353 and 717 poplars transformed with single and double gRNA constructs directed to the *PtaAG1* and *PtaAG2* genes. Somatic mutations are shown with filled squares. Locations and mutation types are shown to the right. **(B)** Somatic mutations in 353 and 717 poplars transformed with single and double gRNA constructs directed to the *PtaLFY* gene. **(C)** Somatic mutations in SP7 eucalypts transformed with single and double gRNA constructs directed to the *EgLFY* locus.

exceeding 10%, mutant allele frequencies were generally higher at the Potri.017G091300 locus (Figure 4A). We observed that off-target edits were usually shared between ramets of the same event, usually at similar allele frequencies. In poplar, off-target mutation sites were observed only in the *PtaAG*-targeting constructs, and none were observed in *PtaLFY* targeting constructs.

FIGURE 7
Validation of off-target edits at the *PtaSAW1* locus. 717 wild type and two off-target events (one in 717 and one in 353) were PCR amplified and analyzed by Oxford Nanopore long-read sequencing of the amplicons. Alleles with frequency over 5% are depicted in the figure. Mutations in each event (homozygous biallelic for #418, heterozygous biallelic for #198) are shown using black bold letters or dashes. Allele frequencies and the number of total reads supporting those frequencies by Nanopore, compared against the initial read depth in each sample by targeted short read, are depicted at right.

In both poplars and eucalypts, off-target mutations were only observed with one of the two gRNAs used to target the *PtaAG* or *EgLFY* genes. Thus, of the six total gRNAs employed in this study, only two were found to have off-target mutagenic potential. The numbers of mismatches and their location within the divergent gRNA spacer sequence are shown in Figure 5A. The numbers of mismatches to the true target sequence ranged from two to five base pairs, with *EgMCSF1* (2bpMM), Potri.017G091300 (3bpMM), *PtaSAW1* (4bpMM), and *EgEndoGluc22* (5bpMM) (Figure 5A). One site, *EgMCSF1*, was induced at a non-canonical NRG PAM location. The overall GC content in the mismatch site relative to the target sequence was less in both poplar off-targets, but higher in one eucalypt off-target site (Figure 5A).

Mutations in some of these events reached fixation, as illustrated in Figure 5B at the Potri.017G091300 locus, with indels in expected locations downstream of the PAM site in the mismatched gRNA. To look at the overall mutation patterns, we mapped the location of induced mutations relative to the PAM site across all off-target mutated ramets and then compared them to the mutations induced at the target loci (Figure 5C). Off-target mutations were preferentially induced at the -3bp site relative to the PAM, the same as at target sites, suggesting the mutations were indeed a result of CRISPR/Cas9 activity (Figure 5C).

We also assessed the allele frequencies in off-target mutated ramets and plotted them by poplar or eucalypt site (Figure 5D). *EgEndoGluc22* and *EgMCSF1* were maximally capped at 50% allele frequency due to only one allele being targeted for off-target editing, while Potri.017G091300 and *PtaSAW1* were edited at or near 100% allele frequency in some ramets due to both alleles having the potential for editing (Figure 5D).

3.5 Somatic mutation analysis

In our manual scoring process, we identified mutations which were greater than 20bp outside of mismatched gRNA spacer sequences, and these were classified as somatic mutations due to the unlikelihood of CRISPR/Cas9 associated mutations that far distally from a gRNA site (Fu et al., 2013). To investigate these mutated sites and how they appear amongst the population of transgenic poplars and eucalypts, we plotted the sites and allele frequencies (Figure 6). In general, they ranged widely in frequency and were associated mainly within individual events or ramets. Only one site, Chr06U:30441501 in eucalypts, was found in multiple events. These somatic mutations were a mix of SNPs and indels, although only SNPs were found in 353, and only one indel was found in SP7. In total, the computed somatic mutation rate for poplars (assuming such mutations are close to a random sample of what is occurring throughout the genome), was 2.5×10^{-8} in poplar and 4.8×10^{-8} in eucalypts.

3.6 Validation of off-target sites using long-read amplicon sequencing

To assess whether the identified off-target mutant sites occurred via a second approach, we amplified the off-target loci at *PtaSAW1* and Potri.017G091300, and resampled in the Spring of 2022. Using Oxford Nanopore long-read sequencing of the amplified regions, we obtained full length reads of the off-target locus. We sequenced two events of the *PtaSAW1* locus and compared them against a 717 wild type control (Figure 7). All events that were identified as mutated at *PtaSAW1* were mutated with the same identified mutations as the targeted sequencing approach. We sequenced one event at the Potri.017G091300 locus and found the same mutations as previously determined (Supplementary Figure S7).

4 Discussion

Using six different gRNAs, we targeted four independent loci and studied their mutation effects within nearly 100 individual trees. The transgenic tissues and derived trees had been growing for approximately 4 years from first transformation to DNA extraction (Supplementary Figures S1, S2). Though off-target mutation rates have been studied using a number of different approaches and in a variety of different plant species, to our knowledge none have looked at a comparable diversity of transgenic events, nor a comparable duration of somatic growth while continually expressing Cas9 and gRNA genes (Supplementary Figures S1, S2).

4.1 Bait-capture vs. other off-target analysis approaches

We chose bait-capture sequencing over other approaches because of the high depth of coverage at selected off-target sites (giving high confidence in our mutation calls, especially for low allele-frequency mutations), and its cost-effectiveness allowed us to study tens of thousands of potential off-target sites and many transgenic events. We investigated nearly 20,000 degenerate sites with up to five base pairs of mismatch relative to the target sequences; this enabled us to detect rare off-target mutable sites, including two that had four or more mismatches. However, this method did not allow us to detect larger structural mutations, which can be common results of some gene editing methods and transformation approaches (Fossi et al., 2019). We were also unable to detect epigenetic modifications. Thus, our conclusions are restricted to small indels and SNPs as off-target and somatic mutations.

4.2 Bait coverage density

In total, the targeted sequencing approach was effective at recovering deep coverage of these identified sites, though access to higher quality, individual reference genomes in the future will likely improve the capture efficiency. In our highly heterozygous tree clones, we saw decreases in bait-capture coverage depth in the 353 and SP7 clones relative to 717, which are likely due to the 717-focused probe design, and the lack of an SP7 eucalypt reference genome at the outset of the study, respectively (Figures 3A, C). Given the high GC content of the designed gRNAs, we hypothesized there off-target sites would be most common in gene rich regions; this prediction was statistically supported by our data, but the correlation with gene density was extremely weak (Figure 2C). We note that for poplars the number of identified mismatch sites was quite low in chromosomes 18 and 19, particularly compared against chromosomes one and 2, for which we have no clear explanation (Figure 2A). In eucalypts we saw no trends corresponding to chromosome/mismatch site abundance. When we compared available methylation and chromatin level data for poplar chromosome 19, we were unable to detect any features that could explain its reduced mismatch site abundance other than random variation in sequence composition (Zhou et al., 2023).

4.3 Mutation detection software

The choice of program to identify off-target sites was very important in our heterozygous genomes, where several initial attempts with other programs—seemingly designed for population genetics or SNP identification in inbred species—failed to identify meaningful signals amongst a sea of noise. Mutect2 was an ideal program for our clonally propagated trees and allowed us to clearly identify off-target mutations with a simplistic manual system in a genome browser. Though false positive rates in poplar ranged from 71 to 100 percent of filtered sites with finalized program settings (Supplementary Figure S4), it was possible to manually score given the number of filtered sites per construct (mean = 59, SD = 51). A function that would improve Mutect2 functionality for this type of study would be to give higher weight to alternative alleles within a window respective to the off-target PAM site. Within the existing program functionalities, setting Mutect2 to investigate very narrow intervals about the PAM at a predicted off-target site could be a simple approach to reduce scoring time or increase throughput. Overall, Mutect2 was serviceable, albeit labor intensive, to complete our analysis in three tree hybrids.

4.4 Identity and location of off-target mutations

We were able to identify four unique off-target loci (two in poplars, two in eucalypts) where unintentional editing occurred. Mutations occurred frequently at some of these sites across many different events (e.g., Potri.017G091300 and *PtaSAW1*). Though in two of these cases (Potri.017G091300 and *EgMCSF1*), some events are predicted knockouts at their respective loci, the poplar gene is expected to be a pseudogene because of its short peptide length compared to the nearest Arabidopsis homolog and the lack of RNA-seq support for the annotated gene region. Only one of the *EgMCSF1* events (66) is a predicted KO in one allele (the *Eucalyptus urophylla* allele is WT), with a frame shift at amino acid 143 resulting in a premature stop codon. The edited eucalypt transgenics did not have detectable growth effects in a greenhouse study of trees lacking the *p409S:AtFT* flower-enhancing transgene (Elorriaga et al., 2021), suggesting *EgMCSF1* does not have a strong phenotypic effect when knocked out. Unfortunately, transgenics without the *AtFT* transgene but edited for *EgLFY* no longer exist to test whether they were mutated at the *EgMCSF1* site or not, and thus whether this gene affects growth and physiology. At least for *p409S:AtFT* events with one mutated allele of *EgMCSF1,* no obvious morphological differences were found. In total, we found that the mutation profile of off-target edited sites exactly resembled edits at the target sites, so we are confident these were induced by CRISPR/Cas9 activity (Figure 5C).

4.5 Timing of mutations during plant development

In most cases, off-target editing was shared amongst events at the 10% allele frequency threshold (84%), but in some cases (16%) one ramet was edited but the other was not (Figure 4). Of these single ramet edited events, two-thirds had allele frequencies within

5% of the other ramet, and just failed to meet the 10% allele frequency threshold, highlighting the rarity of single ramet off-target editing. Thus, in most cases off-target editing is likely occurring in very early stages of transformation and organogenesis rather than during micropropagation and subsequent vegetative growth in the greenhouse or field. Our validation experiments by long-read amplicon sequencing on different branches and tissue types (dormant buds vs leaves) than had been initially sampled for the off-target study implies that off-target mutations were stable in the trees nearly 3 years after initial sampling. Still, the prevalence of a chimeric mutation in one of the surveyed ramets (Supplementary Figure S7) suggests there is a low level of ongoing mutation during vegetative growth, which could be characterized in detail to understand variation in its rate and cellular basis.

4.6 Surprising divergence of on- and off-target sequences

Together, the off-target sites (with their unexpectedly large mismatch of two to five base pairs) and their mutation patterns among events and ramets highlights several gaps in knowledge about off-target mutations induced by CRISPR/Cas9. At the outset of the study, we hypothesized that we would not find any off-target mutations, and that if we did, they would be one or two base pairs mismatched to the target gRNAs, as has been published in other plant species (Young et al., 2019; Wang et al., 2021). The prevailing methodology in the gene editing field is to design gRNAs with high *in silico* predicted activity, higher GC content, and as few as possible sites with one to two base pair mismatches elsewhere in the genome. Our study shows that sites with high affinity for Cas9/gRNA complexes exist that no current bioinformatic workflow would have predicted. Given current models for Cas9 off-target binding, we expected to see that mismatches more distal to the PAM would be more permissive for off-target editing. In fact, one of our most mutagenic off-targets, Potri.017G091300, was mismatched to the target sequence two bases downstream of the PAM (Figure 5A). This further highlights that we lack sophisticated knowledge of the biophysical affinity of gRNA/Cas9 complex to targets in plant cells. This is in agreement with the well-known lack of predictive power of *in silico* gRNA activity tools, suggesting that *in planta* optimization of gRNA choice in protoplasts, hairy roots, or similar systems is a good first step when any off-target mutations are unacceptable (Naim et al., 2020; Hodgins et al., 2024). Still, four out of six gRNAs had no off-target mutagenic potential we could detect.

In our survey of off-target mutations we also found somatic mutations within our surveyed bait-capture regions. These mutations were generally shared amongst events or ramets as expected, and showed anticipated somaclonal drift during the *in vitro* culturing process (Figure 6). Because we opted for a targeted sequencing approach that cannot detect large structural mutations, we cannot reliably estimate the total rate of somaclonal variation across the genome induced by transformation and culture.

4.7 Alignment with governance approaches

Though we did find off-target mutated sites in all three tree hybrids we investigated, the mutations were extremely rare in a whole genome context. At a predicted 1.2×10^{-9} in poplar and 3.1×10^{-10} in eucalypts, the rate of unintentional editing is comparable or less than the rates reported to be induced by a single selfing event in inbred species such as maize or Arabidopsis (3×10^{-8}, 1.36×10^{-9}, respectively) (Yang et al., 2017; Lu et al., 2021). Our observed rate is also less than somatic mutations in differing branches of long-lived trees such as oaks, estimated at $4–5\text{x}10^{-8}$ (Schmid-Siegert et al., 2017). Our results, as well as the long history of highly mutagenic methods during plant breeding, strongly support the decision by USDA to effectively ignore off-target mutation from gene-editing for regulatory purposes (Hoffman et al., 2022).

We surveyed a small number of gRNAs, but over a longer period of somatic growth and with more transgenic events than has previously been reported. However, some genome sites interacted strongly with the applied CRISPR complexes and led to mutations in a manner that was not predicted. A much larger study, with many more gRNAs and interrogation of more target sites, may shed light on the biophysical features of the CRISPR/Cas9 complexes that gave rise to its idiosyncratic behavior.

Our results suggest that there are only minute effects of retaining the editing reagents in the genome of a clonally propagated plant. Where plants are sterile, or for other reasons pose a low potential for gene drive, retention of the CRISPR/Cas9 locus in the genome appears inconsequential. In the trees studied, we expect strong and potentially permanent sterility from biallelic mutation of either *PtaLFY or PtaAG* targets based on gene suppression results in prior field studies (Klocko et al., 2016b; Lu et al., 2019), and due to the highly conserved bisexual functions of these target genes. As Cas9 does not appear to be immunogenic (Nakajima et al., 2016), and other Cas proteins originate from cultures used widely in food production such as yogurts (Horvath et al., 2008) they should also be safe for consumption as food or feed. In addition, the presence of Cas9 in the genome would facilitate further editing using gRNAs alone for diverse traits, perhaps through viral, physical, or transient methods. Nonetheless, where "clean editing" is required for market or regulatory needs, new methods appear capable of accomplishing this, even in clonal and sterile plants (e.g., Huang et al., 2023).

5 Conclusion

In a large field population of CRISPR/Cas9 edited transgenic forest trees that included three diverse genotypes from two genera, *Populus* and *Eucalyptus*, and that had been growing vegetatively for approximately 4 years since transformation, we found extremely low rates of off-target and somatic mutation. We also found that targets that were very different from gRNAs could be mutated, contrary to theory. It appears that, where socially acceptable, retention of CRISPR/Cas9 in gene-edited and transgenic plants could be a useful option, especially in highly sterile, long generation, and clonal plants.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA1109417 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1109417/).

Author contributions

GG: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. IA: Data curation, Formal Analysis, Investigation, Validation, Writing–review and editing. VC: Data curation, Formal Analysis, Investigation, Validation, Writing–review and editing. QX: Data curation, Investigation, Writing–review and editing. KV: Conceptualization, Investigation, Methodology, Supervision, Writing–review and editing. SS: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2024.1412927/full#supplementary-material

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Social license -What's in a name?

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Introduction

Should social license be used in sustainable bioinnovation?

Putting the question in context, all three notions, Social License, sustainability, and bioinnovation are trending and all three sound good, suggesting actions and goals worth pursuing (Purvis et al., 2019). However, that does not mean that Social License is necessary, or even desirable, for successful and responsible sustainable bioinnovation. Taking a closer look at the terminology: sustainability is a concept with a long history, dating back to at least the sixties and seventies of the 20th century, and it is used with different meanings in diverse contexts (Purvis et al., 2019). Biotechnology innovation has been advocated for in a concerted effort since 1993 (BIO Biotechnology Innovation Organization, 1993), and it involves all fields of biotechnology and related areas in, for example, medicine, economics, law, governance, and regulatory sciences. In this short essay I will zoom in on the notion and practice of Social License and discuss whether it is an appropriate instrument to achieve the goals of sustainable bioinnovation, using an example from synthetic biology.

Social license, what's in a name?

"Social License" is short for "Social License to Operate" (SLO) and this notion originally arose in the context of extractive industries—mining—and later became wider used for interventions in natural ecosystems or in social systems, as, for example, public health practices, or even in a combination of those spheres: the assumed license to carry out programs for wide-area vector control. I will get back to that use case further below.

In 1997, James Cooney, executive director of a gold mining company, discussed the problem of "obtaining social license to operate" in a discussion with the World bank (Gehman et al., 2017). Two industry consultants, Joyce and Thomson, developed the concept of SLO further, and added the aspects of legitimacy, credibility, and trust. Their foundational definition reads:

"A social license to operate exists when a mineral exploration or mining project is seen as having the approval, the broad acceptance of society to conduct its activities. Such acceptability must be achieved on many levels, but it must begin with, and be firmly grounded in, the social acceptance of the resource development by local communities" (Joyce and Thomson, 2000).

This definition sounds good, in the moral sense, emphasizing "approval," "acceptability, "social acceptance," but this may be too good to be true if we take the context into account: extractive industries. In other words, an outside third party strives to be granted permission for operations that are their primary for-profit business interest. In this setting, neither the "social" nor the "license" refer to moral values or altruism. Is Social License to Operate "a term largely invented by business for business," as Gehman et al., 2017 suggest? Similarly, Kemp and Owen, 2013, in their thorough critical analysis, conclude "Even through an appreciative read, social license remains a pragmatic calculation of what is required to minimize business risk and win the degree of community support required to avoid delay or disruption to company operations".

Social License was further developed and put into practice in mining projects by Thomson and Boutilier, and they founded socialicense.com (The Social License to Operate, 2020). The widely used "Social License Pyramid" visualizes the "conversion" of a community from initial refusal to allow mining activities on or under their land, to not only approval, but above and beyond that psychological identification with the third party exploitation of their resources. This strategy raises serious ethical, social, legal, and political concerns and has been addressed in detail by Kemp and Owen, 2013, Gehman et al., 2017, and many others.

While the criticism above refers to the use of the term Social License to Operate in mining, and to similarly extractive activities like logging, we need to ask whether the terminology should be adopted in responsible bioinnovation, including projects that aim at providing populations with improved crops or pursue public health goals, as is the case in area-wide vector control, for example, control of malaria or dengue transmission through genetic interventions in mosquitos.

Social license in bioinnovation

In a recent study on the use of "Social License" in synthetic biology (Delborne et al., 2020), we conducted a systematic review of the literature until mid 2019, and found increasing use of "social license to operate" in peer reviewed and non-peer reviewed literature in the synbio field from 2015 till 2019. Similar to the use in the mining context, obtaining social license was used to describe the societal buy-in necessary for the development and use of novel synthetic biology-based technologies. A first reference we found, was in a 2015 meeting report of the de-extinction-focused organization Revive&Restore (Revive and Restore, 2015). We encountered the introduction of this term known from the extractive industries while being involved in research on the social and ethical aspects of gene drives, a novel and highly controversial bioinnovation. A gene drive is a construct occurring naturally or achieved through genetic engineering that enables a trait to spread through a population of animals or plants with higher than Mendelian probability (James et al., 2023). That means that traits of a species—for example, transmitting malaria—can be overpowered or even erased. Not all gene drive research and development is for-profit, and many projects are enabled by philanthropic funding, for example, Target Malaria (Target Malaria, 2021).

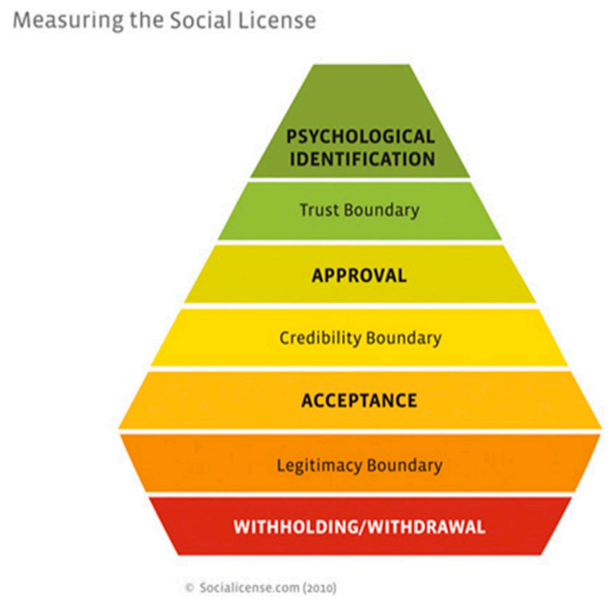

FIGURE 1
Measuring the social license.

One specific feature of gene drives, and of many applications of genetically modified organisms, is that their intended use is in the shared environment and self-propagation in an ecosystem, irrespective of local sovereignty or national borders. And exactly that raises the question of acceptability, and of community consent: who benefits and who is affected by potential harms? Like the case of mining, the question is whose interests are served and who decides about initiating the activity—here the deployment of the genetic constructs.

It is tempting to introduce the concept of "Social License": if successfully obtained, it would change attitudes of the involved population from refusal to embracing the technology and even psychological identification. At least, that is the idea.

The reality, however, is far more complex and there are important differences between mining operations and bioinnovation projects. Deciding about the introduction of synthetic biology-based ecosystem interventions is complex due to a number of features that are inherent to these technologies. These features are different from those in mining. In mining, estimates about the effects on the environment are possible, based on very longtime experience: evidence of earliest mining activities in Ngwenya dates from 43.000 years BC (UNESCO World Heritage Convention, 2008). The many forms of lasting damage to the environment from modern mining are well-documented, mitigation and environmental remediation are lagging behind (MIT, 2016).

Synthetic biology-based ecosystem interventions are new and while *in silico* simulations yield probabilities about the effects—both the intended beneficial outcomes and the potential adverse effects—these remain probabilities. Inherent system effects make predictions difficult: all ecosystems are part of other, larger ecosystems and the scale of extent over time is hard to predict. While reversibility is often intended and is, for example, one of the criteria for gene drive release, it is unknown whether this will be the case indeed. After self-propagating organisms have been released in the wild the further course of events may be hard to influence. The fact that these organisms will cross geographic and national boundaries adds a complication, also in terms of international law. At the same time, the goal of the interventions is to realize the benefits of sustainable bioinnovation to communities and populations.

How can these technologies be introduced in a responsible manner, doing justice to humans and the environment? In my opinion, it cannot be Social License. A model that is inclusive, proactive, integrative, (it still has to be developed and proven) would rather focus on "Social Agreement" and not Social License. There is a substantial difference between reaching agreement or giving/obtaining a license.

Bioinnovation—a window of opportunity

While novel bioinnovation projects are still at early stages of implementation—or even still at the stage of a research setting, as, for example, large cage experiments in gene drive mosquito research—there is a unique window of opportunity to consider possible scenarios for responsible deployment that include all stakeholders.

First, the stakeholders: who are stakeholders in the development of novel technologies that will be deployed in the shared environment?

The potential impact of synthetic biology-based ecosystem interventions could ultimately be global, but does that mean that the global community—if we can even say who they are—have a stake and should have a voice in decision-making? It is an easy claim that sounds good, but it does not recognize that stakes are unequal, some people have larger interests at stake than others. With these technologies it is fair to say that the communities where the technologies are actually being introduced and used have the weightiest interests. They may also receive the largest benefits, like a gene drive-based technology to curb the spread of malaria benefits the people in the regions where malaria occurs. In order to reap those benefits, these communities also bear the risk of possible adverse effects, if any would occur. As with biomedical and public health innovation, to be ethical and qualify as responsible innovation, four criteria must be met: a technology must be Available, Accessible, Acceptable, and Affordable for the users and other stakeholders (the 4A Framework[1]).

The 4A Framework is also a benchmarking tool during research and development. Genuine involvement of the primary stakeholders can already start at the stage of research, setting the research goals in alignment with the needs of the communities who will be the users. The likelihood that a technology is acceptable—socially, culturally—is far greater when the future users are involved at the stage of development. Accessibility, for example, deployment logistics, and affordability will usually depend on higher level structures in a society and those may be a bigger bottleneck in the innovation process. The 4A Framework thus outlines a mixed bottom-up and top-down model.

Communities can proactively seek solutions for their problem, thereby driving research. This is a key difference with the Social License model that is driven by third party—economic—interests to which communities should ultimately agree through a reward-oriented strategy. Social License, notwithstanding the pyramid visualization, is a top-down model. Moreover, history (extensively described by Boutilier and Thomson (2019) in the story of the San Cristobal mine) has shown that the "original" Social License is very difficult to obtain and even more difficult to sustain. Therefore, it may not even be worth pursuing this concept in sustainable bioinnovation. The big question is what the alternative can be. Understanding why not Social License is a first step. The key question in bioinnovation, also from an ethics point of view, is where does innovation or the wish for innovation start, who is the initiator and what is the motivation. The next question is about decision making, who decides and how are decisions made in, e.g., "co-production." Co-production is aspirational, what is the next step to make it a reality? There is no clear answer/solution that is universally valid.

Examples of approaches to bioinnovation with early community and other stakeholder involvement have been the Genetic Biocontrol of Invasive Rodents (GBIRd) project, a global partnership of seven organizations including researchers at North Carolina State University, ATM University, the University of Adelaide, CSIRO (Australia), and the USDA (GBIRd Genetic, 2017).

The Responsive Science initiative, led by a team at MIT Media Lab (of which the author was a member), featured proactive open interaction between researchers and communities, interaction from the earliest stages of ideas and project design, and "adaptive science"—ongoing improvements in research based on new scientific insights and community input. Pilot studies, however, showed some of the real-world obstacles (Najjar et al., 2017; Buchthal et al., 2019; Normandin et al., 2022).

A different approach is found in communitarian values-based models, in the African context, for example, the use of Ubuntu, setting goals, methods and modes of interaction in alignment with regional values. A central element of Ubuntu is the interdependence of the interests of individuals and communities—in their environment—and their reciprocal obligations (Sambala et al., 2020; Shozi and Thaldar, 2023). Ubuntu thereby bridges the bottom-up and the top-down structure of other models. Given the strong interest on the African continent in sustainable bioinnovation (Bizoza, 2024), these authentic ethics approaches may hold great promise and the same may be true for value-based approaches in other areas in the world.

Sustainable bioinnovation does not need Social License, it rather needs innovative models for community agreement and responsible decision-making.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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[1] The 4A Framework was developed by Lunshof in 1989, in the Netherlands, in collaboration with organizations of patients affected by rare genetic disorders

Publisher's note

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Building bio-innovation systems through advanced biotechnology education

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We discuss the role of advanced biotechnology education in fostering sustainable bio-innovation systems. As a case study, we focus on Paraguay's Graduate Diploma in Innovation Management and Biotechnological Projects, which emphasizes interdisciplinary collaboration, stakeholder integration, and professionals skilled in the interplay between biotechnology, society, and governance. We highlight the relevance of educational programs in addressing the gap between academic research and industrial needs, thereby contributing to sustainable growth in the biotechnology sector.

KEYWORDS

sustainable development, stakeholder integration, virtual learning, biotechnology policy and regulation, innovation and entrepreneurship

1 Introduction

To address global challenges such as climate change and food security, a sustainable bio-innovation system is essential. This involves developing solutions that promote economic growth while minimizing environmental impacts, addressing social concerns, and engaging in participatory governance (McCormick and Kautto, 2013; Falcone et al., 2019; Eckardt et al., 2023).

In the last 10 years, with the support of the National Council of Science and Technology (CONACYT), Paraguay has started to develop advanced educational programs with the objective of generating professionals ready to tackle these challenges (Delgado, 2023). Significant resources have been used to develop biotechnology-related programs, and one of their strategic axes is to support projects related to bio-innovation.

An efficient education system provides the necessary knowledge and skills to make informed decisions regarding the development and application of new technologies (Steele and Aubusson, 2004). This is essential for comprehending the intricacies of biotechnology, including business aspects, intellectual property protection, and regulatory environments (Narasimharao, 2010). Furthermore, thorough education fosters biotechnological literacy, which is becoming increasingly essential in contemporary society, due to significant advancements in agriculture, industry, and medicine (Mohd Saruan et al., 2015; De La Hoz et al., 2022).

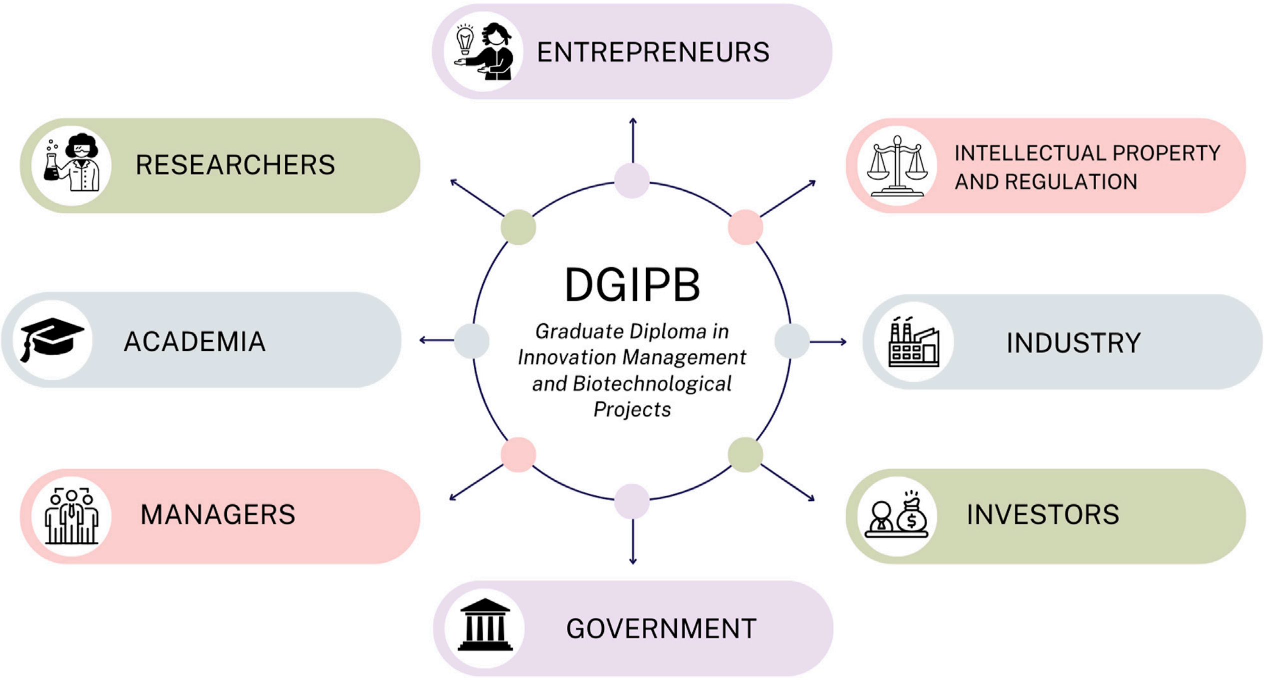

FIGURE 1
Stakeholder Integration in the Graduate Diploma in Innovation Management and Biotechnological Projects (DGIPB). This figure illustrates the DGIPB stakeholder network, reflecting the multidisciplinary collaboration that is essential for advancing bio-innovation.

2 How can a curriculum be developed for a bio-innovation course?

2.1 Aspects that should be considered

The development of an educational program focused on bioinnovation requires planning that considers several factors. For this purpose, we will take the case of a Graduate Diploma in Innovation Management and Biotechnological Projects (DGIPB) developed by the Faculty of Exact and Natural Sciences (FACEN) at the National University of Asunción (UNA) as an example to address the need for biotechnological applications that can solve identified problems and capitalize on opportunities (CONACYT, 2023). This program aligns research outcomes with practical issues, facilitating the development and adoption of bio-innovative strategies while also considering economic factors. The primary goal of the DGIPB is to build bridges between academia, business, regulation, and civil society, ultimately facilitating the development and adaptation of technologies for their transfer to the productive and social sectors through the assistance of *Innovation Managers* (Figure 1).

The Diploma, which was co-funded by FACEN and CONACYT through the Innovation Program in Paraguayan Companies (PROINNOVA), explicitly focuses on biotechnology. The program is designed to equip professionals with the ability to combine scientific, economic, and legislative understanding to recognize the context of biotechnological development initiatives. Furthermore, it offers guidance in identifying the intricacies of international and domestic legislation concerning intellectual property protection.

The DGIPB curriculum includes three modules.

1. Fundamentals of bioeconomy and innovation: This module examines the dimensions and aspects that define biotechnology development systems, providing a comprehensive overview of the industry, including its technology, growth prospects, and economic development. In addition, this module addresses the key elements that impact activities related to the bioeconomy and innovation ecosystems, including public policies and regulatory frameworks.
2. Innovation management and biobusiness: This module is designed to help participants understand the nature of managing innovation in the biotechnology sector and develop appropriate business models that can leverage the benefits of an innovation management system.
3. Formulation of biotechnology projects: The final module focuses on identifying key variables and actions involved in the conception, planning, execution, and evaluation of biotechnology projects. This module culminates in the formulation of an innovative biotechnology project that serves as the capstone of the course.

While there are similar programs in the country that focus on project management and innovation, two factors make the DGIPB unique. First, none of them specializes in bioprojects. This highlights its relevance in the training of professionals in the Paraguayan context. Second, as the regulatory environment has a significant impact on the direction and speed of innovation, the DGIPB also focuses on public policies and regulatory frameworks that govern various biotechnology industries. Unfortunately, this aspect is frequently disregarded in similar courses, which can have severe repercussions on the innovation landscape (Bogner and Torgersen, 2018).

One of the features of the DGIPB is that it was conducted entirely virtually, which facilitated interaction between students and professionals who were geographically distant from one another. Virtual education has been demonstrated to be effective as a flexible and time-efficient means for students to receive guidance (McReynolds et al., 2020), enabling them to establish multiple relationships and seek advice from the entire network during the program. Furthermore, to promote and encourage enrollment, CONACYT provided economic support to organize the DGIPB and full scholarships for all admitted students, granting them the opportunity to attend at no cost.

2.2 Is it possible to measure the course's impact on bio-innovation?

Nineteen students completed the DGIPB in the first edition of the program. Among them, nine belonged to academic institutions, such as universities and research centers; six worked in the private sector for companies involved in pharmaceuticals and agro-inputs; and two were members of the government sector. Students were given the opportunity to create, develop, and refine Research and Developments and Innovation projects (from now on R&D + i) that focused on the application of biotechnology and introduction of bioproducts to the market. Through this process, they were able to identify the key factors involved in each stage of a biotechnology project, and use analytical tools to assist in planning, controlling, and managing these projects.

Nine projects were developed within the DGIPB framework, three of which were presented at the III Paraguayan Biotechnology and Applications Conference. This event, directly linked to the DGIPB as a graduation act, was themed "Innovation and Bio-business," and featured participants from the Ministry of Industry and Commerce, Paraguayan Industrial Union, and private investors focused on Science, Technology and Business (STBs). The initiative was supported by Technological Linkage Managers from the National University of Rosario and the National University of San Martin in Argentina as well as company executives and investor groups from GeneBiome EAS, Paraguayan Association of Venture Capital (PARCAPY), Paraguayan Innovation Investment Fund (FIIP), OpenX in Paraguay, GRIDX in Argentina, and Bioeutectics in Argentina and the United States. The expertise of these individuals and organizations is crucial for the development of a scientific business ecosystem in Paraguay (FACEN, 2023).

The primary focus of projects initiated by DGIPB graduates was on the development and delivery of biotechnology-based goods and services (OECD and Eurostat, 2018a). These projects emphasized R&D + i and entrepreneurial endeavors. One such project, led by a graduate working in the government sector at the Ministry of Industry and Commerce, focused on innovation management. This area involves all-encompassing activities essential for planning, governing, and managing resources to foster innovation (OECD and Eurostat, 2018b). This project also diverged from the typical trajectory pursued by natural science professionals, who generally focus on the technical aspects of product development. This divergence highlights a distinct application of the skills and knowledge acquired through the DGIPB programme, thereby underscoring its versatility.

After completing each module, surveys were conducted among the students to obtain feedback, which was subsequently used to make improvements (Table 1). These results demonstrated that the DGIPB was highly appreciated. Students expressed satisfaction with the content and valued their interactions with actors in the regional innovation environment. The DGIPB facilitated a stronger connection between the academic and business sectors, highlighting potential commercial applications rooted in scientific and technological advancements.

The interactions that took place between professors, mentors, guest speakers, and students were very rich and fruitful in the DGIPB. Professionals from outside academia were eager to collaborate with the program by sharing their experiences through discussions, reflecting the urgency of developing a collaborative system for bio-innovation.

3 Biotechnology ecosystem for sustainable bio-innovation

Paraguay has been identified as one of the countries with the highest growth projections (IMF, 2021). Despite the COVID-19 pandemic, macroeconomic stability remains appealing to investors. However, the percentage of Gross Domestic Product (GDP) allocated to R&D + i in Paraguay is significantly lower than the regional average (0.15% in 2022 compared to 0.61% in Latin America and the Caribbean). Nevertheless, the public sector is the primary source of funding, and primary efforts to conduct R&D + i activities are led by universities and public agencies (CONACYT, 2024).

Paraguay's latest socio-economic development strategy, outlined in the IICA Bioeconomy proposal, calls for the expansion of biomass supply and the creation of sustainable conditions for its reproduction. Additionally, the plan aims to add value to local production, give businesses a competitive edge in the global bioindustry market, and establish connections with dynamic global markets (Productiva, 2022).

Although Paraguay has established a regulatory framework for biotechnology that can facilitate locally developed products (Benitez Candia et al., 2024), the country's scientific–technological ecosystem, consisting of public and private research institutions, universities, and funding organizations, does not seem to provide complete support for local development. While all technological projects are evaluated in terms of R&D + i costs, regulatory aspects, potential benefits, prospective markets, and possible degree of adoption, sufficient funding is often unavailable to advance and complete final product development. This phenomenon also occurs with products obtained through new breeding technologies (NBTs), which may be more readily implemented because of their streamlined regulatory procedures (Fernández Ríos et al., 2024).

Considering this scenario, FACEN has established inter-institutional cooperation agreements with various organizations to enhance education, research, and technological development.

TABLE 1 Outcomes and project engagement of the DGIPB graduates.

Indicator	Degree of compliance (%)	Lessons learned
Percentage of graduates whose subsequent professional practice is linked to innovation management and/or biotechnological projects	70	Graduates remain connected to innovation and/or biotechnological projects, suggesting a link between their professional endeavors and the DGIPB. This continuity highlights the relevance and practical applications of the course
Tools or skills acquired in the DGIPB for professional practice	100	There is a primary interest in planning and management tools as well as in financial administration. The experiences of expert guests (entrepreneurs and investors) were valued
Percentage of graduates who participated in at least one project after completing the DGIPB	62	The graduates participated in at least one project after completing the DGIPB
Percentage of new projects generated by graduates after completing the DGIPB	54	Almost half of the graduates generated at least one new project after completing the DGIPB
Percentage of projects associated with public entities	77	Most projects are associated with the public sector and are funded by research funds. More emphasis should be placed on opportunities for private investment
Percentage of projects linked to foreign companies or institutions	15	Only two projects showed links to foreign capital. Emphasis should be placed on private foreign capital investment opportunities

These agreements facilitate collaboration between domestic and international companies and institutions, promoting strategic alliances to train highly qualified entrepreneurs. This is crucial for addressing the challenges outlined in Paraguay's Vision 2030, which aims to transform the economic model into a knowledge-based one (STP, 2014).

Throughout the DGIPB, investors identified the disconnection between academia and the production sector as a significant issue. The lack of coordination, information, communication, and articulation among different innovation actors is deemed a critical problem. For instance, the DGIPB facilitated discussions between a company led by a diploma graduate and FACEN, with the aim of developing local bioproducts. However, it was observed that many companies in the production sector are still unaware of the existence of biotechnology professionals in the country, resulting in them seeking foreign advice.

This type of collaboration between public and private sectors has historically been viewed as a strategy for enhancing research responsiveness to evolving global challenges, thereby expediting innovation and facilitating broader economic and social benefits from joint investments made by governments and private industries. Furthermore, these collaborations can facilitate the alignment of academics' specialized knowledge with the skills of industry scientists, thereby translating scientific advancements into practical applications within a stable funding environment (Gloger et al., 2021).

4 Discussion

Considering a regional scenario, prior to 2001, scientific research and development in Latin America had not been a priority for governments, in the same manner as in similar-sized economies. However, a period of sustained investment followed with budget allocations for state research agencies and the formation of new Ministries for Science. Additionally, funding was provided for international training programs, leading to an increase in scientific output and the return of scientists who had previously been based overseas to lead research initiatives in their home countries. This investment demonstrates the potential benefits of shifting towards a knowledge-based economy (Catanzaro et al., 2014; Van Noorden, 2014).

Experiences such as GlaxoSmithKline (GSK) introduced a pioneering model for public-private collaborations called the *Trust in Science* initiative (GSK, 2018). Initially concentrated in Argentina and Brazil, this initiative was later expanded to include Uruguay and Mexico. The model stands out because of its emphasis on direct scientific collaboration between academic and industrial scientists, its specialized approach to intellectual property rights for academic collaborators, and its transparent process for project applications and joint funding decisions with governments (Gloger et al., 2021).

Trust in Science has also taken steps to ensure that its advantages extend beyond financial support to encompass mentorship, project guidance and direction, and fostering local scientific talent. This collaborative effort has provided professionals valuable experience in learning how their research ideas can be transformed into commercially viable products. In contrast, Paraguay has not yet reached the same level of success as its Mercosur counterparts and has not been involved in similar initiatives.

Despite the advantages of industry-academia partnerships, there are also challenges that must be considered. For instance, a sector of the academia views with apprehension the private sector´s sponsored research as market driven, at the cost of basic science and academic freedom (Palmer and Chaguturu, 2017). Furthermore, concerns regarding intellectual property rights may arise, emphasizing the importance of addressing these issues to ensure long-term sustainability. Bio-innovation has the potential to affect socioeconomic development significantly, underscoring the need for a skilled workforce to guide progress. Educational programs that provide a solid foundation in biotechnology management can potentially bridge the gap between academia and industry, ensuring that graduates are adequately prepared to fulfill the requirements of the biotechnology sector. This alignment between education and industry demand is critical for the sustained growth and success of the biotechnology industry.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Author contributions

EC: Conceptualization, Investigation, Methodology, Software, Validation, Writing–original draft, Writing–review and editing, Data curation, Project administration, Resources. SQ: Conceptualization, Investigation, Methodology, Software, Validation, Visualization, Writing–original draft, Writing–review and editing. CR: Data curation, Project administration, Writing–review and editing. DF: Conceptualization, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fertilizer management practices in potato cultivation: a baseline study for the introduction of GE potato in Bangladesh

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Genetically engineered (GE) crops have the potential to contribute to agricultural sustainability, food security, and nutritional enrichment. However, these crops cannot be released for commercial cultivation without undergoing environmental risk assessments (ERA), thus biosafety evaluation. ERA assessments are performed comparatively with their natural non-GE counterparts. As Bangladesh is progressing with GE potato research, the present study aims to collect baseline information on non-GE potato cultivation with an emphasis on current agronomic practices focusing on fertilizer management and farmers' knowledge base. The survey had three parts, including information on the farmers, information on potato cultivation practices, especially fertilizer use, and lastly, the farmer's view on GE potato. From 2020 to 2021, data were collected through interviews with experienced growers in four potato-growing regions, the Central and Mid-East, North-West, Mid-West, and South-East regions (n = 1757) of the country. The study revealed that farmers of all regions used more than the recommended amounts of fertilizer; for instance, 67.1% more nitrogen fertilizer was applied as an extra dose during potato cultivation in Munshiganj (Central and Mid-East) than in the Dinajpur region (North-West). This overuse of nitrogen fertilizer can enhance plant vigor but makes the plants more susceptible to insect attraction and allows pests easier access to the plants. As a result, the excess dose of nitrogen fertilizer in Munshiganj may act as a catalyst to increase the probability of late blight. The findings also showed that 73.6% of farmers observed unexpected flowering in certain potato cultivars, which corresponded to the higher application of phosphate and potassium fertilizers aimed at late blight control. Furthermore, this study reported infestations of Solanaceous weeds, specifically *Solanum torvum* and *Physalis heterophylla*, in potato fields. Finally, our findings demonstrated that more than 68.7% of the potato growers intend to adopt disease-resistant GE potato as that may reduce the need for excess fertilizer use and thus reduce cultivation costs.

KEYWORDS

agronomic practices, baseline data, biosafety assessment, fertilizer management, potato

1 Introduction

Potato (*Solanum tuberosum*), belonging to the family Solanaceae, is a staple food and the world's fourth most important crop after rice, wheat, and maize (Islam et al., 2017). Bangladesh ranks as the seventh largest potato-growing country globally and third-largest in Asia (Crops. FAOSTAT, 2018). Recent data indicate that Bangladesh is self-sufficient in potato production, yielding 9.87 million metric tons by cultivating 468.7 thousand hectares of land (BBS, 2023). Despite this impressive production, potato exports are significantly hindered due to their compromised quality, which results from various diseases (Mittler, 2006).

Potato is highly susceptible to various diseases caused by fungi, viruses, and bacteria, resulting in significant crop damage and yield loss (Islam et al., 2022). Farmers commonly use fungicides and insecticides to combat these infestations (Kromann et al., 2009). However, the extensive use of these chemicals raises serious food safety and environmental concerns, highlighting the need for alternative disease management strategies (Islam et al., 2018).

Plant breeders have worked relentlessly for centuries to develop improved crop varieties with enhanced disease resistance. Although most disease-resistant crops have been developed through conventional and molecular breeding approaches, these methods have limitations, for instance, limited genetic resources within the gene pool, cross-incompatibility, and prolonged development phases. Genetic engineering can overcome these barriers by allowing the integration of beneficial genes from any source into targeted crops, providing tolerance and resistance against both abiotic and biotic stresses in a shorter breeding time. Moreover, considering the adverse impacts of chemical treatments, the introduction of disease-resistant potato varieties through genetic engineering offers a feasible and environment-friendly alternative for disease control (Lozoya-Saldana, 2011). Experience from nearly 3 decades of commercial cultivation of various GE crops has demonstrated its benefits to the growers. Therefore, the adoption of GE crops is rising globally (Bhajan et al., 2022), particularly in developing countries like Bangladesh (James, 2017).

In 2013, Bangladesh became a biotech country by adopting Bt eggplant, a genetically modified variety developed by introducing the Cry1Ac gene from *B. thuringiensis*, which provides resistance against eggplant fruit and shoot borer (EFSB) (Shelton et al., 2018). In 2023, commercial cultivation of Bt cotton began, which was developed by inserting the same gene of the Cry family (*Cry1AC*) as Bt brinjal from *Bacillus thuringiensis*, conferring resistance to cotton bollworm (ISAAA, 2023; Sujan et al., 2024). Bangladesh has been engaged in research on GE potato since 2006, and to date, several confined field trials were conducted on late blight (LB) resistant potato developed by introgression of three LB resistance genes, *Rpi-mcq1, Rpi-blb2, and Rpivnt1.1* (Potatopro, 2021; USDA, 2021).

However, the commercial cultivation of GE crops requires prior biosafety assessments to ensure environmental safety. According to the Guidelines for the Environmental Risk Assessment (ERA) of Genetically Engineered (GE) Plants 2016, one key issue to consider is the comparison of cultivation practices between GE and non-GE natural counterparts. Agronomic performance and crop physiology also need to be evaluated during these assessments. Therefore, it is necessary to understand the common cultivation practices employed by Bangladeshi farmers for potato cultivation and to identify potential changes that may occur with the introduction of the GE potato.

Considering the above-mentioned issues, a foundational survey was conducted throughout the potato-growing season to collect information from both potato growers and seed potato producers. The study aimed to understand their approaches to fertilizer management and other agricultural practices during potato cultivation. To achieve this goal, several specific objectives were outlined, including i) Collecting baseline information on potato cultivation, ii) Gathering data on the use of macro and

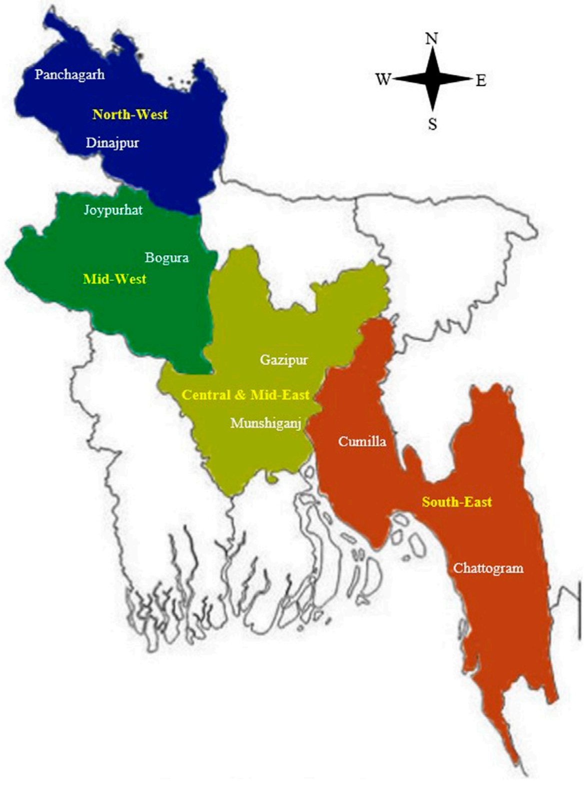

FIGURE 1
Geographic distribution of survey sites across Bangladesh, encompassing four study regions.

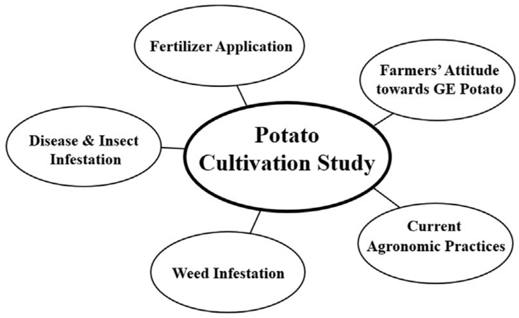

FIGURE 2
Research model of the study.

micronutrients in the form of manures and fertilizers in potato cultivation, and iii) Understanding the attitude of Bangladeshi potato growers toward GE potato.

2 Materials and methods

2.1 Demographic site selection

A total of 1,757 potato growers were randomly interviewed across various upazilas, taking into account the diversity in climate and soil types across regions, with consideration given to their varying levels of academic knowledge and financial circumstances. A total of 16 study locations have been selected from the four major potato-growing regions in Bangladesh, namely, i) Central and Mid-East (Munshiganj and Gazipur districts, n = 404), ii) North-West (Panchagarh and Dinajpur districts, n = 527), iii) Mid-West (Joypurhat and Bogura districts, n = 439), and iv) South-East (Cumilla and Chattogram districts, n = 387) (Figure 1). Each region was represented by two districts, and within each district, two upazilas were chosen for data collection and analysis.

2.2 Survey

This survey was carried out by the Advanced Seed Research and Biotech Centre (ASRBC), ACI Ltd., Dhaka, Bangladesh. In this survey, information and data were collected from farmers owning at least 10 decimals (1 decimal = 435.56 sq. feet) of land for potato cultivation. The potato growers from each region were selected through a standard field survey technique (BBS, 2014). The risk impacts survey consisted of five parts: 1) fertilizer management, 2) agronomic practices, 3) disease and insect infestation, 4) weed infestation, and 5) knowledge and willingness about GE technology among farmers.

2.3 Data collection methods

The data collected through a pre-tested questionnaire were tabulated, processed, and analyzed, keeping in view the goal and objectives of the study. Information and data were collected by a team of 16 designated enumerators, with two enumerators assigned to each district, as part of this comprehensive survey conducted among farmers. The questionnaire included several key data indicators, encompassing the farmer's demographic information such as name, age, sex, and education, as well as details regarding farm size, agricultural practices, and the management of manures and fertilizers. Additionally, the questionnaire also addressed the recommended doses of fertilizer, potential risks, and economic damages stemming from insect or pest infestations in potato cultivation. It also inquired about the effectiveness of the farmers' weed, insect, or pest control measures and assessed their attitudes toward acceptance of advanced technology for potato production. A theoretical working model (Figure 2) was utilized that integrates the innovation adoption model by Rogers et al. (2003) with the construct of attitude from the attitudinal models by Fishbein and Ajzen (1975), as outlined in the research model proposed by Kwade et al. (2019). This approach enabled us to identify factors related to agronomic practices in potato cultivation, explore the risks associated with potato farming, and examine the attitudes of the people of Bangladesh toward genetic engineering (GE) technology.

2.4 Statistical analysis

To evaluate the association between attitudinal variables, bivariate Pearson correlation analysis was used with the help of GraphPad Prism 9.00, United States. A paired sample *t*-test was used to examine the differences between the opinions. Multinomial logistic regression was used to identify significant variables of growers' perceptions of major obstacles in potato production and willingness to adapt GE technology for potatoes. As a result of allocating the response categories, the dependent variable was transformed into a variable with two categories (yes/no). This strategy was used because our primary objective was to estimate the likelihood that people will accept the GE potato (willing or not) while also allowing for the assessment of differences between the indifferent farmers and the intended adapters or non-adapters. Because of the strong correlation between farm size (ha) and potato cultivation area (ha), the latter was not included in the model. Gender was excluded from the socio-demographic variables due to its extremely skewed distribution toward male farmers. Knowledge of GE technology, ethical and socio-economic concerns, and environmental benefits were entered as continuous variables in the model, whereas farm size, farmer education level, number of fertilizer applications, age, and experience in potato cultivation were included as dummy variables. The demographic information about participants (n = 1757) is shown in Table 1, and the questionnaire containing the statements chosen for data collection and analysis is shown in Table 2.

3 Results

The findings of the survey have been categorized and presented in several ways to demonstrate the current state of potato production as well as potential strategies of fertilizer management and agronomic practices for the introduction of GE potato in Bangladesh.

TABLE 1 Gender, age group, and academic qualification of the study population (n = 1757).

Demographic profile	Category	Number	Percentage (%)
Gender	Male	1729	98.40
	Female	28	1.60
Age group	18–20 years	26	1.50
	21–30 years	504	28.70
	31–40 years	761	43.30
	41–50 years	365	20.80
	Over 50 years	101	5.70
Academic qualification	Elementary school	763	44.80
	Secondary school	543	30.90
	Higher secondary school	390	19.20
	University	61	5.10

The demographic characteristics of the study participants, detailing their gender distribution, age groups, and academic qualifications, are presented. The total sample size was 1757 individuals.

TABLE 2 Selected statements on common agronomic practices during potato cultivation in Bangladesh according to the questionnaire.

Statement
1. Both organic (cow dung and ash) and inorganic (chemical fertilizers) fertilizers are frequently used in potato cultivation across the country 2. Basal dosage as the granular form is used to apply TSP (P), magnesium (Mg), gypsum (S), and boron (B), except urea (N) and MOP (K) fertilizers, during the last plowing of the soil preparation 3. Urea and MOP are applied as split doses in the first and second earthing up of potato cultivation 4. Micronutrients: boron (B), copper (Cu), zinc (Zn), and manganese (Mn) are supplied in liquid form 5. High dependence on fertilizers in potato cultivation is not only for plant growth enhancement but also for disease management 6. Several weeds (*S. torvum*, *P. heterophylla*, *A. philoxeroides*, *C. album,* and *A. viridis*) have been identified in potato fields and are primarily managed manually 7. Groundwater dependency for irrigation is very high 8. The advanced agronomic practices that are employed in quality potato production (processing and export quality) globally are largely unknown to farmers 9. After harvesting, plant debris is left in the field 10. Late blight, virus infestation, and scab disease are the most common diseases that significantly affect potato quality and yield in the country 11. Excessive pesticides and insecticides are frequently used to control diseases 12. Farmers have no knowledge about environmental pollution when applying fertilizers and pesticides in the potato field 13. Positive attitudes have been shown among the farmers in the acceptance of disease-resistant and high-yielding GE potato varieties

These statements from the questionnaires reflected common agronomic practices employed during potato cultivation in Bangladesh. These statements are designed to capture key aspects of agricultural methods and techniques used by local farmers.

3.1 Demographic information

According to the demographic profile of the respondents (n = 1757), the sample consisted of a group of participants with characteristics that reflected the diversity of the research population (Table 1). The gender distribution was 98.4% male and 1.6% female, reflecting the typically male-dominated agricultural profession. Most of the total study population had more than 10 years of experience in cultivating potato (data not shown). In addition, the largest age group of participants, at 43.3%, was people between the ages of 31 and 40 years. In terms of formal education, only 5.1% of the participants held a college-level degree, while 44.8% had only elementary education.

3.2 Responses to the statements

In the designated four regions, respondents were interviewed face-to-face regarding the current state of potato production technology, fertilizer management practices, disease and weed infestation, and their attitudes toward adopting GE crops. During the interviews, 13 statements were selected from the questionnaire to reflect the specific aims of the proposed study (Table 2). Most farmers in the survey regions followed conventional fertilizer application strategies (statements 1–5). During land preparation, they applied both organic and inorganic fertilizers as basal doses. Additionally, liquid fertilizers were often applied during the growing stages to address macro- and micronutrient deficiencies. Weed diversity is a vital cause of yield reduction and was managed manually to maintain the nutritional balance in potato plants (statement 6). None of the farmers expressed concern about the biosafety issues that could arise from plant debris residues during haulm pulling or potato harvesting (statement 9). Furthermore, most participants expressed positive attitudes toward adopting disease-resistant genetically engineered (GE) crops, which aim to reduce yield losses due to pests. This adoption would help address challenges in disease management and production losses, thereby supporting farmers in generating higher profits.

TABLE 3 Fertilizer management practices of the four regions compared to the recommended dose for potato cultivation in Bangladesh.

Fertilizer	Recommended dose (kg/ha)[a]	Central and Mid-East (kg/ha)	North-West (kg/ha)	Mid-West (kg/ha)	South-East (kg/ha)
Urea	250	585	350	360	385
TSP	150	298	395	325	243
MOP	250	385	543	487	363
Gypsum	120	115	121	119	118
Zinc sulfate	10	10	10	11	10
Magnesium sulfate	100	95	98	100	112
Boric acid	10	9	8	10	10
Cow dung	9,071	4,580	5,690	4,500	5,600

[a]Recommended doses reported by Azad et al., 2019 in the *Krishi Projukti Hatboi* (*Handbook on Agro-Technology*). Published by the Bangladesh Agricultural Research Institute (BARI). Comparing these fertilizer management practices across four different regions with the recommended fertilizer doses highlighted variations in nutrient application and adherence to agricultural guidelines during potato cultivation in Bangladesh.

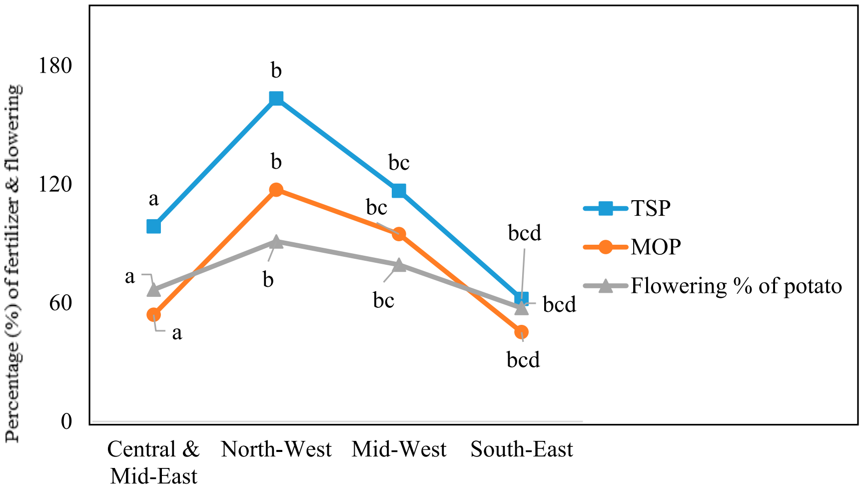

FIGURE 3
Induced flowering (%) in potato resulting from overuse of TSP and MOP across the four study regions; ^{abc} indicates a significant difference at $p = 0.05$ among the regions.

3.3 Assessment of the existing fertilizer management scenario

Generally, farmers in Bangladesh depend on the traditional system of potato cultivation. They follow the recommended doses of fertilizer outlined by Azad et al. (2019) to achieve a significant increase in production (Table 3). Nonetheless, during the survey, it was observed that the major fertilizers, such as urea (N), TSP (P), and MOP (K), were applied excessively in potato cultivation. In the Central and Mid-East region, nitrogen fertilizer was applied at 134.0% of the recommended dose, amounting to 250 kg/ha. Whereas in the North-West, phosphorus was applied at 163.3% of the recommended dose, and potassium was applied at 117.2% of the recommended dose. The recommended amounts are 150 kg/ha and 250 kg/ha, respectively.

In addition, significant differences were found in the excessive application of TSP and MOP during potato cultivation among the four studied regions. During cultivation, early physiological maturity of potato plants followed by induced flowering was observed in these fields. Among our interviewees, 73.6% of farmers reported observing unexpected flowering in some potato varieties when they applied more TSP and MOP fertilizer than the recommended dose to control late blight disease (Figure 3). On the other hand, the potato growers of Central and Mid-east assumed that the excessive use of urea may increase the potato yields. The line graph (Figure 4) illustrates the relationship between the percentage of excess urea usage in the Central and Mid-East region compared to the other three studied regions, namely, the South-East, the Mid-West, and the North-West region. The data revealed that the difference in urea application between the Central and Mid-East

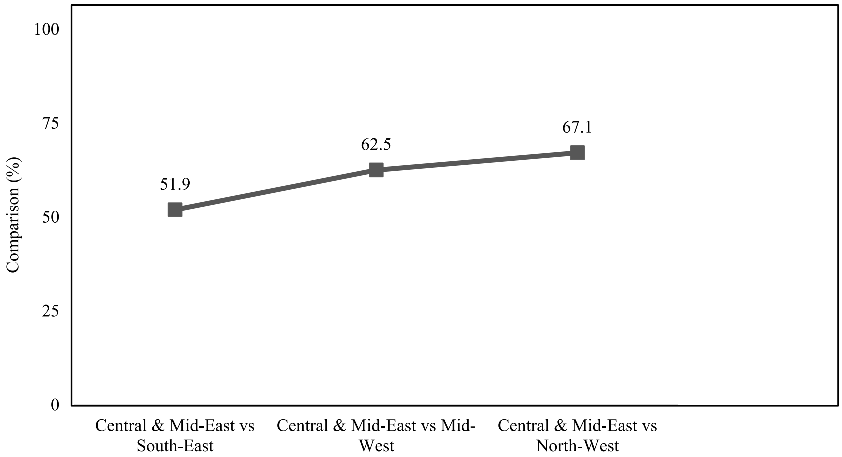

FIGURE 4
Comparison (%) between Central and Mid-East with the other three regions in the case of excessive urea application during potato cultivation.

TABLE 4 Growers' perceptions (%) of major obstacles observed in potato production across the four study regions.

Obstacle	Response (%) to major obstacles				
	Central and Mid-East	North-West	Mid-West	South-East	LSD
1. Disease infection a. Late blight b. Virus c. Scab	29.00 ± 0.447^{a} 12.50 9.40 7.10	35.40 ± 0.435^{b} 19.10 11.60 4.70	32.69 ± 1.249^{bc} 15.80 10.30 6.59	23.57 ± 1.086b^{cd} 10.75 7.60 5.22	2.536
2. Insect attack	14.62 ± 0.388^{a}	23.00 ± 0.471^{b}	19.00 ± 0.453^{bc}	11.70 ± 0.494^{bcd}	1.301
3. Weed infestation	9.35 ± 0.212^{a}	11.25 ± 0.700^{b}	15.25 ± 0.366^{bc}	8.50 ± 0.500^{a}	1.375
4. Lack of irrigation facilities	8.40 ± 0.600^{a}	7.00 ± 0.391^{b}	4.20 ± 0.493^{bc}	5.30 ± 0.251^{bc}	1.298
5. Pest attack in storage	13.80 ± 0.872^{a}	9.10 ± 0.524^{b}	10.70 ± 0.626^{b}	10.75 ± 0.656^{b}	1.956
6. Excessive rainfall	2.16 ± 0.314^{a}	4.75 ± 0.395^{b}	3.56 ± 0.382^{bc}	7.36 ± 0.423^{bcd}	1.093
7. Lack of storage facilities	8.80 ± 0.694^{a}	3.10 ± 0.202^{b}	4.70 ± 0.394^{b}	11.90 ± 0.908^{bc}	1.758
8. Lack of accuracy in pricing	9.89 ± 0.613^{a}	2.00 ± 0.168^{b}	5.50 ± 0.526^{bc}	13.96 ± 0.817^{bcd}	1.666
9. Problems to collect agrochemicals	0.50 ± 0.092^{a}	3.80 ± 0.354^{b}	1.80 ± 0.129^{bc}	2.87 ± 0.316^{bcd}	0.719
10. Others	3.48 ± 0.127^{a}	0.60 ± 0.115^{b}	2.60 ± 0.214^{bc}	4.09 ± 0.259^{bcd}	0.542

^{abc} indicates a significant difference at $p = 0.05$ among the regions.
The data show the percentage distribution of growers' perceptions of major obstacles in potato production across four study regions, highlighting regional differences and localized issues. Significant differences were observed among the regions at $p = 0.05$.

region and the South-East, at 51.9%, is the lowest. In contrast, the discrepancy in urea usage between the Central and Mid-East and the North-West was the highest at 67.1% (Figure 4).

3.4 Obstacles to potato cultivation

A significant portion of the interviewed farmers identified various diseases, such as late blight, virus infection, scab, etc., as the most severe challenge in potato cultivation, leading to substantial crop damage and yield loss. In the North-West, approximately 35.4% of farmers reported significant yield loss and quality deterioration due to these diseases, while 32.7% of farmers in the Mid-West experienced similar issues. Our survey found that late blight was the most devastating disease, reported by approximately 10.7%–19.1% of respondents (Table 4). Approximately 23.0% of respondents reported insect and pest attacks in the North-West region, and 15.25% of respondents in the Mid-West identified weed infestation as a major problem in their potato fields. Moreover, due to adverse calamity, excessive rainfall was also observed in the dry season by 2.2%–7.4% of the farmers. This rainfall caused severe water management problems followed by crop damage due to disease outbreaks, especially late blight. Additionally, inadequate storage facilities were reported by 3.1%–11.9% of respondents; inadequate storage causes massive tuber loss by different fungal and bacterial infections. Moreover, due to rising costs and the

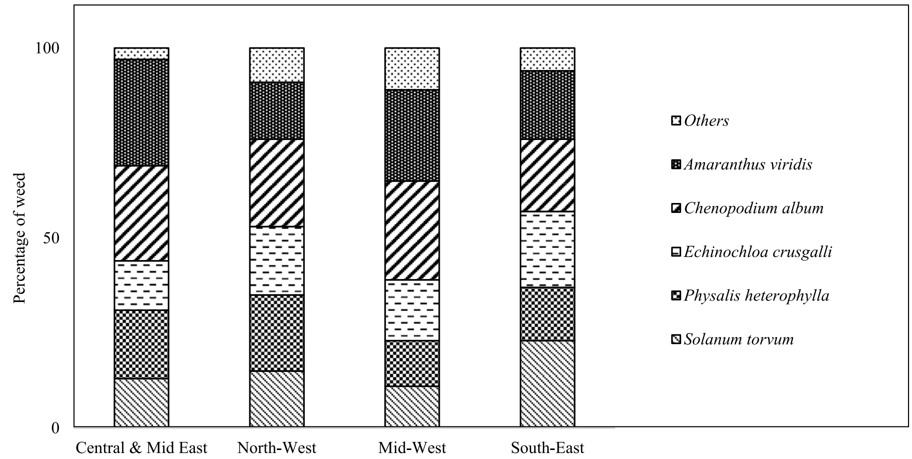

FIGURE 5
Weed diversity during potato cultivation among the four study regions.

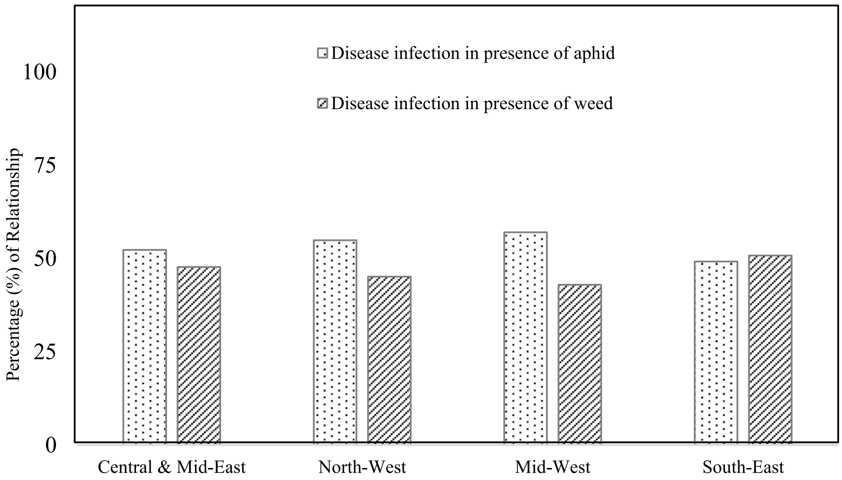

FIGURE 6
Relationship (%) between aphid and weed infestation in potato fields across the four study regions.

scarcity of fertilizers and insecticides in the market, farmers struggled to grow potatoes profitably.

3.5 Weed diversity

The farmers mentioned weed infestation in their potato fields. *S. torvum*, *P. heterophylla*, *Alternanthera philoxeroides*, *C. album,* and *Amaranthus viridis* weeds are generally found in potato fields in Bangladesh (Figure 5). Farmers observed weeds in their potato fields at approximately 30 DAP (days after planting). *Solanum torvum*, *Physalis heterophylla,* and *A. philoxeroides* are from the Solanaceae family. *Chenopodium album* and *A. viridis* are from the Amaranthaceae family. Among the five mentioned weeds, *A. viridis* (28%) and *S. torvum* (23%) were found to have high densities in the Central and Mid-East and South-East regions compared to the other regions. These weeds significantly reduce productivity by competing with potato crops for nutrients. Respondents from the North-West and Mid-West regions reported the existence of vectors, namely, aphids that cause rapid virus transmission, while weed infestation was also observed (Figure 6). These reports indicate there was a strong and positive association between pests and weed infestation.

3.6 Willingness to adopt GE crops

The knowledge and keenness to accept the GE technology were evaluated among the participants interviewed, as illustrated in Figure 7. In this study, we observed that more than 50% of interviewees were willing to integrate GE potatoes into their potato cultivation system as they think GE crops will have high yield potential and their disease resistance may also reduce cultivation costs. The Central and Mid-East region showed the

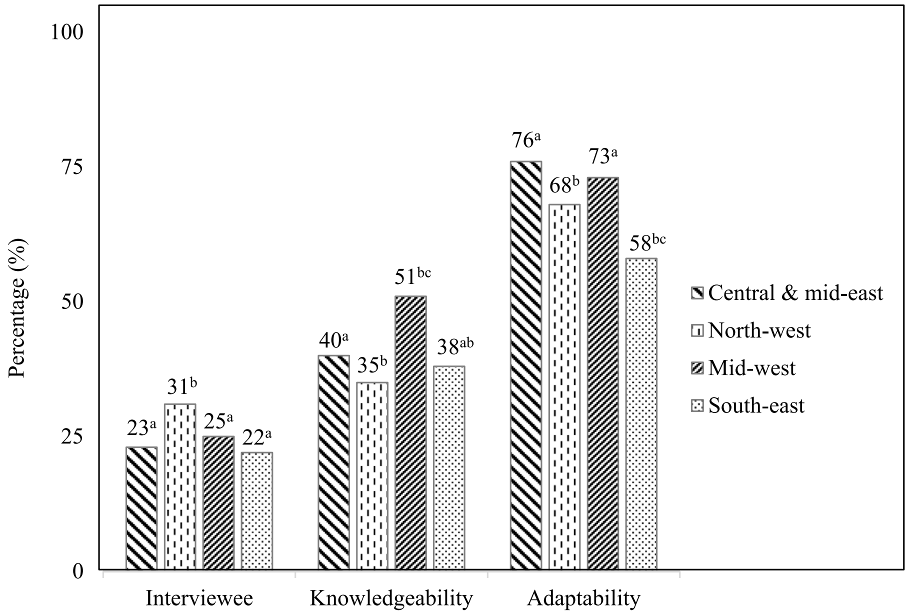

FIGURE 7
Willingness to adopt GE technology among farmers in the four study regions (^{abc} indicates a significant difference at $p = 0.05$ among the regions).

highest enthusiasm for adopting GE technology, with 76% of participants expressing willingness, while the South-East region had the lowest at 58%.

4 Discussion

The statistical populations for this survey in the four regions were mostly regular farmers with more than 10 years of experience. Respondents (n = 1757) were grouped based on their gender, age, and educational qualifications, as reported by De Steur et al. (2019) (Table 1). To ensure the effectiveness of the study, a purposeful random sample technique was utilized to choose respondents from various regions. This approach aimed to determine fertilizer management procedures, cultural practices, and farmer attitudes toward genetically engineered (GE) potato, which was reflected through the 13 statements in Table 2. This methodology was supported by Areal et al. (2011), who summarized farmers' attitudes toward genetically modified herbicide-tolerant crops with 24 statements.

Though Bangladesh produces a huge amount of potato, the quality of potato has undergone several challenges due to biotic and abiotic stresses. In terms of the use of fertilizers, we have recorded that all of the interviewed farmers applied more fertilizer than the recommended dose with an expectation of higher yield and better disease management (Table 3). Similar phenomena were observed in a study where the farmers of Munshiganj applied 3–4 times higher doses of urea, TSP, and MOP fertilizers than was recommended for a potato field (Choudhury et al., 2006). We observed that this attitude was more frequent among farmers who did not have a high school or college diploma. Due to a lack of proper education and understanding of fertilizers, including their mode of action, most of the farmers in Bangladesh believe that a higher application would benefit them with a higher yield and, thus, more profit.

Consequently, in this survey, 73.6% of farmers reported that an extra amount of TSP and MOP fertilizer could reduce the devastating damage to potatoes by controlling late blight diseases in the North-West and Mid-West regions. The high cost of pesticides or herbicides might be one reason behind the using extra fertilizers to tackle pests and pathogens. Apart from this, potato plants that received an excessive amount of TSP and MOP for a prolonged period experienced an unexpected flowering and physiological maturity compared to plants that received the recommended rate of fertilizer (Figure 3). The result of this survey is consistent with that of Setu et al. (2018), who observed the same effect of TSP and MOP. They reported these fertilizers did not affect potato phenotype, growth parameters, or tuber yields; however, they had a significant impact on days to unexpected flowering, physiological maturity, plant height, leaf area, above and underground dry biomasses, and marketable yield.

In addition to TSP and MOP, we observed that 67.1% of excess urea as a nitrogen source was applied in Munshiganj (Central and Mid-East) compared to Dinajpur (North-West) during potato cultivation. Moreover, a relationship between the percentages of excessive use of urea in the Central and Mid-East region compared with three other regions, namely, the South-East, the Mid-West, and the North-West regions, was studied. Given that the recommended dose of urea application was 250 kg/ha, each region was noted to overuse this fertilizer, especially the Central and Mid-East region. Excessive amounts of urea produce more succulent vegetative parts, leading to increased plant vigor with darker green coloration, which in turn attracts more insects and pests (Singh and Sarkar, 2021). This may act as a catalyst to increase the probability of late blight and boost insect infestation and virus infection.

Apart from the disease infestations, several other obstacles were recorded during potato cultivation, including the absence of suitable germplasm, difficulties in water management, lack of storage facilities, unexpected weather, etc. (Table 4). Similar obstacles were also reported during potato cultivation in Bangladesh by Hoque et al. (2023). In the case of water management, farmers faced obstacles to managing groundwater for irrigation and unexpected rainfall, which directly hampered the yield. Similarly, Sun et al. (2015) found that the frequency of watering at different phases of potato growth and development impacts the production and quality of the crop.

Weeds also pose significant challenges in potato production in Bangladesh through nutrient uptake and playing host to pathogens (Showler and Granberg, 2003; Faruq et al., 2021). Potato yield reduction due to weed competition was extensively documented by Rana et al. (2004) and Attri et al. (2022). The present study observed that *S. torvum*, *P. heterophylla*, *A. philoxeroides*, *C. album,* and *A. viridis* are some of the weed species in potato fields. Similarly, Khan et al. (2008) demonstrated that *C. album* and *A. viridis* are the main weed species in the potato fields of Bangladesh. Cuda et al. (2002) and Khokon et al. (2017) showed that *S. torvum* and *P. heterophylla* are major weeds of the Solanaceae family. Moreover, many insects, including aphids, thrips, whiteflies, and leafhoppers, transmit plant diseases from weeds (Capinera, 2005). Future studies may be needed to assess the potential outcrossing among these weeds and potato.

The current survey aimed to study the agronomic practices of potato cultivation in Bangladesh along with the effect of fertilizer on crop performance, the weed population, and overall challenges during cultivation. The final goal is to understand farmers' willingness to adopt GE potato. The study reveals a willingness among farmers to adopt genetically engineered (GE) potatoes. However, the acceptance level varies among the study regions. Several studies have shown that the variation in acceptance due to the presence and absence of effective science communication about the benefits (Farid et al., 2020; Abdul Aziz et al., 2022), cultural attitudes, support systems, and local agricultural services (Koralesky et al., 2023). In the future, the reason for the variation in the study regions may be assessed.

5 Conclusion

The study was conducted in four major potato cultivation areas of Bangladesh. Farmers with more than a decade of experience in potato cultivation were interviewed. The growers' responses showed that fertilizers are usually applied at higher-than-recommended doses to ensure optimum yield while controlling disease infestation by making the plants vigorous. This overdose was found to correspond to early flowering in the field. Common weed populations were also recorded in the study. Several cultivation challenges, including diseases and pest infestations, were mentioned in response to the questions asked during the survey. A large percentage of farmers opined in favor of the cultivation of GE potato due to their understanding that it will give higher yield and disease resistance. However, the questions also revealed their lack of in-depth knowledge of GE crops. The present study deals with farmers' agronomic practices and observations about potato cultivation. This can partially fulfill the required baseline data for ERA prior to the introduction of GE potato in Bangladesh.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants OR patients/participants legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

AN: conceptualization, supervision, writing–original draft, and writing–review and editing. SI: methodology, project administration, writing–original draft, and writing–review and editing. AI: conceptualization, formal analysis, and writing–review and editing. MR: data curation, resources, and writing–original draft. MH: data curation, methodology, and writing–original draft. TE: funding acquisition, resources, and writing–original draft. MA: data curation, funding acquisition, and writing–original draft. FA: investigation, validation, and writing–review and editing. TK: methodology, resources, and writing–original draft. AU: supervision, validation, and writing–review and editing.

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Conflict of interest

Authors AN, SI, MR, MH, TE, MA, and FA were employed by ACI Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

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Nutritional and anti-nutritional compositional analysis of transgenic potatoes with late blight resistance

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Late blight, caused by the pathogen *Phytophthora infestans*, is a devastating disease affecting potato production globally, with adverse effects in Africa where limited access to fungicides exacerbates its impact. Outbreaks of late blight lead to reduced yields and substantial economic losses to potato farmers and agricultural systems. The development of resistant potato varieties, tailored to African agroecological conditions, offers a viable solution in mitigating the devastating effects of late blight on potato cultivation. Leading to this study, two consumer-preferred varieties, Victoria and Shangi, with high susceptibility to late blight were targeted for conferring late blight resistance through genetic engineering. This was achieved by inserting *R* genes from wild relatives of potato displaying resistance to the disease. The intended effect of conferring resistance to the late blight disease has been consistently observed over twenty experimental field trials spanning 8 years at three locations in Uganda and Kenya. In this study, we assessed whether the genetic transformation has led to any significant unintended effects on the nutritional and anti-nutritional composition of potato tubers compared to the non-transgenic controls grown under the same agroecological conditions. The compositional assessments were conducted on commercial-size potato tubers harvested from regulatory trials at three locations in Uganda and Kenya. Statistical analysis was conducted using two-way analysis of variance comparing transgenic and non-transgenic samples. Overall, the results showed that the transgenic and non-transgenic samples exhibited similar levels of nutritional and antinutritional components. Variations detected in the levels of the analysed components fell within the expected ranges as documented in existing literature and potato composition databases. Thus, we conclude that there are no biologically significant differences in the nutritional and anti-nutritional composition of transgenic and non-transgenic potato tubers engineered for resistance to late blight.

KEYWORDS

potato, transgenic, composition, unintended effect, safety assessment

Introduction

Potato (*Solanum tuberosum* L.) ranks third in global food crop production after rice and wheat with global total crop production exceeding 300 million metric tons (Devaux et al., 2014). Production and consumption of potatoes have been declining in the developed regions, while in the developing regions, it surpassed the developed regions' production in 2005 and continue to rise (Devaux et al., 2014; Haverkort and Struik, 2015). Potatoes are consumed by more than a billion people worldwide, making it a critical crop for food security in the face of population growth and increased hunger rates. Potatoes are typically consumed cooked, either from fresh tubers or from processed products that can already be pre-cooked. Consumption of raw tubers is rare due to their unappealing texture, low digestibility, and presence of anti-nutrients and toxicants which are partially destroyed during the cooking process. The consumption of processed potato products is a growing global trend, especially in urban areas where consumers tend to opt for convenient, ready-to-eat foods. Due to the overall importance of potatoes in diets worldwide, the characterization of their nutritional and anti-nutritional properties is crucial for public health (Camire et al., 2009). Though potatoes can also be used for industrial products (starch) and as livestock feed, the primary use in developing countries remains as food (OECD, 2002; OECD, 2021).

Potatoes are rich in carbohydrates, making them an excellent energy source with low fat content. Although low in protein, potatoes also contain a good amount of essential amino acids, dietary and crude fibers, vitamin C, several B vitamins, and potassium. According to Burton (1989) and Ooko (2008), potatoes are a good source of iron (2.5–10 mg/100 g), phosphorus and magnesium (60–140 mg/100 g), copper (0.06–2.83 mg/100 g), calcium (30–90 mg/100 g) and an excellent source of potassium (320–450g/100 g of potato). About 200 g of potatoes provides 10% of the recommended daily intake for phosphorus and magnesium, and up to 20% for copper, iron, and iodine. In addition, approximately 200 g will supply 2–4 g of dietary fiber which is equivalent to about half that supplied by other commonly eaten vegetables (Burton, 1989). Potato tubers have been reported to contain up to 46 mg/100 g ascorbic acid (fresh weight basis) depending on the variety, maturity of the tubers at harvest, and the environmental conditions under which they were grown (Haase and Weber, 2003; Nourian et al., 2003; Han et al., 2004; Burlingame et al., 2009). The variety is the greatest determinant of variation of ascorbic acid concentration in potatoes (Hamouz et al., 2009; Hemavathi et al., 2010).

Apart from the nutritional benefits of potato tubers, they also contain anti-nutrients such as protease inhibitors and lectins, but both are inactivated during cooking. Potato possesses natural toxicants of which the most important are the glycoalkaloids (GA) composed mainly of α-Solanine and α-Chaconine. Together they form 95% of the total glycoalkaloids (TGA) present in potatoes, α -chaconine being present in relatively higher proportions (Friedman, 2006). GA are thought to function in the chemical defense of the plant, as non-specific protectors or repellents against potential pests and predators (Roddick, 1989). In addition, stress tolerance is correlated to GA content (Veilleux et al., 1997). The highest concentration of GA is found within the green parts and in the sprouts. The TGA concentration is high within the peel and just below it and generally decreases with an increase in tuber size. The levels of TGA vary significantly between cultivars (Hellenas, 2001; Friedman, 2006) and are influenced by both genetic and environmental factors as well as various pre- and post-harvest stresses (Dale et al., 1998). TGA in tubers ranging from 20–100 mg/kg or below are of no food safety concerns (FAO/WHO, 1999). Excess levels of GA give potatoes a bitter taste and consumption of such tubers can result in poisoning with several symptoms ranging from gastrointestinal disorders, hallucinations, and partial paralysis to convulsions, coma, and death (Smith et al., 1996). Acrylamide is another anti-nutrient formed in potatoes during high-temperature cooking processes, however, multiple post-harvest strategies have successfully reduced the acrylamide content of processed potato products (Amrein et al., 2003; Liyanage et al., 2021; Kumari et al., 2022). Allergens are not prominent in potatoes, and it is generally considered to be a food source with low, if any, allergenicity potential. A more detailed description of the nutrient, antinutrient, toxicant, and allergen contents of the potato can be found in Camire et al. (2009) and OECD (2002, OECD, 2021).

Leading to the current study, transgenic events were produced by genetic transformation of several varieties by introducing three late blight resistance (*R*) genes and the *nptII* selectable marker gene. The intended effect was to confer resistance to the late blight disease which has indeed been observed in multiple seasons and locations (Ghislain et al., 2019; Webi et al., 2019; Byarugaba et al., 2021). None of these genes and their products are expected to cause changes in the nutritional and anti-nutritional components of the potato. Previous studies evaluated insect and virus-resistant potatoes which were commercially released in the late 90s for compositional analyses, revealing their equivalence to their isogenic non-transgenic counterparts (Rogan et al., 2000; El Sanhoty et al., 2004; Zdunczyk et al., 2005; El-Khishin et al., 2009; Khalf et al., 2010; Chen et al., 2020).

In this study, compositional analysis has been conducted to establish if the genetically engineered plants have significant changes in essential nutritional and anti-nutritional components compared to the plants they are derived from (Codex, 2003). Two types of changes are considered in a food safety assessment of genetically engineered plants: (i) the expected changes resulting from the expression of the new genes added to the plant which are assessed for allergenicity and toxicity specific to the gene products; and (ii) the unexpected changes observed without a clear causal origin. In general, it is assumed that these unexpected changes can arise due to *in vitro* cell and tissue culture, or insertional effects of the T-DNA in the plant genome. Compositional analysis is one method to assess these changes. Hence, if any changes in composition are identified, expected or unexpected, these are then assessed for their potential to cause harm. Worth mentioning, a 20-year review of this approach for safety assessment has revealed that genetic modification has not led to unintended compositional changes (Herman and Price, 2013). This conclusion is supported by extensive evaluation of 148 transgenic events by the U.S. FDA, 189 submissions evaluated by the Japanese regulators and over 80 peer reviewed publications on compositional safety of GM crops. The genetic modifications targeted traits such as drought tolerance, nutrient enhancement, insect resistance, amongst others in a wide range of crops including potato, tomato, cabbage

TABLE 1 Transgenic potato events and non-transgenic varieties, years and locations of trials, number of replications (blocks) per trial, number of samples per replicate, and total sample number (N) used for compositional analysis.

Potato variety	Transgenic event	Year	Location of confined field trials	Replicates per trial	Samples per replicate	Total sample (N)
Victoria	Vic.1	2018	Uganda: Kashwekano	4	2	8
	Vic.172	2020	Uganda: Kashwekano, Rwebitaba, Buginyanya	3	2	18
		2021	Uganda: Kashwekano, Rwebitaba, Buginyanya	3	2	18
		2023	Kenya: Muguga, Njabini, Molo	1–3[a]	1	8 (7)
	Vic.185	2023	Kenya: Muguga, Njabini, Molo	2–3[a]	1	8
Shangi	Sha.105	2023	Kenya: Muguga, Njabini, Molo	2–3[a]	1	8

[a] In the 2023 Kenya Trials: one sample was collected from three replicates at two sites and from two replicates at one site, for Victoria, Vic. 185, Shangi and Sha.105 (N = 8); one sample was collected from three replicates at two sites and from one replicate at one site for Vic.172 (N = 7).

and mushrooms. Additionally, a metabolomics study on five potato cultivars showed that the GM plants exhibited changes in metabolite abundance comparable to conventionally bred varieties (Catchpole et al., 2005).

Compositional analyses were conducted on several transgenic events from two varieties (Victoria, Shangi) to assess whether the gene construct is conducive to unexpected effects and whether any of the transgenic events differ significantly from potatoes recognized as safe for consumption. The study was conducted to ensure compliance with regulatory frameworks set by the national biosafety authorities regulating GM crops in Kenya and Uganda in accordance with the internationally accepted standards for food safety assessment of GM plants established by the Codex Alimentarius (Codex, 2003). A two-step process is used to determine the similarity between the transgenic event and the variety. First, the difference between values for each component is tested statistically for significance. Those found significant are then compared to those from other potato varieties. Should one or more components fall markedly outside the range known from the literature, additional food safety investigations will be advisable.

Materials and methods

Plant materials

Two potato varieties produced transgenic events of interest for release in African countries (specifically Kenya, Nigeria, Rwanda, and Uganda). The variety Victoria, also known as Asante in Kenya, has generated three transgenic events, Vic.1, Vic.172, and Vic.185, considered as suitable for release based on molecular characterization and agronomic performance. Vic.1 tubers were obtained from a regulatory trial (confined field trials) at one location in Uganda in 2018, Vic.172 tubers were from trials in three locations in Uganda in 2020 and in 2021 and from three locations in Kenya in 2023, and Vic.185 tubers were from three locations in Kenya in 2023 (Table 1). The variety Shangi, widely grown and popular in Kenya, has generated one transgenic event considered for release in Kenya, Nigeria and Rwanda. Sha.105 tubers were obtained from confined field trials at three locations in Kenya (Table 1).

Sampling tubers for compositional analysis

Tubers from the transgenic event(s) and the comparator (the non-transgenic variety it derives from) were harvested from each location and samples collected for compositional analysis. The design of each confined field trial was a randomized complete block design with three blocks (replications) (Byarugaba et al., 2021) except the Vic.1 trial in 2018 Kashwekano, Uganda where there were four replications (Table 1). The total number of samples for each genotype combined across trial sites are shown in Tables 1, 2 and indicated in parenthesis in the results section (Tables 4–8). At harvest, for each sample, four medium-sized tubers, each weighing at least 100g, were selected as free of any defect (no regrowth, no mechanical injuries, no damage due to pests or diseases) and clean of any soil.

Tubers from Kenya and Uganda were approximately 1-month and two-month-old, respectively, before being processed by the International Potato Center's (CIP) Food And Nutritional Evaluation Laboratory (FANEL) at the Bioscience for east and central Africa (BecA) at ILRI in Nairobi, Kenya. The age difference is due to the process for handling and exporting these transgenic tubers over borders since they are considered as restricted materials and thus classified and handled following UN3245 norms. Movement of this material was fully compliant with biosafety and phytosanitary regulations in place for both countries.

All fresh samples were kept at room temperature until use. Samples were coded and handed over to the FANEL team without the key code that provides the full identity of the sample. Samples were screened for morphological anomalies, for any damage resulting from pest, disease, and handling. In all cases, tubers were peeled before use. Tubers from the same sample were pooled together and three technical replicates taken for all analyses. Results are presented on fresh weight basis. All lyophilized samples were milled to a fine powder and kept at −80°C for the duration of analyses to preserve sample quality.

The samples for Vic.1 from one trial site harvested in 2018 (Table 1) were analyzed for five components as recommended in OECD (2002) (Table 4). The samples for Vic.172 were from three trial sites and 3 years (Table 1). Samples from three sites harvested in 2020 were analyzed for the five components in OECD (2002) and analyzed for 12 components as recommended in OECD (2021) from three sites harvested in 2021 and in 2023 (Table 2). The samples

TABLE 2 Sample sizes and nutritional components measured for Event Vic.172 and Victoria collected from trials in Uganda 2020 following OECD (2002) recommendations and Uganda 2021 and Kenya 2023 following OECD (2021) recommendations.

Component		Uganda 2020	Uganda 2021	Kenya 2023	Total
Moisture	Vic.172	18	18	7	43
	Victoria	18	18	8	44
Protein	Vic.172	18	18	7	43
	Victoria	18	18	8	44
Total Sugars	Vic.172	18			18
	Victoria	18			18
Fat	Vic.172		18	7	25
	Victoria		18	8	26
Carbohydrate	Vic.172		18	7	25
	Victoria		18	8	26
Ash	Vic.172		18	7	25
	Victoria		18	8	26
Dietary Fiber	Vic.172		18	7	25
	Victoria		18	8	26
Starch	Vic.172		18	7	25
	Victoria		18	8	26
Vitamin C	Vic.172	18	18	7	43
	Victoria	18	18	8	44
Vitamin B6	Vic.172		18	7	25
	Victoria		18	8	26
Potassium	Vic.172		N/A	7	7
	Victoria		N/A	8	8
Magnesium	Vic.172		18	7	25
	Victoria		18	8	26
Total Glycoalkaloids	Vic.172	18	18	7	43
	Victoria	18	18	8	44

N/A not available.

from Vic.185 from three sites harvested in 2023 (Table 1) were analyzed for 12 components as in OECD (2021) (Table 6).

Determination of moisture content

The method is based on drying the sample under controlled temperature conditions until a constant weight is obtained as per the method described by AOAC (2012b).

Determination of sugar (reducing and total) content

The quantification of individual and total sugars in potato tubers was done according to the method described by Sesta (2006). Briefly, sugars were extracted from lyophilized potato material using 85% ethanol. The ethanol was then evaporated, leaving the sugars in an aqueous solution. The individual sugars were separated on the HPLC using a Eurospher 100–5 NH2 column (Knauer, Berlin) and detected by a refractive index detector.

Determination of protein content

Protein content was determined using the Kjeldahl method as described by AOAC (2012b). The analysis involves the digestion of potato samples in sulfuric acid in the presence of a Kjeldahl tablet, containing catalysts that facilitate the release of nitrogen from protein-bound nitrogen and free amino acids. The amount of nitrogen is then used to calculate crude protein content.

Determination of fat content

Fat and oils (total fat) were determined by hydrolyzing 3 g of the lyophilized samples containing 1 g of Celite using 4N HCl followed by solvent extraction using petroleum ether as per AOAC (2012b).

Determination of ash content

Ash content was determined by AOAC (2012b) method. The method involves the oxidation of the organic matter in potato samples in a furnace at 550°C for at least 4 h.

Quantification of carbohydrates

Carbohydrate content was obtained by calculation by removing moisture, protein, fat, and ash content in %.

Determination of dietary fiber

Dietary fiber was determined according to AOAC (2012b). The method involves the sequential digestion of lyophilized potato samples with dilute acid and alkali solutions, leaving behind the indigestible components that make up the dietary fiber content.

Determination of total starch

This was measured using the Total Starch Assay kit (Megazyme, Ltd), as per manufacturer instructions.

Determination of vitamin C

The method detects vitamin C (ascorbic acid) using the HPLC method as described in Gazdik et al. (2008). The lyophilized potato samples are homogenized in 3% metaphosphoric acid before being filtered and chromatographed using the HPLC.

Determination of vitamin B6

The extraction of Vitamin B6 was done as per the method in Zand et al. (2012). The lyophilized potato samples were solubilized and Vitamin B6 was extracted using 1% acetic acid, heated at 70°C for 40 min in a water bath. The solutions were centrifuged and filtered before analysis using the HPLC.

Determination of minerals: Potassium (K) and magnesium (Mg)

The mineral analysis was done by ICP-OES equipment, Perkin Elmer, Optima 2100. 0.3 g of the sample was digested in nitric acid and hydrogen peroxide at 150°C in a microwave digestor for 20 min. The solution was cooled and diluted to 20 mL with 1% nitric acid and the minerals values were read out in the ICP-OES machine using the WinLab software. The calibration curves in a range of 0–2 mg/L were developed using the standards and blanks from Perkin Elmer.

Determination of glycoalkaloids

The glycoalkaloids were extracted as per the method by Tomoskozi-Farkas et al. (2006). Extraction is done on lyophilized potato samples using dilute acetic acid. The extract is concentrated and cleaned up on disposable solid-phase extraction cartridges. The final separation and measurement of α-solanine and α-chaconine was done by HPLC (AOAC, 2012a).

Literature range of potato components

The OECD (2021) used an extensive survey of publications, private and national database to establish ranges among conventional varieties of potato for the components of the study. These ranges were broader than those from AFSI (2024). We expanded most of them using data from other publications not included in the OECD survey (Burlingame et al., 2009; Dhingra et al., 2012; Chen et al., 2020; Tatarowska et al., 2023). The new ranges were established by using the lower and upper limits from these publications (Table 3).

Statistical analysis

The analysis of variance was made assuming the effects of Genotypes, Locations, and the interaction of genotypes with locations, such as fixed effects, to the effect of blocks within locations, such as random effects. We used the ANOVA: Two-Factor with Repetition function of the Analysis ToolPak Excel for Microsoft 365 except for Vic.172 which combined 3 year data with different numbers of samples. In this case, we used Tukey's honesty significant difference (HSD) using R statistical package (version 4.4.1) to obtain the *p* values. Significance was declared at $p < 0.05$.

Results

Transgenic events from the variety Victoria

Three transgenic events derived from the variety Victoria were analyzed using the five or the 12 components recommended by OECD 2002; OECD, 2021 respectively.

The five components analyzed in tubers from Vic.1 and its comparator, the variety Victoria, had essentially the same value and the small differences were not statistically significant (Table 4). Moisture was quite high, around 82% for both genotypes but typically within the literature range. Protein content was around 2 g/100 g for both genotypes which is the most commonly reported value in potato tubers. Total sugar of 0.36 and 0.51 g/100 g for Vic.1 and Victoria respectively was also within the literature range. Vitamin C of 7.44 and 6.22 mg/100 g was also within the literature range and the differences were not statistically significant. Finally,

TABLE 3 Ranges for the components analyzed using literature on a fresh weight basis.

Component	Literature range	Units	References
Moisture	63–87	%	Burlingame et al. (2009)
Protein	0.85–4.2	%	Burlingame et al. (2009)
Total sugar	0.05–8	%	OECD (2002)
Fat	0.00–0.30	%	OECD (2021)
Carbohydrates	12.9–19.6	%	AFSI, 2024; Chen et al., 2020
Ash	0.62–1.36	%	AFSI (2024)
Dietary fiber	1.30–3.6	%	Dhingra et al., 2012; OECD, 2021
Starch	7.3–22.6	%	Burlingame et al., 2009; Chen et al., 2020
Vitamin C	1.0–42	mg/100 g	Burlingame et al., 2009; Tatarowska et al., 2023
Vitamin B6	0.11–0.26	mg/100 g	Chen et al., 2020; OECD, 2021
Potassium	239–693.8	mg/100 g	Burlingame et al. (2009)
Magnesium	7.7–37.6	mg/100 g	Burlingame et al., 2009; OECD, 2021
Total glycoalkaloids	0.071–175	mg/100 g	Burlingame et al. (2009)

TABLE 4 Compositional analysis of tubers of Vic.1 *versus* Victoria from Uganda in 2018 following OECD (2002).

Component	Vic.1		Victoria		*p*-Value	Literature range	Units
	Mean(N)	Range	Mean(N)	Range			
Moisture	82.14 (8)	78.38–84.89	81.62 (8)	78.23–83.75	0.599	63–87	%
Protein	2.04 (8)	1.80–2.34	2.026 (8)	1.68–2.60	0.930	0.85–4.2	%
Total sugars	0.36 (8)	0.31–0.42	0.51 (8)	0.26–1.00	0.124	0.09–4.3	%
Vitamin C	7.44 (8)	4.62–11.54	8.48 (8)	6.22–11.86	0.319	2.8–42	mg/100 g
Total glycoalkaloids	0.305 (8)	0.082–0.994	0.364 (8)	0.083–0.688	0.701	0.071–175	mg/100 g

(N) = total number of samples.

total glycoalkaloids were 0.31 and 0.36 mg/100 g for Vic.1 and Victoria, a relatively low value compared to other potato varieties but within the literature range.

Tubers from the transgenic event Vic.172 were analyzed separately on three occasions (Table 1, 2), once with the OECD (2002) components and twice with the OECD (2021) components. This transgenic event was also assessed for its agronomic performance (Byarugaba et al., 2021). The 13 components analyzed on tubers from Vic.172 and Victoria were similar between the two genotypes except for three (Table 5). Ash, vitamin C, and total glycoalkaloids were higher for Vic.172 compared to Victoria and the differences were statistically significant ($p < 0.05$). However, Vic.172 values were still within the literature range.

The third transgenic event from the variety Victoria, Vic.185, was analyzed for its 12 components from tubers grown in Kenya (Table 1). The results support the conclusion that none of the differences were statistically significant (Table 6). All component values were within the literature range except for protein which was higher for Vic.185 than the literature range but not statistically significantly higher than Victoria (*p*-value of 0.117).

Transgenic event from the variety Shangi

One transgenic event from the variety Shangi (Sha.105) was analyzed for the 12 components recommended by OECD (2021). Tubers from Sha.105 were obtained from three locations in Kenya (Table 1). Small differences in the values of the 12 components were observed but none were statistically significant (Table 7). All of these values were within the literature range. Moisture for both Sha.105 and Shangi was lower compared to the previously analyzed genotypes whereas starch and carbohydrate seemed higher. This prompted us to compare the two varieties since tubers were harvested from the same trials. Interestingly, out of the 12 components, the differences of three components (moisture, carbohydrate, and starch) were statistically significant (Table 8).

Discussion

Compositional analysis of tubers from transgenic events and the variety they are derived from was conducted to assess unintended

TABLE 5 Compositional analysis of tubers of Vic.172 *versus* Victoria from Uganda in 2020 following OECD (2002), in 2021 following OECD (2021), and from Kenya in 2023 following OECD (2021).

Component	Vic.172		Victoria		*p*-Value	Literature range	Units
	Mean(N)	Range	Mean(N)	Range			
Moisture	76.24 (43)	70.41–80.57	76.75 (44)	70.35–82.83	0.354	63–87	%
Protein	2.49 (43)	1.71–4.94	2.38 (44)	1.44–5.12	0.547	0.85–4.2	%
Total sugars	1.28 (18)	0.61–2.46	1.27 (18)	0.68–1.88	0.967	0.05–8	%
Fat	0.02 (25)	0.01–0.05	0.03 (26)	0.01–0.06	0.064	0.0–0.3	%
Carbohydrates	19.47 (25)	16.61–24.23	19.32 (26)	14.75–23.49	0.778	12.9–19.6	%
Ash	1.05 (25)	0.77–1.34	0.96 (26)	0.68–1.29	0.043[a]	0.62–1.36	%
Dietary fiber	1.98 (25)	1.51–2.97	2.07 (26)	1.40–3.50	0.463	1.30–3.6	%
Starch	12.73 (25)	10.28–16.08	12.44 (26)	9.45–14.70	0.481	7.3–22.6	%
Vitamin C	3.84 (43)	0.14–11.14	2.98 (44)	1.01–11.79	0.131	1.0–42	mg/100 g
Vitamin B6	0.83 (25)	0.01–1.60	0.42 (26)	0.01–1.24	0.005[a]	0.11–0.26	mg/100 g
Potassium	383.71 (7)	244–491	338.5 (8)	228–469	0.328	239–693.8	mg/100 g
Magnesium	18.02 (25)	14.57–23.13	16.64 (26)	9.48–22.28	0.204	7.7–37.6	mg/100 g
Total glycoalkaloids	11.28 (43)	0.42–2.98	7.66 (44)	0.17–1.83	0.007[a]	0.071–175	mg/100 g

[a] $p < 0.05$; (N) = total number of samples.

TABLE 6 Compositional analysis of tubers of Vic.185 *versus* Victoria from Kenya in 2023 following OECD (2021).

Component	Vic.185		Victoria		*p*-Value	Literature range	Units
	Mean(N)	Range	Mean(N)	Range			
Moisture	75.03 (8)	67.82–82.34	76.27 (8)	73.40–77.20	0.376	63–87	%
Protein	4.64 (8)	3.05–5.23	3.91 (8)	2.56–5.12	0.117	0.85–4.2	%
Fat	0.02 (8)	0.01–0.04	0.03 (8)	0.01–0.04	0.400	0.0–0.3	%
Carbohydrates	19.36 (8)	13.96–25.23	18.78 (8)	16.62–21.23	0.607	12.9–19.6	%
Ash	1.12 (8)	0.65–1.70	1.02 (8)	0.68–1.29	0.267	0.62–1.36	%
Dietary fiber	2.42 (8)	1.77–3.58	2.50 (8)	1.89–3.50	0.761	0.39–3.6	%
Starch	12.77 (8)	9.24–16.12	12.00 (8)	10.84–14.02	0.276	7.3–22.6	%
Vitamin C	5.07 (8)	1.43–11.03	4.75 (8)	1.01–11.79	0.774	2.8–42	mg/100 g
Vitamin B6	0.24 (8)	0.02–0.67	0.11 (8)	0.03–0.26	0.102	0.11–0.26	mg/100 g
Potassium	393 (8)	241–612	339 (8)	228–469	0.125	239–693.8	mg/100 g
Magnesium	14.72 (8)	11.61–22.42	11.90 (8)	9.48–15.49	0.054	7.7–37.6	mg/100 g
Total glycoalkaloids	1.21 (8)	0.25–2.48	0.91 (8)	0.45–1.45	0.251	0.071–175	mg/100 g

(N) = total number of samples.

compositional effects in the transgenic events following the recommended components by OECD (2002, OECD, 2021). The plant materials were produced from regulatory trials with up to three repetitions, and up to three locations per country. The compositional analyses revealed small differences between the transgenic events and the variety they derived from. For three transgenic events, Vic.1, Vic.185, and Sha.105, none of these differences were statistically significant. For one transgenic event Vic.172, eight of the 12 components were higher than those of Victoria and statistically different for three of them. Still, all component values were within the range of values published in the literature and database. Such small differences with statistical significance between a transgenic event and the variety it derives from have been observed previously but values stayed within the

TABLE 7 Compositional analysis of tubers of Sha.105 *versus* Shangi from Kenya in 2023 following OECD (2021).

Component	Sha.105		Shangi		*p*-Value	Literature range	Units
	Mean(N)	Range	Mean(N)	Range			
Moisture	72.76 (7)	63.01–84.57	71.84 (8)	64.67–77.10	0.727	63–87	%
Protein	3.91 (7)	2.35–5.21	4.01 (8)	2.81–5.31	0.822	0.85–4.2	%
Fat	0.03 (7)	0.01–0.05	0.02 (8)	0.01–0.06	0.512	0.0–0.3	%
Carbohydrates	23.14 (7)	12.30–31.04	22.99 (8)	19.02–29.39	0.938	12.9–19.6	%
Ash	1.05 (7)	0.65–1.58	1.14 (8)	0.58–1.64	0.508	0.62–1.36	%
Dietary fiber	2.56 (7)	1.17–4.08	2.78 (8)	2.26–3.39	0.459	0.39–3.6	%
Starch	14.48 (7)	9.12–20.45	15.53 (8)	11.50–18.84	0.505	7.3–22.6	%
Vitamin C	6.74 (7)	1.47–12.66	4.95 (8)	1.52–10.10	0.137	2.8–42	mg/100 g
Vitamin B6	0.10 (7)	0.04–0.20	0.13 (8)	0.02–0.40	0.414	0.11–0.26	mg/100 g
Potassium	359 (7)	236–483	383 (8)	229–513	0.540	239–693.8	mg/100 g
Magnesium	11.53 (7)	8.62–16.52	12.61 (8)	8.51–24.68	0.496	7.7–37.6	mg/100 g
Total glycoalkaloids	0.80 (7)	0.36–1.76	1.04 (8)	0.56–2.19	0.206	0.071–175	mg/100 g

(N) = total number of samples.

TABLE 8 Compositional analysis of tubers of Victoria *versus* Shangi from Kenya following OECD (2021).

Component	Victoria		Shangi		*p*-Value	Literature range	Units
	Mean(N)	Range	Mean(N)	Range			
Moisture	76.27 (8)	73.40–77.20	71.84 (8)	64.67–77.10	0.009[a]	63–87	%
Protein	3.91 (8)	2.56–5.12	4.01 (8)	2.81–5.31	0.802	0.85–4.2	%
Fat	0.03 (8)	0.01–0.04	0.02 (8)	0.01–0.06	0.879	0.0–0.3	%
Carbohydrates	18.78 (8)	16.62–21.23	22.99 (8)	19.02–29.39	0.004[a]	12.9–19.6	%
Ash	1.02 (8)	0.68–1.29	1.14 (8)	0.58–1.64	0.171	0.62–1.36	%
Dietary fiber	2.50 (8)	1.89–3.50	2.78 (8)	2.26–3.39	0.175	0.39–3.6	%
Starch	12.00 (8)	10.84–14.02	15.53 (8)	11.50–18.84	0.002[a]	7.3–22.6	%
Vitamin C	4.75 (8)	1.01–11.79	4.95 (8)	1.52–10.10	0.849	2.8–42	mg/100 g
Vitamin B6	0.11 (8)	0.03–0.26	0.13 (8)	0.02–0.40	0.600	0.11–0.26	mg/100 g
Potassium	339 (8)	228–469	383 (8)	229–513	0.109	239–693.8	mg/100 g
Magnesium	11.90 (8)	9.48–15.49	12.61 (8)	8.51–24.68	0.617	7.7–37.6	mg/100 g
Total glycoalkaloids	0.91 (8)	0.45–1.45	1.04 (8)	0.56–2.19	0.451	0.071–175	mg/100 g

[a]$p < 0.05$; (N) = total number of samples.

literature range (Rogan et al., 2000; Khalf et al., 2010; Chen et al., 2020).

Our results align with those of other compositional analyses comparing transgenic events with their near isogenic lines demonstrating that there are no unintended biologically significant differences in the nutritional composition between the transgenic events and the non-transgenic variety due to the addition of new genes through genetic transformation. Moisture ranged between 72% and 82%, which is within the literature range of 63%–87% (Burlingame et al., 2009). Protein content was close to 2 g/100 g for the samples from Uganda whereas samples from Kenya were closer to 4 g/100 g. This difference might be related to differences in soil nitrogen content as observed in potato tubers (Rosyidah et al., 2021). Fat for all genotypes was on the lower end of the range for other varieties. Indeed OECD (2021) reports that potato tubers are generally recognized as naturally very low in fat (undetectable to 0.3 g/100 g). Carbohydrates, ash, dietary fiber, and starch were within the range reported in the literature. Vitamins C and B6 were in the low end of the literature range for each genotype. Vitamins are components which may have been underestimated in our analyses due to various factors. During preparation and processing of tubers, water soluble vitamins may be washed out, and/or partially destroyed by heat and oxidation. Ascorbic acid content may also decrease during storage and be affected by environmental conditions (OECD, 2002; Burgos et al., 2009; OECD, 2021). Minerals, potassium, and magnesium were always within the literature range with magnesium being in the lower end of the literature range. Finally, total glycoalkaloids were also in the low end of the literature range. For this component, the low values might be related to the peeling depth. Indeed, the bulk of glycoalkaloids is produced and accumulates within 1.5 mm below the skin (Valkonen et al., 1996).

When the two varieties, Victoria and Shangi, were compared, the differences were significant only for starch and carbohydrates. These components are related to traits breeders focus on. The number one trait for potato breeders is yield. Dry matter content is always assessed because it determines the essential qualities for consuming potatoes as fresh food or processed products. Hence, these differences may be the result of different breeding priorities. Potato varieties may also have differences in their composition in relation to the germplasm it derives from. Cultivated tetraploid potatoes have two germplasm origins, one of the Andigena type well adapted to tropical latitudes and short-day conditions, and the other of Tuberosum type well adapted to temperate latitudes and long day conditions (Spooner et al., 2014). Victoria and Shangi varieties are of the Andigena type whereas most of the compositional data published in databases and the literature are from potato varieties of the Tuberosum type.

Conclusion

The compositional analyses conducted on tubers grown from several locations, years, countries, and varieties reveal that there were no biologically significant differences in nutritional and anti-nutritional components between the transgenic events and the variety they derived from. In a few cases where differences were observed, the values were within the range reported in literature and compositional databases. The transgenic events, therefore, did not result in unintended compositional changes to the variety they derived from and should be regarded as safe as any other non-transgenic potato variety. Our study also supports the findings of the 20-year review of the use of compositional analysis to detect unintentional changes in transgenic plants, indicating that the introduction of the new genes of our gene construct did not prompt unintended effects (Herman and Price, 2013). Ten years after that study, it is also our firmly held belief that compositional analysis going beyond the standard parameters potato breeders typically evaluate (dry matter and total glycoalkaloids) is not scientifically justified.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

MM: Writing–review and editing, Writing–original draft, Methodology, Data curation. EM: Writing–original draft, Conceptualization, Writing–review and editing, Supervision, Investigation. LM: Writing–review and editing, Methodology, Data curation. ABy: Writing–review and editing, Investigation. ABa: Writing–review and editing, Investigation. MN: Writing–review and editing, Investigation. CT: Writing–review and editing, Investigation. MG: Writing–review and editing, Writing–original draft, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Application of CRISPR/Cas-based gene-editing for developing better banana

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Banana (*Musa* spp.), including plantain, is one of the major staple food and cash crops grown in over 140 countries in the subtropics and tropics, with around 153 million tons annual global production, feeding about 400 million people. Despite its widespread cultivation and adaptability to diverse environments, banana production faces significant challenges from pathogens and pests that often coexist within agricultural landscapes. Recent advancements in CRISPR/Cas-based gene editing offer transformative solutions to enhance banana resilience and productivity. Researchers at IITA, Kenya, have successfully employed gene editing to confer resistance to diseases such as banana Xanthomonas wilt (BXW) by targeting susceptibility genes and banana streak virus (BSV) by disrupting viral sequences. Other breakthroughs include the development of semi-dwarf plants, and increased β-carotene content. Additionally, non-browning banana have been developed to reduce food waste, with regulatory approval in the Philippines. The future prospects of gene editing in banana looks promising with CRISPR-based gene activation (CRISPRa) and inhibition (CRISPRi) techniques offering potential for improved disease resistance. The Cas-CLOVER system provides a precise alternative to CRISPR/Cas9, demonstrating success in generating gene-edited banana mutants. Integration of precision genetics with traditional breeding, and adopting transgene-free editing strategies, will be pivotal in harnessing the full potential of gene-edited banana. The future of crop gene editing holds exciting prospects for producing banana that thrives across diverse agroecological zones and offers superior nutritional value, ultimately benefiting farmers and consumers. This article highlights the pivotal role of CRISPR/Cas technology in advancing banana resilience, yield and nutritional quality, with significant implications for global food security.

KEYWORDS

banana, CRISPR/Cas, gene editing, disease resistance, nutrition enhancement

1 Introduction

Banana including plantain (*Musa* spp.) plays a pivotal role in global agriculture and food security, provides a reliable source of affordable and nutritious food. With its year-round availability and adaptability to diverse climates in the tropics and subtropics (Jones, 2000), banana emerge as a steadfast source of essential nutrients, particularly potassium, vitamin C, vitamin B6, and dietary fiber (Kumari et al., 2023). It not only provides a swift and convenient energy boost but also assumes a central role as a staple in the diets of millions of people in regions, especially within tropics.

In addition to its nutritional value, the economic significance of banana cannot be overstated. Serving as a critical export commodity for numerous tropical nations, banana contributes substantially to international agricultural trade, with vast plantations spanning over 140 countries and islands, covering over 12 million hectares globally, and 7.5 million hectares in Africa (FAOSTAT, 2021). The undeniable ubiquity and economic impact of banana underscore their pivotal role in sustaining the wellbeing of populations worldwide.

However, despite its importance, banana production faces a multitude of challenges, including biotic and abiotic stresses, declining soil fertility, limited genetic diversity, and insufficient availability of clean planting material, particularly among smallholder farmers. The prevalence and co-existence of various pathogens and pests further exacerbate these challenges, leading to significant yield gaps and threatening the sustainability of banana cultivation in affected regions (Tripathi et al., 2020). In response to these pressing challenges, the application of cutting-edge technologies such as CRISPR/Cas-based gene editing emerges as a promising avenue for revolutionizing banana production. By harnessing the power of gene editing, researchers are developing improved banana varieties with enhanced resistance or tolerance to biotic and abiotic stresses, thereby bolstering productivity and resilience in the face of environmental adversities. This article provides a comprehensive overview of recent advancements and future prospects in the utilization of gene editing technologies for the development of better banana varieties. This article seeks to elucidate the potential of CRISPR/Cas-based gene editing in driving innovation and sustainability within the global banana industry.

2 Challenges in banana production

Banana production grapples with an array of biotic and abiotic challenges that pose significant threats to both yield and fruit quality. These challenges are multifaceted and contingent upon factors such as geographical location, climate conditions, and specific agricultural practices. Among the notable biotic constraints, bacterial, fungal, and viral diseases stand out as major threats to banana crops.

Particularly menacing are diseases like Fusarium wilt disease, caused by the *Fusarium oxysporum* f. sp. *cubense* (Foc) fungus, and black Sigatoka, attributable to the *Mycosphaerella fijiensis* fungus. These diseases have the potential to inflict substantial yield losses, with Fusarium wilt, in particular, proving to be one of the deadliest biotic constraints (Ploetz, 2015). Fusarium wilt, also known as Panama disease, damages the plant's vascular system, causing wilting and, ultimately, death. Compounding the challenge is the soilborne nature of Foc, which can persist in the soil for decades, posing significant challenges for disease management (Hennessy et al., 2005).

Furthermore, the emergence of Foc tropical race 4 (TR4) poses a grave threat to banana production globally. This deadly fungal strain has been identified in various regions worldwide, including key banana-producing countries like Mozambique, Colombia, Israel, Jordan, Turkey, Mayotte, Peru, and Venezuela. The spread of TR4 in Africa is particularly alarming, given the continent's status as the world's second-largest banana producer and consumer (Viljoen et al., 2020). With limited control, prevention, and management tools currently available, addressing the spread of TR4 remains a critical challenge for sustaining banana production (Ploetz, 2015). The only viable option is disease-resistant banana varieties that ensures increase productivity with high nutritional value by application of genetic engineering (Dale et al., 2017). These TR4 resistant banana are recently approved for environmental release in Australia (OGTR, 2024).

Second, the most important fungal disease is Black Sigatoka, affecting mainly the lower leaves of banana plants and directly reducing the yield of the crop and poor quality of fruits (Arango Isaza et al., 2016).

In addition to fungal diseases, bacterial infections such as banana Xanthomonas wilt (BXW), caused by *Xanthomonas campestris* pv. *musacearum*, pose significant threats to banana production in Africa. The impact of BXW disease on banana yield losses is particularly severe in the Democratic Republic of Congo (83%), Uganda (71%), and other East African countries like Burundi, Kenya, Rwanda, and Tanzania, where losses range from 39% to 51% (Ainembabazi et al., 2015). Notably, the lack of disease-resistant banana varieties exacerbates the economic repercussions of BXW, with estimated losses ranging from USD 2 to 8 billion over a decade (Abele and Pillay, 2007; Biruma et al., 2007; Tripathi et al., 2009). No cultivated banana varieties have yet to demonstrate resistance except for the wild-type diploid banana *Musa balbisiansa,* which is native to Southeast Asia (Nakato et al., 2019).

Several viruses, including *banana streak virus* (BSV, genus Badnavirus) and *banana bunchy top virus* (BBTV, genus Babuvirus) also affect banana production worldwide because of their effects on yield, quality, and limitations to the international germplasm exchange due to presence of viruses in planting materials, posing a severe threat to food and nutrition security in banana-growing regions (Kumar et al., 2015).

Banana also face pressure from various pests, including aphids, mites, nematodes, and weevils, further complicating pest management in banana cultivation. Plant-parasitic nematodes and weevils pose a significant global threat to banana cultivation, resulting in severe yield losses varying from 40% to 50% (Gold et al., 2001). Various nematodes, such as *Radopholus similis*, *Pratylenchus goodeyi*, *Pratylenchus coffeae*, *Helicotylenchus multicinctus*, and *Meloidogyne* spp., are prevalent either alone or in combination in banana fields (Coyne et al., 2013). Similarly, banana weevils (*Cosmopolites sordidus*) stand out as the most challenging insect pest on a global scale, inflicting severe damage to both roots and pseudostems (Twesigye et al., 2018). The escalating impact of these pests underscores the urgent need for sustainable and accessible pest management strategies.

Moreover, abiotic factors such as soil erosion, nutrient deficiencies, and climatic variability pose additional challenges to banana production. The susceptibility of banana to climatic conditions, coupled with the looming threat of climate change, further exacerbates production challenges, potentially altering traditional growing regions and exposing banana to new risks.

Addressing these multifaceted production constraints requires a holistic approach encompassing improved agricultural practices, disease-resistant varieties, sustainable soil management, and strategies to enhance genetic diversity. By adopting such a comprehensive approach, the banana industry can mitigate risks, enhance productivity, and ensure the long-term sustainability of banana cultivation.

3 Genetic diversity in banana germplasm

Edible banana were originated through the natural hybridization of two wild progenitors, Musa acuminata (AA genome) and *Musa balbisiana* (BB genome), boast a rich tapestry of genetic diversity. This diversity is reflected in the multitude of cultivars, which are classified into various genome groups based on their genetic makeup. Among these groups are diploid banana genomes, denoted as AA or AB, and seedless triploid genomes, encompassing AAB, AAA, and ABB variations (Hinge et al., 2022). The global count of banana cultivars ranges from an estimated 300 to 1,200, showcasing the wide array of genetic variations that have emerged through natural processes and human interventions (Ploetz et al., 2007; Thierry, 2020). Diploid seeded banana are about 290 cultivars grown in Southeast Asian countries, and edible seedless triploid banana are about 650 cultivars grown worldwide (Thierry, 2020).

The importance of genetic diversity in banana cannot be overstated, particularly in the context of adapting to environmental stresses. A diverse genetic pool equips banana plants with the resilience needed to navigate through challenges posed by biotic and abiotic factors. In contrast, a lack of genetic diversity renders banana crops vulnerable to extinction, especially in the face of rapidly changing environmental conditions. Within individual genomic groups, banana exhibit varying degrees of genetic diversity, shaped by mutations and decades of selective breeding efforts aimed at enhancing desirable traits (Thierry, 2020). Diversity in plant genetic resources allows plant breeders to develop new and improved banana cultivars with desirable characteristics, including farmer-preferred traits and disease-resistance high-yielding varieties (Govindaraj et al., 2015).

Despite the inherent challenges in preserving genetic diversity within genomic groups, it remains a crucial endeavor for the long-term sustainability of banana cultivation. The conservation of diverse genetic resources serves as the foundation for breeding programs aimed at developing new banana cultivars with enhanced traits, including resistance to diseases and pests, improved yield, and better adaptation to changing environmental conditions.

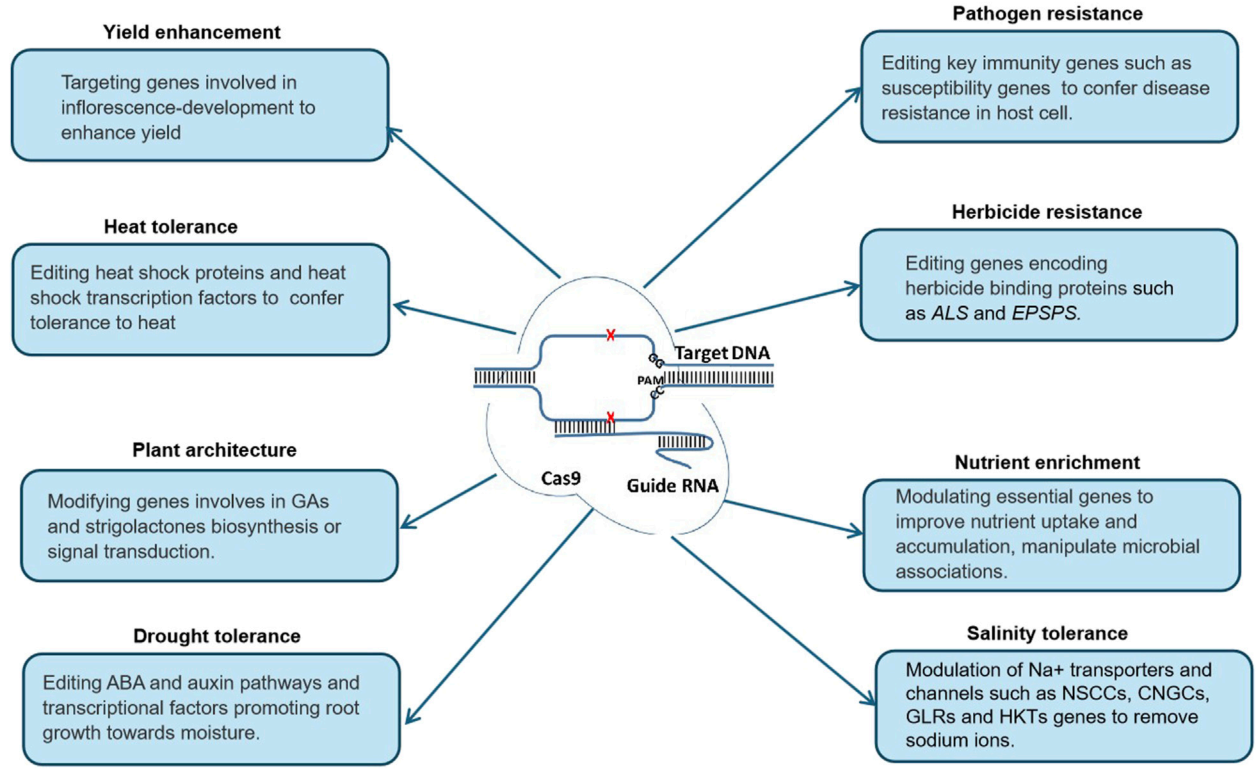
The domestication of banana spans over a millennium, resulting in the emergence of numerous parthenocarpic varieties. These varieties, characterized by the absence of seeds and developed through natural hybridization, have been propagated vegetatively by farmers over generations. However, it's noteworthy that the initial domestication process likely tapped into only a fraction of the available genetic diversity present in wild banana species (De Langhe et al., 2009). Understanding the genetic diversity of *Musa* species is not only crucial for the preservation of biodiversity but also for addressing future food security challenges (Ortiz, 1997). The advent of CRISPR technology represents a significant leap forward in genetic manipulation, offering unprecedented precision and speed in breeding efforts. This revolutionary tool has the potential to expedite the breeding cycles of banana and facilitate the development of cultivars with tailored traits, thereby contributing to the resilience and sustainability of banana cultivation in the face of evolving environmental and agricultural landscapes.

4 Overview of CRISPR/Cas gene-editing technology

The field of gene editing, encompassing technologies that enable precise alterations to an organism's DNA, has witnessed significant advancements. These tools empower scientists to add, remove, or modify genetic material at specific genomic locations with unparalleled accuracy. Among the various gene editing tools, clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein (Cas) systems have emerged as a cornerstone of genetic manipulation due to their speed, cost-effectiveness, precision, and efficiency, surpassing previous techniques like meganucleases, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) (Tripathi et al., 2022; Ntui et al., 2023).

CRISPR/Cas9, derived from a naturally occurring bacterial immune defense mechanism against viruses, functions by leveraging RNA segments generated from CRISPR arrays to identify and bind to specific DNA sequences (Koonin and Makarova, 2009). Upon binding, the Cas9 enzyme cleaves the DNA at precise locations, initiating the process of editing. Scientists have harnessed this bacterial defense system to edit DNA by designing short guide RNA (gRNA) sequences that guide the Cas9 enzyme to target DNA sequences in cells. Once the desired DNA sequence is identified, Cas9 cuts the DNA, allowing for the deletion, addition, or substitution of nucleotides, thereby altering the genomic DNA of cells.

The CRISPR/Cas9 system encompasses two classes (Class 1 and Class 2), six types (I to VI), and several subtypes, each with distinct characteristics (Xu and Li, 2020). The CRISPR/Cas9 technology primarily comprises two essential components: the Cas9 nuclease and the gRNA. The gRNA guides the Cas9 enzyme to induce precise double-stranded breaks (DSB) at target sites in the DNA. Moreover, it detects the protospacer adjacent motif (PAM), a three nucleotides sequence, and initiates editing upstream. Subsequently, the cell's endogenous repair mechanisms, namely non-homologous end joining (NHEJ) and homology-directed repair (HDR), come into play to repair the DNA damage. The NHEJ pathway, an error-prone mechanism, leads to random insertions or deletions (indels) at the cleavage sites, resulting in frameshift mutations and targeted gene knockouts. On the other hand, the HDR pathway enables precise genomic alterations, such as gene knock-in, gene replacement, or insertion of foreign genes or DNA sequences, by employing a homologous DNA repair template. Furthermore, the type of repair determines the classification of editing into three categories: SDN1, SDN2, or SDN3 (Modrzejewski et al., 2019). SDN1 involves random mutations in the host genome, altering gene function or causing gene silencing or knockout. SDN2 utilizes a repair template matching the DSB, leading to nucleotide substitution or targeted indels via HDR. SDN3 facilitates the targeted insertion of



Yield enhancement

Targeting genes involved in inflorescence-development to enhance yield

Heat tolerance

Editing heat shock proteins and heat shock transcription factors to confer tolerance to heat

Plant architecture

Modifying genes involves in GAs and strigolactones biosynthesis or signal transduction.

Drought tolerance

Editing ABA and auxin pathways and transcriptional factors promoting root growth towards moisture.

Pathogen resistance

Editing key immunity genes such as susceptibility genes to confer disease resistance in host cell.

Herbicide resistance

Editing genes encoding herbicide binding proteins such as *ALS* and *EPSPS*.

Nutrient enrichment

Modulating essential genes to improve nutrient uptake and accumulation, manipulate microbial associations.

Salinity tolerance

Modulation of Na+ transporters and channels such as NSCCs, CNGCs, GLRs and HKTs genes to remove sodium ions.

FIGURE 1
Application of CRISPR/Cas9 based editing in crop improvement.

foreign genes by repairing the DSB with a longer repair template than the homologous sequences.

5 Advances in CRISPR technology

Since the advent of CRISPR technology, several CRISPR-based tools have been developed, with broadened targeting ranges, enhanced editing specificity and efficiency, and other unique functionalities, revolutionizing crop engineering (Figure 1). CRISPR/Cas9 remains the most widely utilized system due to its stability, adaptability, ease of design, and capacity to multiplex gene editing Originating from the type II CRISPR immune system in bacteria, CRISPR/Cas9 comprises the Cas9 endonuclease from *Streptococcus pyogenes* and a synthetic single guide RNA (sgRNA). The sgRNA directs Cas9 to a specific DNA sequence, guided by the protospacer adjacent motif (PAM), with Cas9 demonstrating a higher affinity for NGG compared to NAG. This system enables precise gene editing and simultaneous modification of multiple genes (Tripathi et al., 2023).

In addition to Cas9, several other Cas variants, including Cas12a, Cas13, Cas14 and CasX have been developed. Cas12a, also known as Cpf1, is a type V, class 2 CRISPR that only harbors the RuvC domain. It possesses RNase activity for crRNA synthesis and DNase activity for single strands. Cas12a recognition of PAM sequences like TTN/TTTN/TTTV and T-rich motifs allows for efficient multiplex gene modifications using a single sequence array on the selected gRNA (Tripathi et al., 2022). Cas13a, a class 2 type VI-A ribonuclease, targets and cleaves single-stranded RNA, offering higher accuracy in viral detection compared to traditional methods. PAM fragments are not necessary for Cas13a activity.

Cas14, an RNA-guided nuclease, uniquely recognizes DNA without PAM dependency, displaying versatile ssDNA cleavage capabilities and high specificity for single-nucleotide polymorphisms (SNPs) (Wu et al., 2022; Zhou et al., 2023). In contrast to Cas12a, Cas14 recognises SNPs with great specificity and sensitivity, which has been used for pathogen discrimination and genotyping. This characteristic has been harnessed for pathogen discrimination and environmental monitoring applications. Indirect sensing of non-nucleic acid targets using Cas14 has been investigated, allowing for the sensitive detection of antibiotics with low nanomolar sensitivity. LC-MS and the usage of metal isotopes, however, made it less straightforward. Wu et al. (2022) created a CRISPR/Cas14-based aptasensor that achieved excellent sensitivity in environmental monitoring by detecting microcystin-LR with nanomaterials-assisted fluorescence generation. However, the use of complementary DNA to block the aptamer's binding capacity may result in signal loss in target recognition. Furthermore, the potential of CRISPR/Cas14 for aptasensing has never been investigated. Thus, it is worthwhile to try to create an affordable, quick, and direct Cas14-based biosensor for flexible aptasensing.

CasX, identified through metagenomic analysis of groundwater-derived bacteria, represents another promising variant. It functions as an RNA-guided DNA nuclease with a distinct PAM recognition pattern (5′-TTCN) (Liu et al., 2019). CasX is smaller than Cas12, with a unique structure that includes a RuvC domain. Its features include PAM-independent ssDNA trans-cleavage activity, making it a versatile tool for genetic modification. Studies have shown CasX's potential in gene editing across different organisms, including human cells (Yang and Patel, 2019).

Recently, a new family of RNA-guided endonucleases was found that shares a core domain with the CRISPR/Cas12 family. Evolutionarily conserved, RNA-guided DNA endonucleases carry out a variety of functions *in vivo*, ranging from the prokaryotic TnpB effector to the eukaryotic Fanzor effector. The transposable elements TnpB and Fanzor effectors, together referred to as the OMEGA system, include a CRISPR/Cas9 like domain (RuvC) that functions as an RNA-guided endonuclease (Karvelis et al., 2021). Using uRNA that is complementary to the target DNA, TnpB helps the TnpA module to facilitate the transposition of a particular locus. This RNA guidance allows for the reprogramming of DNA targeting, which is widely employed in gene editing. It is believed that the CRISPR/Cas12 system present in prokaryotes arose from TnpB by introducing extra domains, given that these TnpBs only have a minimal core domain that performs the CRISPR/Cas12 family's function. From the first classified ISDra2 TnpB, K, and racemifer TnpB types to the most recent database-screened ISDge10, ISAam1, and ISYmu1, the features of target DNA recognition of TnpB have been reported (Altae-Tran et al., 2021; Sasnauskas et al., 2023; Xiang et al., 2023; Badon et al., 2024). Fanzor effectors are mostly found in plants, fungi, protists, arthropods, and eukaryotic viruses. At the molecular level, they exhibit a great deal of similarities to the TnpB system. Fanzor is mainly divided into Fanzor 1 and 2 types. It has been observed that both forms use TAM sequence recognition and uRNA complementary to target DNA, much like TnpB, to produce RNA-DNA heteroduplexes on target DNA. Like TnpB and Fanzor endonucleases, IscB recognises TAM and has a small size of 496 aa (OgeuIscB). Nonetheless, it has comparable functionality, nucleic acid binding, and domain organisation (RuvC, BH, and HNH domains) (Badon et al., 2024).

Prime and base editing technologies represent significant advancements in CRISPR-based gene editing. These editing methods make use of dead Cas9 (dCas9), a modified version of Cas9. To produce a base editor that allows base substitution at single nucleotide resolution without the need for a DNA donor template, a DNA deaminase is coupled to the dCas9 in base editing. The effectors permit C:G-to-T:A or A:T-to-G:C substitution, depending on the kind of DNA deaminase, and the RNA-guided CRISPR system locates the targeted locus in the genome that has to be altered. Prime editing mediates DNA base pair swaps, minor insertions, and tiny deletions (indels) by a process similar to that of classical CRISPR/Cas systems (Matsoukas, 2020; Chen et al., 2021). In contrast, primer editing doesn't require a donor template or result in DSB; instead, it removes off-target effects and fixes frameshifts brought on by indels. The genome can only be altered by a fusion protein made up of a longer-than-usual gRNA called pegRNA and Cas9 H840A nickase linked to a modified reverse transcriptase (RT) enzyme. Prime and base editing are SDN1 types of editing because they don't need a DNA donor template. This suggests that they may be treated similarly to non-transgenic crops and may not be subject to stringent biosafety regulations.

These advanced CRISPR technologies offer tremendous potential for improving crop resilience, enhancing nutritional quality, and addressing global food security challenges.

6 CRISPR/Cas applications for improvement of banana

Banana is one of the most consumed fruits globally playing a crucial role in food security. However, challenges such as pests, diseases, and nutritional deficiencies pose significant threats to banana production and sustainability. To address these challenges, researchers worldwide are harnessing the power of CRISPR/Cas technology to develop resilient, high-yielding, and nutrient-enriched banana varieties (Table 1).

6.1 Targeted gene editing for disease resistance

Researchers at the International Institute of Tropical Agriculture (IITA) in Kenya have spearheaded efforts to enhance banana and plantain varieties through gene editing techniques. By targeting disease susceptibility "S"genes in banana, they have generated hundreds of gene-edited events, many of which have exhibited enhanced resistance to bacterial diseases after rigorous screening in controlled environments (Tripathi et al., 2021; Ntui et al., 2023). These promising events are now slated for field trials before eventual deployment to farmers' fields.

The availability of reference genome sequences and sophisticated CRISPR/Cas9 gene editing tools has greatly facilitated the development of banana resistant to BSV and BXW (Tripathi et al., 2022). By meticulously targeting endogenous genes, researchers have achieved significant success in conferring resistance to these devastating diseases.

For instance, BXW resistance has been effectively demonstrated in banana through the precise knockout of S-genes. These host genes play a crucial role in enabling pathogen invasion, thereby facilitating pathogen proliferation and symptom emergence. Editing these pivotal S-genes has unlocked broad-spectrum resistance against bacterial pathogens, providing a promising avenue for disease management (Zaidi et al., 2020). Editing S-genes can confer broad-spectrum resistance in certain scenarios and resistance tailored to the specific pathogen (Peng et al., 2017; Kim et al., 2018). Several disease susceptibility genes linked to bacterial resistance have been identified and targeted for editing in banana plants (Tripathi et al., 2020).

In a specific example, *MusaDMR6* gene in the banana cultivar 'Sukali Ndiizi' was knocked out at two sites using a multiplexed CRISPR/Cas9 system via *Agrobacterium*-mediated transformation of embryogenic cells. This resulted in *dmr6* mutants displaying increased resistance to BXW without any morphological defects (Tripathi et al., 2021). DMR6 functions as a negative regulator of plant defense, encoding 2-oxoglutarate Fe (II)-dependent oxygenase (2OGO) that hydrolyzes the plant defense signaling molecule salicylic acid (SA), and is upregulated during pathogen infection (Zhang et al., 2017; Low et al., 2020). Loss of function of *DMR6* gene in other crops, such as tomatoes, has shown to confer resistance to various pathogens (Thomazella et al., 2021).

Similarly, CRISPR/Cas9 was utilized to knockout *early nodulin-like 3* gene (*MusaENOD3*) in the banana cultivar "Gonja Manjaya" to confer resistance to BXW (Ntui et al., 2023). Nodulins and nodulin-like genes are typically induced in legumes upon

TABLE 1 Summary of gene editing in banana.

S. No.	Trait	Editing system	Target	References
1.	_	CRISPR/Cas9	*Musa phytoene desaturase (MusaPDS)*	Kaur et al. (2020), Naim et al. (2018), Ntui et al. (2020)
2.	Xanthomonas wilt resistance	CRISPR/Cas9	*Musa downy mildew resistance 6 (MusaDMR6)*	Tripathi et al. (2021)
3.	Xanthomonas wilt resistance	CRISPR/Cas9	*Musa early nodulin-like 3 (MusaENOD*3)	Ntui et al. (2023)
4.	Banana streak virus resistance	CRISPR/Cas9	*Endogenous Banana Streak Virus* in the B genome of banana	Tripathi et al. (2019c)
5.	Shorter height	CRISPR/Cas9	*gibberellin 20ox2* (*MaGA20ox2*)	Shao et al. (2020)
6.	Increase β-carotene	CRISPR/Cas9	*Musa lycopene epsilon-cyclase* (*LCYε*)	Kaur et al. (2020)
7.	Delayed ripening	CRISPR/Cas9	*Musa acuminata aminocyclopropane-1-carboxylase oxidase (MaACO1)*	Hu et al. (2021)
8.	—	Cas-CLOVER	*MusaPDS*	Tripathi et al. (2023)

nodulation by *Rhizobium* bacteria and play a pivotal role in symbiotic interactions. Many nodulin-like proteins, particularly early nodulin-like proteins (ENODL), have been found in various non-leguminous plants, indicating their involvement in processes beyond nodulation, including growth control and nutrient transfer (Denance et al., 2014). Plant defense against infections has been associated with some ENODL proteins. Editing of *MusaENOD3* resulted in edited events exhibiting resistance to BXW. The sequencing data showed several types of mutations, including deletions, insertions, substitutions. Most of the detected deletions were large, ranging from 23 bp to 180 bp (Ntui et al., 2023), underscoring its significance in plant-pathogen interactions and offering novel opportunities for enhancing resistance to bacterial diseases in crops.

Additionally, apart from *MusaDMR6* and *MusaENDOL*, several potential genes identified through comparative transcriptomic studies comparing RNAseq of BXW-susceptible cultivars with BXW-resistance wild progenitor could be targeted for editing using CRISPR/Cas9 to develop resistance against BXW (Tripathi et al., 2019a).

BSV, a member of the badnavirus family (Harper et al., 1999), integrates into the host plant's genome. It exhibits two forms: the integrated BSV, known as endogenous BSV (eBSV), and the episomal form,. Stress factors like temperature, drought, crossing, and micropropagation cause the integrated viral sequences to become activated, resulting in the infectious episomal form of BSV, which causes symptoms in plants. Drought and extremely high temperatures are two aspects of climate change that might exacerbate BSV disease. A multiplex CRISPR/Cas9 system was employed to inactivate the integrated eBSV by targeting all three open reading frames (ORF) of the virus in "Gonja Manjaya" (Tripathi et al., 2019). The regenerated gene edited events displayed mutations in the target regions that hindered the transcription of viral protein into functional viral episomal proteins. Under water stress, most of the edited events remained asymptomatic compared to the non-edited control plants, demonstrating inactivation of integrated eBSV into infectious viral episomal proteins (Tripathi et al., 2019).

BBTV is a single-stranded DNA (ssDNA) virus with a multipartite genome comprising six circular components with an approximate size of 1.1 kb each (Tripathi et al., 2021). As of now, no instances of CRISPR/Cas-mediated resistance against BBTV have been documented. However, various genes have been edited for resistance against ssDNA viruses, presenting potential targets for developing resistance against BBTV. Here, we describe some promising potential targets for exploration in the quest to establish resistance against BBTV.

Targeting viral proteins has shown promise for tackling DNA viruses. For instance, CRISPR/Cas9 targeting the viral replication-associated region or intergenic region (IR) of *cotton leaf curl Multan virus* (CLCuMuV) and *bean yellow dwarf virus* (BeYDV) have demonstrated effective DNA interference, providing resistance against *beetroot severe curly top virus* (BSCTV) in transgenic *Nicotiana benthamiana* or *Arabidopsis thaliana* plants in bioassay experiments. These mutant viruses were unable to synthesis viral coat proteins and rendered them inactive (Baltes et al., 2015; Ji et al., 2015; Yin et al., 2019). Gene editing of the coat protein (CP) or replicase (Rep) of *tomato yellow leaf curl virus* (TYLCV) by CRISPR/Cas9 resulted in efficient virus interference, as evidenced by the low accumulation of the TYLCV DNA genome in the transgenic tomato and N. benthamiana plants (Tashkandi et al., 2018). Similarly, Ali et al. (2015) observed that sgRNAs targeting the stem-loop sequence were more effective at interfering with multiple geminiviruses, such as the monopartite geminivirus *Cotton Leaf Curl Kokhran virus* (CLCuKoV), the bipartite geminivirus *Merremia mosaic virus* (MeMV), and various severe and mild strains of TYLCV geminivirus in comparison to sgRNAs targeting the viral CP region and the replication-associated region within IR. Viral movement protein (MP) has proven to be a valuable target for developing resistance to viruses. For example, a multiplexed CRISPR/Cas9 system with sgRNAs targeting MP or CP region established resistance to *wheat dwarf virus* (WDV) (Kis et al., 2019).

TR4 is one of the major fungal diseases of banana, which scientists are still battling to control. Gene editing could be a valuable tool for controlling this disease by targeting susceptibility genes. While there is currently no documented evidence of using gene editing to develop resistance to TR4, various susceptibility genes such as *alcohol dehydrogenase* 1 (ADH1), *mildew resistance locus* O (MLO), *LATERAL ORGAN BOUNDARIES* (CsLOB1), *DMR6*, and *ERF922* could serve as potential targets for developing resistance against TR4. These susceptibility genes are induced and overexpressed by pathogens as demonstrated by transcriptome analysis (Tripathi et al., 2019a). Knocking out of these susceptibility genes are proven strategy to generate disease resistant plantls. *ADH1* disruption in tomatoes resulted in reduced ethanol production and impaired growth and development of *F. oxysporum*, indicating its role as a susceptibility gene facilitating pathogen colonization and symptom development, making it a promising target for developing TR4 resistance in banana (Pathuri et al., 2011; Zhang E. et al., 2018).

6.2 Improving plant architecture

The plant architecture encompasses the growth and development of a plant from its meristems to the development of stems, leaves, inflorescences and roots. This architecture plays a crucial role in determining their performance and ability to thrive in challenging environments (Wang et al., 2018). One crucial aspect of plant structure is its height, which is influenced by factors like genetic makeup, environmental conditions, and hormone levels such as gibberellins, brassinosteroids, and strigolactones.

Recent breakthroughs in gene editing have provided exciting opportunities to manipulate plant structure for improved outcomes. For example, using CRISPR/Cas9 technology, researchers have successfully targeted genes, GA20ox2, involved in gibberellic acid (GA) signaling in rice, resulting in plants with reduced height by 22.2% and yet yielding 6% more, without affecting other important traits (Han et al., 2019). Similarly, editing the gene CLEAVAGE DIOXYGENASE 7 (CCD7), which controls a crucial step in strigolactone biosynthesis, by CRISPR/Cas9, produced mutants with reduced height and a striking increase in tillers, demonstrating the potential of genetic editing to shape plant morphology (Butt et al., 2018).

In banana cultivation, researchers utilized the CRISPR/Cas9 system to target *MaGA20ox2* gene in the "Gros Michel" variety. This resulted in the development of semi-dwarf mutants with thicker, darker, and greener leaves compared to non-edited plants, highlighting the effectiveness of gene editing in altering plant structure for desired characteristics (Shao et al., 2020).

6.3 Targeting genes related to nutrient biosynthesis

A fundamental objective of gene editing in agriculture is enhancing the nutritional content of crops.

Nutritional improvement in cultivated crops is one of the significant goals of gene editing. This can be achieved by augmenting the expression levels of genes involved in nutrient biosynthesis. Editing targets situated upstream of the coding sequences (CDS) or in untranslated regions, like the 5′UTR, which regulate expression, can induce frameshifts leading to premature termination codons, ultimately modulating nutrient production (Nagamine and Ezura, 2022). Furthermore, manipulating metabolic pathway enzymes through gene editing can boost nutrient functionality or aid in metabolizing toxic substances, thereby improving overall crop quality (Nagamine and Ezura, 2022).

Various strategies have been employed to enhance diverse nutrients in crops, including carotenoids, γ-aminobutyric acid (GABA), iron, and zinc. Carotenoids, renowned for their antioxidant properties and role in preventing eye-related diseases, have been a major focus. Beta-carotene, a primary dietary precursor of vitamin A, crucial for eye health and immunity, has been targeted for enhancement using CRISPR/Cas9 in rice, tomato, and banana (Dong et al., 2020; Liu et al., 2021).

Increasing carotenoid levels involves overexpressing *phytoene synthase* genes, like *CrtI* and *PSY*, to redirect carbon flux into the biosynthetic pathway. Conversely, silencing genes such as *LCYe, BCH, ZEP,* and *CCD4* can inhibit precursor conversion. For instance, in banana, Kaur et al. (2020) utilized CRISPR/Cas9 to enhance β-carotene content in "Grand Naine" cultivar by editing the *lycopene epsilon-cyclase* (*LCYε*) gene, resulting in a substantial 6-fold increase (~24 μg/g) in β-carotene accumulation in the fruit pulp compared to the non-edited plants.

Another area of interest is GABA, an inhibitory neurotransmitter with potential health benefits. Gene editing has enabled the development of GABA-rich foods like the "Sicilian Rouge High GABA" tomato, which accumulates four to five times more GABA than ordinary tomatoes, achieved through targeted deletion of the C-terminal of glutamate decarboxylase (GAD) (Nonaka et al., 2017). Additionally, biofortification of micronutrients like iron and selenium has been demonstrated through gene editing. Targeting genes such as *Vacuolar Iron Transporter* (VIT) and *arsenite tolerant* 1 (*astol1*) in crops like rice has resulted in increased iron and selenium content, respectively, offering potential health benefits to consumers (Che et al., 2021).

6.4 Targeting genes related to shelf life

Banana, as a typical climacteric fruit, ripen and decay within a week after exposure to exogenous ethylene. This short shelf life significantly limits their storage, transportation, and marketing, leading to substantial postharvest losses. By editing the *aminocyclopropane-1-carboxylase oxidase* (*MaACO1*) gene, it is possible to delay the ripening process by reducing endogenous ethylene production (Hu et al., 2021). This approach demonstrates significant potential in enhancing banana quality and resilience.

Further, researchers at Tropic Biosciences developed non-browning banana by rendering a key gene responsible for polyphenol oxidase production nonfunctional. This breakthrough holds immense potential to dramatically reduce food waste and carbon dioxide emissions along the supply chain, with projections indicating a potential decrease of over 25%. This is particularly significant given that more than 60% of exported banana currently go to waste before reaching consumers (source: Tropic Biosciences, 2023).

These gene-edited banana have received a non-GMO exemption from the Philippines Department of Agriculture-Bureau of Plant Industry. This marks a significant milestone as the first gene-edited product to undergo the newly established regulatory determination process in the Philippines. As a result of this determination, Tropic's non-browning banana can now be freely imported and propagated within the country.

7 Future prospects for gene editing in banana

7.1 Advancements in CRISPR-based gene activation or inhibition

In the context of banana improvement, CRISPR-based gene activation or inhibition holds immense potential for enhancing specific traits. Our research at IITA focuses on activating endogenous banana genes associated with antimicrobial properties, pathogen resistance, and disease tolerance through CRISPRa. By targeting genes identified through transcriptomic analysis (Tripathi et al., 2019a), we aim to confer resistance to diseases like BXW, which poses a significant threat to banana cultivation. Initial screenings of regenerated plants have shown promising levels of gene activation, paving the way for further characterization of their resistance to BXW and other banana diseases.

Moreover, CRISPRi presents a promising strategy for developing virus-resistant banana. When viruses attack plants, they incorporate their genetic material into the genome to reproduce and generate the building blocks for new virus particles. In response, plants activate their RNAi machinery to defend themselves against invading viruses. However, many viruses could inhibit the plant RNAi silencing pathway by releasing a suppressor protein to prevent siRNAs from initiating the defense process (Karlson et al., 2021). By targeting viral RNA, CRISPRi could disrupt viral invasion and enhance plant immunity. Zhang Y.-Z. et al. (2018) produced transgenic Arabidopsis plants resistant to CMV using CRISPRi technology. They showed that the resistance could be detected up to T6 generation. Similarly, Aman et al. (2018) developed a CRISPR/dCas9 construct containing Cas13a, which could innately process pre-crRNA into functional crRNA to target the viral mRNAs and deliver them to tobacco plants. When the plants were inoculated with a recombinant TuMV expressing GFP (TuMV-GFP), they found that the intensity of GFP-expressing TuMV in tobacco was reduced up to 50%, indicating the successful control over the spread of the viral GFP signal (Karlson et al., 2021). Previous studies have demonstrated successful virus resistance in plants like Arabidopsis, and tobacco using CRISPR technology, indicating its potential in banana virus management. With further optimization and refinement, CRISPRi could emerge as a powerful tool for conferring robust viral resistance in banana, safeguarding their production from devastating viral infections.

7.2 Alternative CRISPR tool for gene editing in banana

CRISPR/Cas9 has emerged as a pivotal tool for gene editing, holding immense promise for revolutionizing agriculture and addressing critical issues such as climate resilience and food security. However, one of the challenges researchers face is navigating the complexities of intellectual property (IP) protection and licensing to enable the release of gene-edited crops for widespread use by growers. Securing licenses for CRISPR/Cas9 technology can be challenging due to high demand and intricate legal frameworks.

To circumvent these challenges and facilitate the development of gene-edited crops, alternative gene editing approaches with clearer IP issues are being explored. Researchers at IITA have pioneered an alternative gene-editing tool tailored specifically for banana, known as the Cas-CLOVER system (Tripathi et al., 2023). This innovative technology is founded on dual-guide RNA and the programmable clover endonuclease Clo051, which induces double-strand breaks at the target site (Madison et al., 2022).

The Clo051 endonuclease functions as a binding protein at the DNA target site, while the fusion protein comprises an inactivated or dead Cas9 (dCas9) protein. Unlike CRISPR, the Cas-CLOVER system employs two gRNAs along with the Clo051 endonuclease, requiring the dimerization of subunits associated with each gRNA. This dual-guide RNA mechanism ensures highly targeted and precise gene editing, as Clo051 generates double strands only when both gRNAs are simultaneously engaged.

Researchers successfully validated the efficacy of the Cas-CLOVER technology in banana gene editing, particularly targeting mutations in the banana *phytoene desaturase (MusaPDS)* gene (Tripathi et al., 2023). Banana mutants generated through this technique exhibited an albino phenotype, indicative of disrupted *PDS* gene function. This demonstration underscores the precision and versatility of the Cas-CLOVER system for precise gene editing in banana, offering a promising alternative to conventional CRISPR/Cas9 technology.

7.3 Potential applications of base editing and prime editing in banana

Base editing and prime editing offer transformative potential for banana improvement, though their application in this crop is still emerging. Base editing, a technique that enables precise nucleotide substitutions, could significantly enhance banana traits, particularly in areas such as nitrogen use efficiency and the production of DNA-free plants. For instance, in rice, a CRISPR/Cas9-xyr5APOBEC1-based system was employed to replace a cytosine with a thymine in the NRT1.1B gene, resulting in improved nitrogen use efficiency (Hu et al., 2015). Similarly, in banana, base editing could be used to enhance nitrogen utilization.

One notable application of base editing is the development of herbicide-resistant, transgene-free plants. A*cetolactate synthase* (*ALS*) gene modulates herbicide resistance in plants. In watermelon, single-base substitutions in *ALS* gene enabled the production of herbicide-resistant, transgene-free plants (Tian et al., 2018). A similar approach could be applied to bananas, where base editing could simultaneously edit the ALS gene and other genes of interest, facilitating the generation of transgene-free plants resistant to herbicides while enabling trait enhancement through multiplexing (Zhang et al., 2019).

Prime editing, which allows precise insertions, deletions, and base substitutions without requiring double-strand breaks or donor DNA, has also demonstrated significant potential in crop breeding. For instance, Jiang et al. (2023) utilized the PPEmax system to generate TAP-IVS mutant rice plants with glyphosate resistance, and Qiao et al. (2023) applied a similar strategy to maize. In addition, efficient insertion of protein tags has been achieved using optimized PE techniques (Li et al., 2023), which could be beneficial for functional genomics in banana.

Prime editing has also shown promise in regulating protein expression through the manipulation of upstream open reading frames (uORFs) in eukaryotes (Zhang et al., 2020). Xue et al. (2023) developed methods to fine-tune uORF expression, which could be applied to banana to precisely regulate target gene expression.

Moreover, prime editing has been successfully employed to confer disease resistance in plants. For example, Gupta et al. (2023) engineered resistance to bacterial blight in rice using enhanced PPE systems, PE5max. Techniques such as knocking in resistance elements or generating resistance alleles could be adapted to develop resistance to BXW, a major disease affecting bananas.

Both base editing and prime editing hold significant promise for advancing banana improvement by enhancing traits such as nutrient efficiency, disease resistance, and enabling the production of transgene-free plants. These innovative editing techniques could address critical challenges in banana cultivation and improve overall crop resilience and productivity.

8 Regulatory challenges regarding the commercialization of gene-edited banana and strategy to develop transgene-free banana

The pursuit of transgene-free gene editing in banana aims to create non-GMO plants with desired traits while addressing regulatory constraints associated with GMOs. Despite the broader array of transformation approaches available, achieving transgene-free plants, especially in clonally propagated crops like banana, remains a challenge.

Currently, gene editing in banana involves plasmid delivery, where plasmids containing the Cas9 protein, selection marker genes, promoters, and terminators are introduced into plant cells via *Agrobacterium*-mediated transformation. These gene sequences integrate into the banana genome. Given that banana is vegetatively propagated crop, segregating out these sequences through crossing is impractical. Consequently, gene-edited banana is classified as GMOs by regulatory bodies and are subject to stringent biosafety regulations, which can hinder commercialization and acceptance (Tripathi et al., 2019c). To address this regulatory hazel and increase the commercialization of gene edited banana, it is imperative to produce DNA-free products. Several strategies are being explored to produce transgene-free gene-edited banana plants.

One approach involves utilizing ribonucleoproteins (RNPs), where a preassembled complex of Cas9 protein and gRNA is delivered into the plant cell (Liang et al., 2017). This complex facilitates gene editing at target sites immediately after transfection and is rapidly degraded by endogenous proteases, minimizing off-target effects and preventing the integration of foreign DNA elements (Woo et al., 2015). Various delivery methods such as electroporation, particle bombardment, and protoplast transfection have been explored for direct delivery of the RNA-guided engineered nucleases- ribonucleoproteins (RGENs-RNPs) into plant cells, with protoplast transfection being the most versatile. While some authors have reported the regeneration of complete plants from banana protoplast (Panis et al., 1993; Matsumoto and Oks, 1998; Assani et al., 2001), regenerating plants from banana protoplasts remains challenging (Tripathi et al., 2022).

Another strategy involves transiently delivering the editing machinery into plant cells via *Agrobacterium* without applying selection. This method has been demonstrated in other crops, resulting in the production of transgene-free plants. For example, Chen et al. (2018) produced transgene-free tobacco plants by transient expression of CRISPR/Cas9 containing gRNAs targeting the PDS gene. They obtained up to 8.2% non-transgenic mutants. Using a similar approach, Veillet et al. (2019) modified the *acetolactate synthase* (ALS) gene via a cytidine-based editor and obtained transgene-free potato and tomato plants with mutation efficiency of 10% and 12.9%, respectively. However, challenges such as high off-target effects and the need for extensive screening remain.

To address these challenges, researchers are designing plasmids with mechanisms for T-DNA excision and removal following editing (Dalla Costa et al., 2020). Techniques such as the Flp/FRT system and synthetic cleavage target sites (CTS) have been developed to remove T-DNA from CRISPR-edited plants. Although challenges with trimming at T-DNA boundaries exist, these approaches represent significant progress toward producing transgene-free plants.

In ongoing research, efforts are underway to refine the process of producing transgene-free banana through transient delivery of the Cas9-gRNA reagent by *Agrobacterium*. Additionally, procedures for regeneration, PEG transfection, and protoplast isolation are being developed to streamline the process.

While transferring CRISPR/Cas9 plasmids into germ lines or protoplasts presents technical challenges and inefficiencies, innovative approaches such as *de novo* induction of meristems offer promising avenues for overcoming these limitations in dicotyledonous plants. This approach involves delivering developmental regulators and gene-editing components into somatic cells of entire plants, resulting in the transmission of desired DNA modifications to the next-generation. The graft-mobile gene editing system can be another strategy to the production of transgene-free plants in one generation without the need for transgene segregation (Yang et al., 2023).

9 Ethical issues of gene editing in agriculture

Gene editing is predicted to usher in a new Green Revolution, enhancing food and nutritional security worldwide and mitigating the effects of climate change. However, the technology also raises significant ethical concerns that encompass environmental, social, and economic dimensions.

One major concern is the possibility of off-target effects, edits occurring in unintended locations, which can result in unwanted phenotypes. The aspect of safety has been one of the critical issues of gene editing. There are questioned surrounding the safety of gene editing, especially when the technology is used for gene drives, since off-target effects in gene editing are not fully understood. There is also concern that gene-edited crops could reduce biodiversity if they outcompete natural species or if large-scale monoculture practices are adopted.

Another significant issue is the regulatory framework for gene editing. There is ongoing debate over whether gene-edited crops should be categorized as GMOs (Karalis et al., 2020). While some countries have clarified their regulations regarding gene edited crops, others, such as the European Union, South Africa and New Zealand, maintain very strict regulations, leading to variations in national regulatory strategies. Policy and regulation development for gene editing in plant breeding must also consider factors like farmers' rights and public acceptance (Idris et al., 2023). As with many new technologies, there is concern of Intellectual property rights (IPR). Gene-edited products will inevitably be patented, providing owners with IPR rights—typically agri-food corporations—with what amounts to monopolistic control over the gene-editing process' output (Sprink et al., 2022). The patenting of gene-edited crops by corporations can lead to concerns about farmers' rights and their dependency on a few large companies for seeds, potentially driving up costs and limiting traditional farming practices.

Moral and religious objections also play a significant role in the ethical debate. Many people believe that gene editing interferes with natural creation, equating it to "playing God." These objections highlight the need for inclusive and culturally sensitive discussions when considering the widespread adoption of gene editing in agriculture.

10 Conclusion

CRISPR/Cas based gene editing stands as a transformative technology with vast potential for enhancing crop productivity and nutritional quality, thus bolstering global food security amidst mounting environmental challenges. However, the clonal propagation of banana presents unique challenges in integrating gene-edited traits due to the seedless nature of the fruit. While plasmid-based delivery systems and embryogenic cell methods offer feasible pathways for generating gene-edited banana plants, the task of segregating transgenes through conventional breeding is hindered by the lack of seeds in banana. Overcoming these hurdles requires optimization of techniques like using RNPs for generating plants from protoplasts or microprojectile bombardment of cell suspension. Additionally, robust protoplast regeneration systems must be developed through further research to facilitate the creation of transgene-free plants in banana cultivars with shorter breeding cycles. Disease resistance trait is successfully targeted by knocking off the susceptible genes in the banana genome, like DMR6 and Early Nodulin gene. However, challenges persist in targeting complex polygenic traits like abiotic stress tolerance, necessitating the simultaneous knockout of multiple genes or targets.

Nevertheless, the diligent exploration of innovative technologies such as CRISPR/Cas holds promise for delivering high-yielding better banana with enhanced nutritional content and disease resistance. By integrating precision genetics with traditional breeding programs and adopting transgene-free strategies, researchers can unlock the full potential of gene-edited banana. The future holds exciting prospects for the development of banana that not only thrive in diverse environments but also offer superior nutritional value, benefiting farmers and consumers alike.

Author contributions

LT: Conceptualization, Funding acquisition, Project administration, Supervision, Writing–original draft, Writing–review and editing. VN: Writing–original draft, Writing–review and editing. JT: Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Understanding public perspectives on genetically engineered Brinjal and the adoption of modern biotechnology in Bangladesh

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The agricultural sector in Bangladesh is currently facing numerous challenges. The country is currently endeavoring to adopt modern biotechnological tools, such as genetic engineering, to modify crops with the aim of ensuring food security. Notably, Bt Brinjal represents a significant milestone as the first genetically engineered (GE) food crop commercially cultivated in South Asia. Public perception and awareness are crucial steps forward for accepting and commercializing GE crops within society. The study discussed here aims to assess public perception and awareness regarding modern biotechnology and GE crops, focusing mainly on Bt Brinjal in Bangladesh. A random survey considered demographic factors such as age, gender, hometown, educational qualification, and occupation to explore the public attitudes towards Bt Brinjal and modern biotechnology. Approximately one-third of those surveyed considered Bt Brinjal safe for consumption, and a third expressed a willingness to buy Bt Brinjal, while nearly two-thirds believed it would gain popularity in the market alongside other crops. Most respondents recognized the necessity of utilizing modern biotechnology for crop improvement beyond Bt Brinjal, and respondents with science backgrounds displayed higher awareness and a more positive attitude than those with limited education or non-science backgrounds. This study explores the public perceptions of Bt Brinjal and the adoption of modern biotechnology in Bangladesh by examining factors such as knowledge dissemination, acceptance levels, and concerns related to GE crops, and offers a meaningful perspective that can shape decision-making processes to promote agricultural sustainability and achieve relevant sustainable development goals in Bangladesh.

KEYWORDS

Bt Brinjal, consumer attitude, modern biotechnology, public perception, science communication

Introduction

Bangladesh, situated in South Asia, has a rich agricultural landscape that has significantly contributed to its economy for centuries. Most people still rely heavily on agriculture as their primary source of income (Rahman, 2017). However, Bangladesh's agricultural sector faces numerous challenges including salinity intrusions, pests, diseases, land fragmentation, water scarcity, and vulnerability to natural disasters like floods and cyclones frequently impeding crop productivity (M. H. Mondal, 2010). Modern biotechnology holds significant potential for transforming agricultural practices in Bangladesh. Advances in this field can help to overcome urgent difficulties by increasing nutrient efficiency, raising crop yield, and strengthening resistance to pests and diseases (Shohael and Hefferon, 2023).

To fully realize the potential of modern biotechnology in agriculture, it is imperative to understand and comply with the regulatory frameworks, biosafety concerns, and public acceptance while promoting inclusive and equitable access to biotechnological innovations among smallholder farmers (Shohael and Hefferon, 2023). While Bangladesh has made significant progress in developing a robust biosafety regulatory system to safely implement biotechnology advancements in agriculture (Khanam and Hasan, 2019), understanding public perspectives on GE crops and the broader adoption of modern biotechnology is essential for informed decision-making and sustainable agricultural progress (Siddiqui et al., 2022). Despite the significant implications of GE crop adoption for agriculture, food security, and environmental sustainability, there has been limited effort to engage the public in meaningful dialogue and understand their perceptions, concerns, and knowledge regarding GMOs. This lack of communication hampers the product's performance in the market (Abdullah et al., 2018).

Bangladesh has been engaged in advanced crop biotechnology research since the late 1970s by applying plant tissue culture to different plant varieties (Choudhury and Islam, 2004). The application of genetic engineering in crop improvement started after 1990, while the formulation of biosafety regulations also started (Khanam and Hasan, 2019). Brinjal cultivars genetically engineered for insect resistance (Bt Brinjal) developed by the Bangladesh Agricultural Research Institute (BARI) with the support of the United States Agency for International Development (USAID) were given authorization for cultivation in Bangladesh by the National Committee on Biosafety on 30 October 2013 (A. M. Shelton et al., 2018).

Eggplant (*Solanum melongena L.*) is a popular plant species grown worldwide for its edible fruit with multi-dimensional use in cooking (Rotino, Sala, and Toppino, 2014). Popularly known as Brinjal in South Asia, it has become a part of the regular diet, a source of nutrition, and an essential source of income for many farmers (Frary, Doganlar, and Daunay, 2007). In Bangladesh, Brinjal, locally known as Begun, is a staple diet, and it ranks third after potato and rice in terms of consumption quantity, which makes it an essential component of food security (Ahsanuzzaman and Zilberman, 2018). However, the overall production of Brinjal is relatively low because of insect infestation, which damages the yield by two-thirds, despite efforts to introduce insecticide and other management practices (Ahsanuzzaman and Zilberman, 2018).

Since the approval, Bt Brinjal has been cultivated by the farmers and sold to consumers. The introduction of Bt Brinjal in Bangladesh marks a significant development for several reasons. It has encouraged more research and development using modern biotechnology, opening doors for creating more GE products, and presented farmers with the decision to adopt GE or non-GE crops by observing the benefits, while consumers can have their own choices between GE and non-GE crops by their quality.

Despite various studies examining the performance and benefits of Bt Brinjal, there has been a noticeable gap in understanding public perception towards this GE crop in Bangladesh. Little is known about public attitudes regarding Bt Brinjal and its background. The study discussed here explores the public perceptions of Bt Brinjal and the adoption of modern biotechnology in Bangladesh by examining factors such as knowledge dissemination, acceptance levels, and concerns related to GE crops. We aim to offer a meaningful perspective that can shape decision-making processes to promote agricultural sustainability and achieve relevant sustainable development goals in Bangladesh.

A survey of public perceptions

We prepared a questionnaire to gather insights from selected participants about their perceptions of Bt Brinjal and modern biotechnology. Questions were designed to obtain information on some socio-demographic variables and structured questions, including respondents' knowledge of Bt Brinjal, understanding of the technology and its potential, consumption history, market impact, and opinions on the need for crop improvement through modern biotechnology (Figure 1). The survey was conducted randomly on 1000 willing participants, and their identities were kept anonymous. Ten data collectors conducted one-to-one interviews, each lasting approximately 20 min per individual.

Demographics

A total of 1000 respondents participated in this study, comprising 637 males and 363 females. The age of the participants ranged from 18 to 75, with a mean age of 29.5 ± 11 years. Participants were sorted out based on their home districts. Bangladesh has 64 home districts. These districts encompass diverse ethnicities, cultures, agricultural practices, and food habits. The education level of participants varied, with 0.5% reporting to have a Ph.D., 13.0% reporting completion of postgraduate studies, 26.8% reporting completion of graduate degrees, 34.5% reporting completion of higher secondary certificate degrees, 7.8% reporting completion of secondary certificate degrees, 11.0% reporting completion of primary education and 6.4% were illiterate. Of 826 respondents who have studied above secondary studies, 39.9% had a science background, 23.2% had a business studies background, and 19.5% had a humanities background. Regarding employment status, 56.7% of respondents were students, 13.2% were in public or private service, 9.0% of participants were businessmen, 2.1% of participants were farmers, and 19.0% of participants were in other sectors of occupations (rickshaw pullers, labor, unemployed).

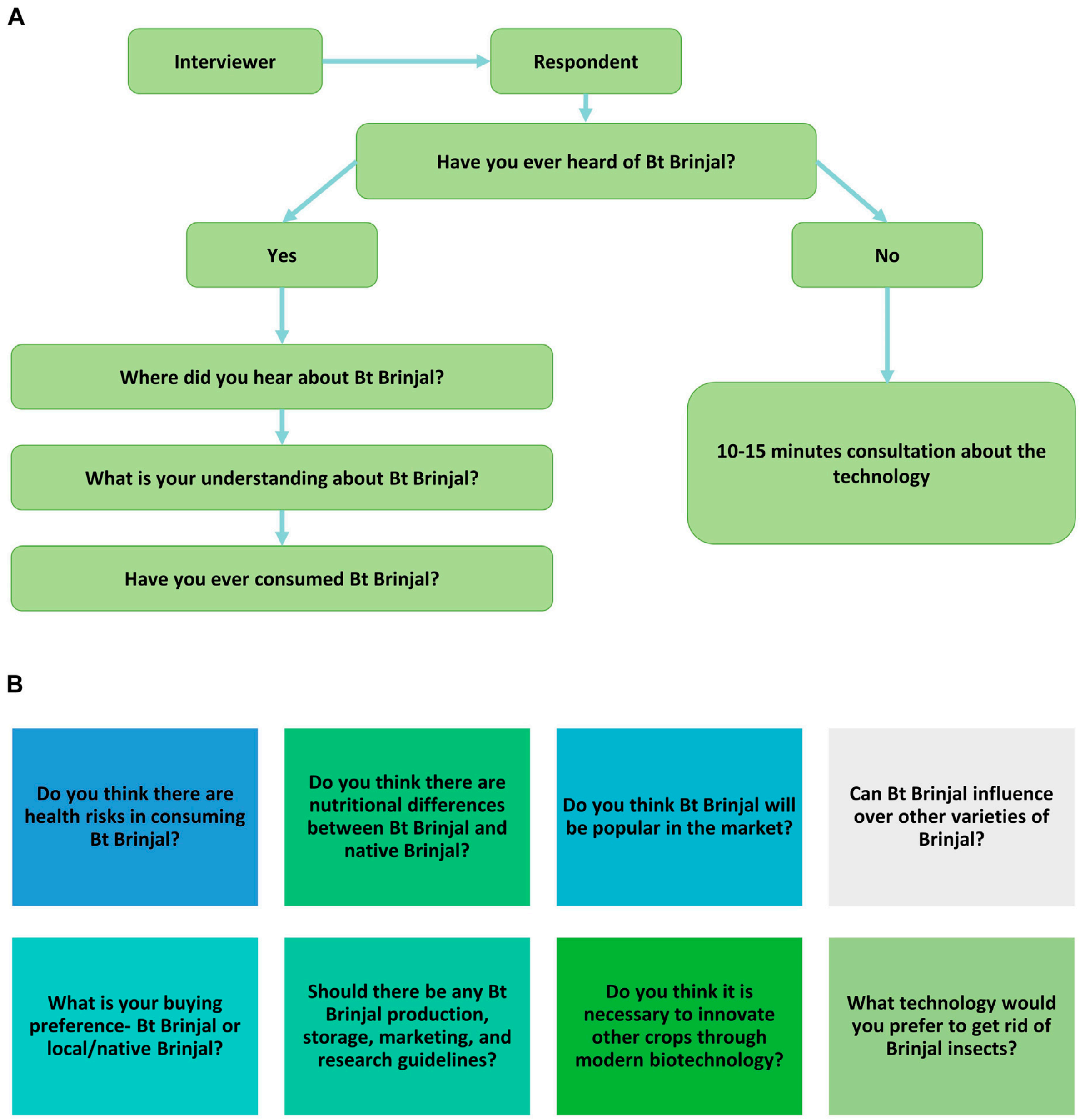

FIGURE 1
(A) The flow diagram of data collection; **(B)** The questions asked during the data collection process.

Familiarity with Bt Brinjal, source of knowledge and idea about the trait

Among 1000 respondents, around 50.1% heard about Bt Brinjal, 44.4% did not, and 5.5% were unaware of the term. Of those who previously heard about Bt Brinjal, 70% had a science background in their study. 79% of respondents were students. 90.8% completed higher secondary education or above. Among the 501 respondents who were familiar with Bt Brinjal, 29.1% knew the term from coursework, 12.1% heard the term from mass media (Television, newspaper, etc.), 6.1% heard the term from their friends or relatives, and 2.8% knew from other sources. When asked about their knowledge of the trait, 22.9% of the respondents said that Bt Brinjal is insect-resistant, and 3.3% thought that Bt Brinjal is a high-yielding Brinjal variety. In comparison, 21.7% of the participants thought Bt Brinjal encompasses both insect resistance and high-yielding properties. Additionally, 2.2% of respondents thought Bt Brinjal has neither insect resistance nor high-yielding properties.

Consumption, health risks and nutritional differences

Among the 501 respondents who were familiar with Bt Brinjal, about 15.5% respondents confirmed that they consumed Bt Brinjal at least once, 50.5% did not consume it, and 34.0% were unaware of whether they ever consumed Bt Brinjal or not. Among the respondents

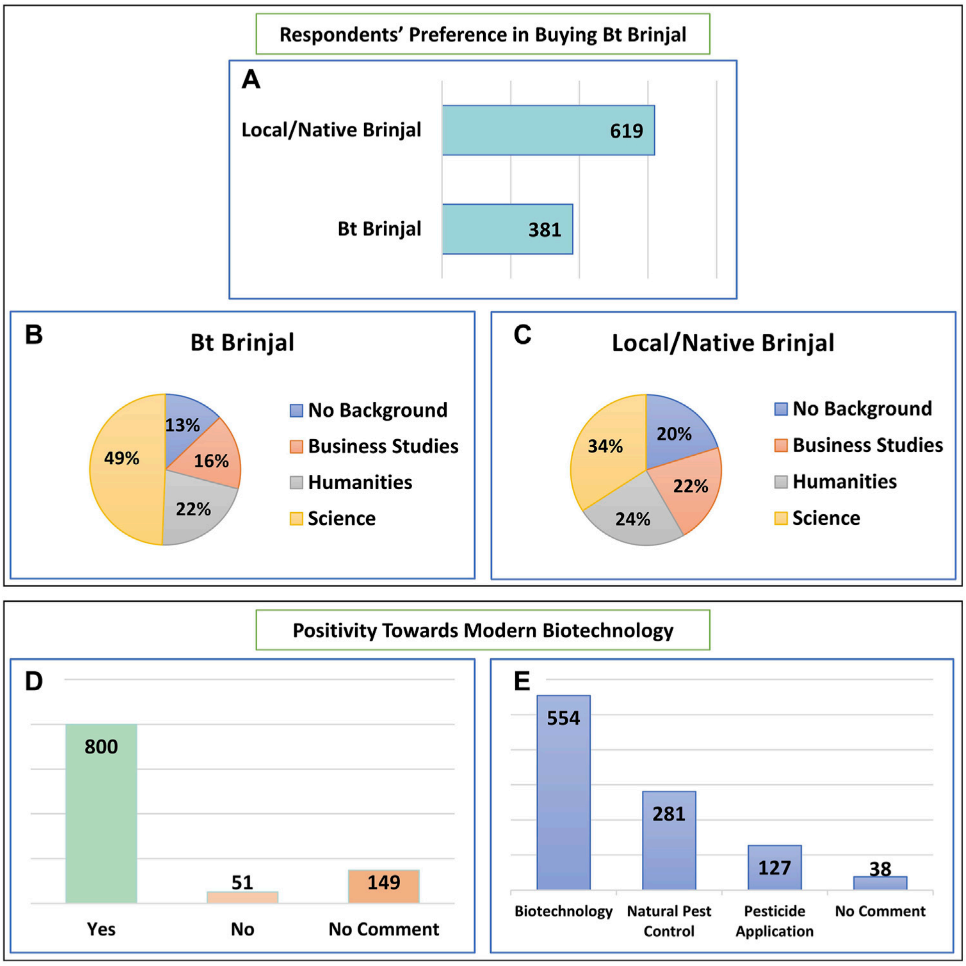

FIGURE 2
Respondents' preference in buying Bt Brinjal and positivity towards modern biotechnology. **(A)** Number of respondents who would buy local/native Brinjal or Bt Brinjal while both are available in the market; **(B)** Educational background of the respondents who would prefer to buy Bt Brinjal; **(C)** Educational background of the respondents who would prefer to buy local/native Brinjal; **(D)** Respondent's opinion regarding improvement of popular crops through modern biotechnology; **(E)** Respondent's preference for controlling pests.

who already knew about Bt Brinjal, 24.1% thought there might be health risks, 52.1% thought there were no health risks associated with Bt Brinjal consumption, and 23.8% were uncertain. 73.3% thought there were nutritional differences between Bt Brinjal and local Brinjal, while 22.5% knew there were no nutritional differences, and 24.2% were uncertain. Among the 444 respondents who were consulted, 15.8% of respondents opined that Bt Brinjal may pose a health risk, while 21.4% thought there were no health risks, and 62.8% refused to pose any comment regarding the risk. 27.5% of the consulted respondents opined Bt Brinjal may have nutritional differences. In comparison, 14% of respondents opined Bt Brinjal is not nutritionally different from local varieties, and 58.6% refused to comment on Bt Brinjal's nutritional composition.

Preference in buying, popularity in the market, and positivity towards modern biotechnology

Figure 2 illustrates the respondent's preference in buying Bt Brinjal and outlines the respondent's positivity toward modern biotechnology. Among the respondents, more than 58.8% agreed that Bt Brinjal is likely to be popular in the market, 8.4% were not in agreement, and 32.8% did not provide any opinion. Of those who thought Bt Brinjal would be popular, 53% of the respondents were from a science background. 48.2% of respondents think that Bt Brinjal may influence and lead to the loss of popularity of other local/native varieties. 22.2% opposed this, and 29.6% were unwilling to comment on this. Among the respondents who thought Bt Brinjal might influence the loss of popularity of other varieties, 54% were from a science background.

Respondents were optimistic about the technology, but their preference skewed toward native/local Brinjal varieties. 38.1% of the respondents agreed they would buy Bt Brinjal if available in the market, while 61.9% think they preferred buying local Brinjal varieties. Of those who opined to buy Bt Brinjal, almost half (49%) were from a science background. On the other hand, of the participants whose preference was aligned with local Brinjal, 66% were from non-science backgrounds. While discussing the technology, the respondents were eager to see its benefits. If biotechnology is the answer, they would like to embrace it. Most respondents (80.0%) agreed that modern biotechnology should be used to improve crops in Bangladesh. 5.1% did not think innovation through modern biotechnology is needed, while 14.9% did not pose any comments. Those who support the technology think that rice, potatoes, and tomatoes should be improved through modern biotechnology. 55.4% respondents opined biotechnology should be the method to get rid of eggplant insects. The respondents also felt the necessity of guidelines. 82.7% of respondents opined that there should be guidelines for Bt Brinjal cultivation, storage, marketing, and modern biotechnology research. 2.9% said the guidelines are not required, and 14.4% of respondents did not provide any opinion.

Discussion

Modern biotechnology demonstrates great potential in numerous fields, providing inventive solutions to urgent issues in agriculture, healthcare, sustainable livelihoods, and industrial applications (Nawaz et al., 2022). GE crops were first commercialized in the mid-1990s and tested or adopted in more than 30 countries, with various benefits (Smyth, Kerr, and Phillips, 2015). Society cannot realize the potential of technology without consumer acceptance. Thus, understanding the factors that contribute to customers' reluctance is crucial for developing GE products in the future (Verdurme and Viaene, 2003). While a negative attitude toward GE crops is perpetuated, it is often associated with insufficient knowledge of modern technologies, a lack of trust in regulators, inadequate communication regarding the risks and/or benefits, and personal values. The cumulation of these factors can seriously impact food security issues (Shohael and Hefferon, 2023).

With around 170 million people living in a land area of 148,460 square kilometers, Bangladesh is the eighth most populous country globally and one of the most densely populated (Wikipedia contributors, 2024). Being a predominantly agricultural nation, Bangladesh's economy depends on agricultural production, which generates 19.6% of the country's GDP and employs 63% of its labor force. The introduction of Bt Brinjal in Bangladesh marked a significant event as agricultural biotechnology implications moved forward. Bt Brinjal varieties have been cultivated since 2014 in Bangladesh; so far, no evidence of any unaccepted or undesirable effects that might harm human health, animals, or the environment has been reported. Farmers cultivating Bt Brinjal are pleased with the performance and profit (M. R. I. Mondal and Nasrin, 2018). Bangladesh's deployment of GE crops to boost agricultural productivity and less pesticide use could serve as a model for other developing nations facing similar challenges (Ahmed et al., 2019). However, the public should be sensitized and informed with science and evidence-based information to proceed further.

Most people are unaware of the frequency of insecticide spraying during Brinjal cultivation. It is common practice in Bangladesh for conventional Brinjal crops to be sprayed with insecticides more than 80 times during the 4–5-month growing season in all the main cultivation regions (Meherunnahar and Paul, 2009). Farmers have noted that growing Bt Brinjal has led to better insect control, lower labor and chemical expenses, higher yields, and increased income. They are pleased with the quality of Brinjal they produce, which they can offer at a lower price. With fewer pesticides needed, farmers feel that Bt-Brinjal is safer for human health (Haque and Saha, 2020). This information should be appropriately communicated to the public so that they can realize the actual benefits that Bt Brinjal aims to provide. The Government of Bangladesh has demonstrated a willingness to adopt and implement modern agricultural policy frameworks and guidelines. The country is mandated to support the safe and appropriate use of science and technology, including modern biotechnology, to help meet agricultural challenges, as implicated by the National Agricultural Policy (2018).

Before discussing the findings, it should be noted that the demographics of the present study are skewed by highly educated individuals because the surveys were conducted primarily at the university and nearby areas, and this does not represent the general population. However, the present study observed some interesting facts and beliefs among this group of respondents. The present study showed that most of the people who knew about the crop were students (higher secondary or above), and a majority of them had a science background. This is because the curricula contain chapters regarding biotechnology from secondary schools, with information about GE crops and Bt Brinjal. Moreover, efforts included information campaigns conducted through various channels, including mass media, radio, television, and printed materials such as pamphlets and posters. It was evident from the study that the familiarity of the Bt Brinjal was mostly from coursework or mass media. In addition, this implies that laypeople who don't have access to the curriculum or promotional materials are not familiar with it. Therefore, more innovative measures such as combining government support, extension services, demonstration plots, information campaigns, success stories, and research efforts may increase people's familiarity with Bt Brinjal in Bangladesh. Though familiarity is demonstrated, the idea or proper knowledge of the technology is not accurate. Therefore, disseminating the science behind the technology may not have been appropriately addressed. The success of any technology requires proper communication among laypeople.

Though the success of Bt Brinjal has been demonstrated in many previous studies (M. R. I. Mondal and Nasrin, 2018; Ahmed et al., 2019; Shelton et al., 2020), a large portion of the consumers in the present study could not confirm that they ever consumed Bt Brinjal. There are practical challenges in labeling the product, as Brinjal is a highly consumed and cheap vegetable sold in bulk in every corner of the country. Therefore, alternative measures may help create a positive appeal so that people can buy and eat without hesitation and make an informed choice.

The interview revealed that those familiar with the crop were also aware of the absence of health risks and nutritional differences. Research indicates that significant portions of consumers lack awareness or a clear understanding of GMOs and their traits and effects (Ribeiro, Barone, and Behrens, 2016; Hwang and Nam, 2021). People express their favorable impression of the technology as they expect the Bt Brinjal to be increasingly popular in the market and may influence other non-GE varieties. However, many respondents did not provide any insights in response to the questions regarding the popularity and influence of Bt Brinjal in the market. This implies a lack of confidence in giving any opinion, as they had no concrete knowledge about the matter. This uncertainty can stem from various factors, such as limited access to reliable information, conflicting sources, or complexity of the subject matter. As a result, individuals may refrain from engaging in discussions or taking positions until they have acquired sufficient knowledge and understanding to form informed opinions. The lack of confidence was also evident when people's preference for buying skewed to local/native Brinjal varieties. It was also apparent that people with previous scientific knowledge were more inclined to buy Bt Brinjal. Therefore, continuous counseling may help increase the confidence of consumers.

Many consumers also express dissatisfaction with their own knowledge on the subject, highlighting a need for broader consumer education efforts (Wunderlich and Gatto, 2015). Therefore, creating awareness about any GE crops is critical, as it provides balanced information in accessible language through various channels, emphasizes scientific consensus, encourages critical thinking, and respectfully addresses concerns. As the current study revealed people were eager to receive information and embrace good science, the source of information should be accurate and authentic. Negative perceptions significantly impact how GE foods are viewed (Giordano et al., 2018). These perceptions are resistant to change, even when consumers are presented with new information (Grunert, Bredahl, and Scholderer, 2003). Moreover, the scarcity of information about GE products partly stems from scientific uncertainty caused by conflicting sources of information (Palmieri et al., 2020).

These interviews demonstrated positivity toward modern biotechnology. This is supported by their idea of different problems associated with different types of crop cultivation in Bangladesh. They think technological interventions could solve the problems. This implies that a supportive stance toward biotechnology and GM crops may help realize the potential benefits and address pressing issues.

Conclusion

The study highlights a gap in public knowledge and awareness about Bt Brinjal and modern biotechnology. Individuals with science backgrounds have a better understanding and appreciation of biotechnology. Therefore, enhancing communication with scientific evidence and improving science education can address misconceptions and improve community perception. To leverage biotechnology for a sustainable agricultural sector that meets Bangladesh's growing population needs, it is essential to educate the public, enabling them to make informed decisions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/ participants and patients/participants legal guardian/ next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

SiA: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing–original draft, Writing–review and editing. AS: Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing–review and editing. TA: Resources, Supervision, Validation, Writing–review and editing. RA: Formal Analysis, Investigation, Methodology, Writing–review and editing. ShA: Formal Analysis, Investigation, Methodology, Writing–review and editing. HH: Data curation, Formal Analysis, Software, Visualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Beyond yield: Unveiling farmer perceptions and needs regarding weed management in Bangladesh

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More than 3.5 billion people depend on rice for more than 20% of their daily calories. Globally, Bangladesh is the third largest rice producer. With 171 million people, Bangladesh is also among the top consumers. Local rice production not only affects the country's food security but also influences the global rice trade. A large yield gap has been reported due to weeds. Traditional hand weeding is very costly because of labor shortages resulting from industrialization. Limited data showed a higher yield and profits when using herbicides. However, quantitative data on various aspects of weed management and associated issues representing the country's variable rice ecosystem, which is characterized by 30 agroecological zones, are lacking. We collected data on weed management practices from 865 farmers and 69 agrochemical shops covering all 30 agro-ecological zones (AEZs) through a structured survey. We observed a significant regional variation among various parameters. Approximately 82% of farmers use herbicides, and few rely solely on either manual weeding or herbicides. Pre-emergence herbicides are the predominant. Application procedures are almost the same across the country. Although 40% of farmers had secondary and higher-level education, most depend upon local sellers' suggestions rather than reading the product label regarding the dose. Few farmers consider herbicides hazardous, and respondents rarely perceive any environmental impact. Pyrazosulfuron ethyl (35%) and acetochlor-containing bensulfuron methyl (27%) are the most-used chemical species. Approximately 45% of farmers observed that herbicides suppress early seedling growth. Additional fertilizer is required to compensate for this. Multiple weed species that are difficult to control through presently used herbicides were noted in all AEZs. Around 64% of farmers observed that herbicide application contributes to higher yields as a function of timely weeding. Cost comparisons showed that high labor prices will make rice cropping unprofitable in most parts of the country if herbicides are eliminated. Clear adverse effects of pre-emergence herbicides on early crop growth implied the potential benefits of broad-spectrum herbicide-tolerant genetically engineered (GE) rice to sustain the country's food security. Additionally, such GE rice could incentivize the adoption of alternate wet and dry irrigation methods, leading to water and cost savings.

KEYWORDS

baseline survey, herbicide, rice, toxicity, weed management representative site, number of surveyed villages and shops

1 Introduction

In well-managed agroecosystems, weeds grow spontaneously and challenge cultivated plants in various ways, eventually reducing crop yield. In general, weeds have evolved with a much higher ability to survive and flourish than domesticated crops. Competition for resources such as nutrients, light, and spaces from a shared, finite pool by neighboring individuals is the leading cause of weed-induced yield loss. Recent evidence also suggested that crop plants negatively respond to weeds (and reduce yield) even when resources are not limited (Linu and Girija, 2020; Gu et al., 2022; Horvath et al., 2023). Thus, effectively controlling weeds is essential for sustainable crop cultivation.

Rice is the most significant and widely cultivated cereal crop in tropical and subtropical regions worldwide. It occupies approximately 10% of all arable land, providing a staple for more than 3.5 billion people. The global population is projected to reach 9 billion by the year 2050, leading to concerns about food security. Rice-dependent countries seek their future food security through a projected increase in rice productivity—specifically, by yield increment per unit area of land (Arifin et al., 2021; Kabir et al., 2015; ICAR, 2013).

Most cultivated rice is grown in tropical and subtropical regions in the warm season and thrives in waterlogged soil. Such an environment is highly favorable for many persistent weeds. Shallow flooded land maintained during early seedling growth also favors the development of numerous weeds. Thus, weeds are among the leading causes of reduced rice productivity and impose a significant management cost. In addition, climate change threatens weed management in cropping systems worldwide (Ramesh et al., 2017; Marambe and Wijesundara, 2021). Advantaged from their more efficient physiological traits, climate change often favors prolonged growing seasons. Reduced water availability due to recurrent and unforeseen droughts would alter the competitive balance between crops and some weed species, intensifying the crop-weed competition pressure.

In rice cultivation, competition from weeds is one of the main biophysical yield constraints (Waddington et al., 2010). A study showed that approximately 12%–18% yield loss occurred because of weed infestation in upland and lowland rice fields (BRKB, 2011). Severe yield loss has been reported in various climates, up to 49% in the Sahel (Johnson et al., 2004) and 50% in Indonesia, irrespective of rice production system or season (Zoschke, 1990). Other studies estimated that uncontrolled weeds caused rice crop losses of 40% in China before widespread herbicide adoption. Severe yield loss has also been reported in other rice-growing countries, including India (national average ~25.6%; Hossain et al., 2020).

Research on weeds and weed management in Bangladesh is less extensive than in other countries. On average, the gap in rice yields in farmers' fields due to poor weed control was estimated to be 43%–51% (Rashid et al., 2012), and the yield gap was as high as 1 t/ha, with 30% of farmers losing more than 500 kg/ha (Ahmed et al., 2001). However, it is possible to reduce the cost of rice production and the yield gap by improving weed management technologies.

Bangladesh is the third largest rice producer after China and India (FAOSTAT, 2023). Most recent statistics show that the consumption of rice and products is 260 kg/capita/year, the highest in Asia and much higher than the global average (81 kg/capita/year; FAOSTAT, 2023). Bangladesh is in a deficit of rice with a significant import value. Food security in Bangladesh is synonymous with rice productivity in a given year. Due to the large population and extremely high dependency on the rice-centric diet, local rice shortages influence the global rice trade. To satisfy its 171 million population (and annual growth of two million), Bangladesh must increase rice yield from the current 2.74 t/ha to 3.74 t/ha over the next 20 years (BRKB, 2011).

Traditionally, hand weeding is the most widely used practice in Bangladesh and many developing countries. Industrialization has drawn a large labor force away from agriculture, leading to a noticeable shortage of workers in the agricultural sector. The introduction of herbicides offers reduced labor requirements for weed control. One study showed that herbicide reduces weed control time to 84 person-hours/ha compared to 590 person-hours/ha, considering the requirement of two rounds of hand weeding (Mazid et al., 2006). Herbicides are a newer introduction for weed control and appear as a potential solution for reducing yield losses caused by weeds and meeting the growing population's demand for food (Kashem et al., 2009). However, proper species, dosage, and timely application are essential for economic benefits and food security.

Herbicides are high-tech solutions that can negatively impact crop growth if not applied properly. The choice of weed management practices depends on the weed composition, availability of tools, and workforce. Farmers adopt weed control methods based on availability and cost-effectiveness. Thus, understanding the extent of different weed management practices across all agroecological zones is essential for effective policy making.

Bangladesh is divided into 30 diverse agroecological zones. Soil type, topography, and water availability are different. Hence, agricultural practices, cropping patterns, and the occurrence of weeds are also diverse. Sporadic studies showed the potential benefits of herbicides (Hossain, 2015; Mia et al., 2021). However, comprehensive nationwide data on weed control practices, herbicide usage, advantages and disadvantages, and potential economic benefits are currently unavailable. The disadvantages of weed control and associated costs were not reported. A countrywide survey of present weed management practices would enable the scope of future interventions and analysis of economic aspects of weed control, providing a basis for the strategic use of manual weeding or herbicides in managing diversified weeds across various agroecological systems (Beltran et al., 2011; Rodenburg et al., 2019; Boyd and Reuss, 2022).

This study aimed to establish baseline data on identifying the current diversified practices in weed management in Bangladesh. It will measure the relative contribution of different methods, their cost-benefits, contribution to productivity enhancement, and accessibility of farmers to herbicides. It will also consider perceived drawbacks, farmers' knowledge, and accessibility. Additionally, a list of weeds showing resistance to herbicides identified by farmers under current rice farming practices will be included. We aim to assess the current knowledge level, attitudes, and practices of Bangladeshi farmers regarding the safe use of herbicides. Data-based decisions would contribute to an improved weed management strategy, regional needs, and the possibility of herbicide-tolerant GMOs. Field information is crucial to understanding the likelihood of placing GMOs in existing weed control strategies.

2 Materials and methods

2.1 Area and farmer selection

Bangladesh comprises 30 agroecological zones (AEZ) with distinct ecology and cropping patterns. This farmer survey was conducted among randomly selected rice farmers from all 30 AEZs. Location information of survey sites was taken using a portable GPS reader with the assistance of Google Maps. Stratified random sampling was chosen to ensure that the sample reflects the diversity of AEZs and agricultural practices across the country. In each AEZ, we interviewed farmers in multiple village communities to provide further randomization. Commonly used standard procedures (Krejcie and Morgan, 1970; Daniel, 1999) were considered regarding sample size. A minimum of 20 farmers were selected from each of the 30 agroecological zones for the survey, except for AEZ-24, where data on 10 farmers were obtained due to the smaller population size. The zones are characterized based on definite attributes such as soil physiography (soil parent materials and landforms of a particular area), hydrology (water holding capacity of soil and the water level of agricultural land), season, soil types, and tidal activity. The survey also included data on cropping patterns and the sources of irrigation for rice cultivation. Characteristics and data of the 30 AEZ of Bangladesh are given in Table 1.

2.2 Survey design

Farmers growing rice on at least 0.33 acres of land were included in this survey. An effort was made to choose farmers representing the average land size. In most cases, we interviewed at least 20 farmers from each agroecological zone. The survey was carried out during 2021–2023 by using a structured questionnaire. Primary information collected from each farmer in each site includes (1) zone and area name, (2) gender of participating farmers, (3) participant age, (4) educational qualification, (5) pre-cultivation rice-growing environment of the participating farmer, (6) cultivated rice varieties, (7) land size of respondents, and (8) number of crops per year (cropping pattern). Follow-up questions were: (1) what kind of weed management strategies did they apply (hand weeding/mechanical weeding/herbicide application/or combination, (2) what number of weed management intervention sessions are needed during a season, (3) in which stage of weed growth is herbicide applied (pre-emergence/post-emergence), (4) what kind of herbicide is used, its availability and the price of herbicide in local market, (5) years of herbicide adoption, (6) herbicide application procedure (spray/mixing with fertilizers/ others), (7) source of information on type and dose (product label/extension workers/neighbor/colleague/shopkeeper/other), and (8) knowledge of active ingredients of herbicide. We also followed up on some questions for farmers who did not adopt herbicides. These were (1) reasons behind not using herbicides and (2) which issue needed to be addressed for the adoption of herbicides. In this survey, hand weeding refers to the practice of manually uprooting weeds or using any combination strategy that farmers employ to remove weeds by hand.

2.3 Market survey

An additional survey was conducted at marketplaces regarding the accessibility of herbicides to farmers. We included retail agrochemical shops from all AEZs. At least two agrochemical shops were surveyed in each zone, and available herbicides were listed. Other collected information includes brand name, active ingredients, price, seasonal variation on availability, company information, and dose recommendations on the label.

2.4 Yield observation and cost analysis

To assess the effects of herbicide adoption on rice productivity, we asked farmers how much yield increment they observed. The analysis was conducted based on their perception of increasing rice yield. Cost analysis was performed on different aspects of weeding and yield. We collected data from each farmer on weed control costs in the entire cultivation period, including labor wage, herbicide price, and application cost. All prices were converted from local currency to US dollars ($).

2.5 Farmers' response to herbicide impact and future intervention

We collected data from farmers' observations regarding the impact of herbicides. The questions include (1) any impact on crop yield by using herbicide application, (2) any adverse effect on rice or soil, (3) any health issues they noticed while using herbicides, (4) whether a herbicide is less harmful than a pesticide, (5) any side effects they observed during herbicide application, (6) is there any weed species that cannot be effectively controlled by regular herbicides, (7) is the alternate wet and dry (AWD) method of irrigation useful, and (8) knowledge of GMO and whether herbicide-tolerant rice is helpful for them.

2.6 Statistical analysis

Descriptive analysis was performed for weed management practices, weeding timing and frequency, herbicide types, herbicide prices, and information sources of herbicide use. Point-biserial correlation was used to find the relationship between herbicide adoption and a farmer's total land size. Pearson chi-square (X^2) test of independence variable was performed to determine whether there were any significant relationships among the data of herbicide use and some other factors (hand weeding, cultivation experience, education, yield, the negative impact on rice, human health, and environment). A one-way ANOVA test was performed to

TABLE 1 Basic survey information: agroecological zones (AEZ), GPS coordinate of the representative site, number of surveyed villages and shops.

AEZ	GPS coordinate	No. of nearby villages	No. of shops	Zone description
1	25.823588, 88.393359	3	2	Old Himalayan piedmont plain
2	26.103672, 89.127459	3	2	Active Tista floodplain
3	25.091179, 88.873756	2	4	Tista meander floodplain
4	24.4161120, 895445217	2	2	Karatoya-Bangali floodplain
5	24.5315981, 89.0417007	2	2	Lower Atria basin
6	24.789749, 88.705645	2	2	Lower Purnabhaba floodplain
7	24.890149, 89.571578	2	3	Active Brahmaputra-Jumana floodplain
8	24.315065, 90.164876	1	2	Young Brahmaputra floodplain and Jamuna floodplain
9	24.760292, 90.248920	2	2	Old Brahmaputra floodplain
10	23.758134, 88.936266	2	2	Active Ganges floodplain
11	23.0688162, 89.0791822	2	3	High Ganga river floodplain
12	23.571218, 89.800433	1	3	Low-high Ganges river floodplain
13	22.7683139, 89.5953063	2	2	Low Ganges river floodplain
14	22.969923, 89.815824	2	3	Gopalgonj-Khulna bil
15	23.499138, 90.424097	2	2	Arial bil
16	23.702475, 90.719734	3	2	Middle Meghna river floodplain
17	23.513068, 89.135748	1	3	Lower Meghna river floodplain
18	22.454336, 90.819306	2	3	Young Meghna estuarian floodplain
19	22.9957533, 90.1105094	3	3	Old Meghna estuarian floodplain
20	24.915433, 91.824180	1	2	Eastern Surma Kushyara floodplain
21	25.102277, 91.195001	1	2	Sylhet basin
22	24.415787, 91.428516	2	2	Northern and eastern piedmont plains
23	21.420553, 92.058327	3	2	Chittagong coastal plain
24	20.622227, 92.325893	1	0	Martin's coral island
25	24.9661833, 89.2816454	2	3	Level Barind tract
26	24.566243, 88.365809	2	2	High Barind tract
27	25.808760, 89.037603	3	2	Northeastern Barind tract
28	24.028235, 90.362919	2	2	Madhupur tract
29	23.168823, 92.203608	3	3	Northern and eastern hills
30	23.959548, 91.176951	1	2	Akhaura terrace

find the cost difference regarding weed management in our studied AEZs. A paired sample *t*-test was conducted to determine the effect of herbicide adoption on reducing costs. The latent structure of the weed management strategy (WMS) was examined using principal component analysis with varimax rotation. Multinomial logistic regression was performed to assess the impact of several factors affecting respondents' adoption of herbicides. The model contained seven independent variables (total cost of herbicide, total cost of weed management, number of herbicide applications, number of hand weeding sessions, whether there is any negative effect of herbicide on rice, do herbicides increase yield, and any negative effect of herbicide on the environment). All the data were analyzed using IBM SPSS Statistics software, version 25.

3 Results

3.1 Location and farm characteristics

Bangladesh comprises 30 distinct AEZs, all of which were considered in this survey. Climatic parameters and natural resource characteristics are diverse and influence various aspects of agriculture. AEZs are distinct in seasonality, topography, soil type, soil fertility, drainage, temperature, availability of both surface and groundwater, and flood patterns. Cropping patterns are, therefore, different. Being positioned around the Tropic of Cancer, sunshine hours and intensity did not vary much except for microclimates such as open coastlines and hilly areas. The altitude of the rice production areas ranged from sea level to 268 feet above sea level. However, 57% of our survey sites ranged from 3 to 60 feet. A summary of the characteristics is provided in Table 2.

TABLE 2 Cropping pattern of the surveyed area in 30 agroecological zones (AEZs). Multiple cropping patterns were observed in some AEZs. The numbers of respondents are given for water availability. Timeline may vary by 2–4 weeks, depending on local situations.

Zone	Elevation (feet)	Pattern	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
AEZ-1	210–220	1	Fallow		Irrigated rice (20)				Fallow					
		2	Vegetable (20)		Irrigated rice (20)									Vegetable (20)
AEZ-2	223–233		Tobacco/Maize (14)			Irrigated rice (20)				Rain-fed rice (20)				Tobacco /maize (14)
AEZ-3	130–140			Irrigated rice (20)					Rain-fed rice (20)					
AEZ-4	27–33			Irrigated rice (20)						Rain-fed rice (20)				
AEZ-5	70–80		Fallow	Irrigated rice (24)				Fallow						
AEZ-6	79–85		Vegetable (18)		Irrigated rice (40)					Rain-fed rice (35)				Vegetable (18)
AEZ-7	53–59		Irrigated rice (15)				Jute (12)				Rain-fed rice (20)			
AEZ-8	60–65			Irrigated rice (20)						Rain-fed rice (20)				
AEZ-9	56–66		Fallow	Irrigated rice (15)					Rain-fed rice (41)					Fallow
AEZ-10	65–78			Irrigated rice (40)					Rain-fed rice (40)					
AEZ-11	118–130		Vegetable (25)		Irrigated rice (40)					Rain-fed rice (45)				
AEZ-12	16–20		Wheat		Irrigated rice (20)				Jute (20)				Wheat (20)	
AEZ-13	3–6		Irrigated rice (20)						Rain-fed rice (20)					
AEZ-14	16–23	1	Irrigated rice (20)			fallow								Irrigated rice (20)
		2	Irrigated rice (20)			Jute (12)								
AEZ-15	13–23		Irrigated rice					Rain-fed Rice						
AEZ-16	30–43													
AEZ-17	16–23	1	Irrigated rice (29)					Rain-fed rice (49)				Mustard (16)		
		2	Irrigated rice (29)					Rain-fed rice (49)						
AEZ-18	3–7		Irrigated rice (40)			Soybean (19)				Rain-fed rice (43)				Irrigated rice (40)
AEZ-19	7–13	1	Irrigated rice (23)			Fallow								
		2	Irrigated rice (23)				Jute (20)							
AEZ-20	33–40		Vegetable (15)						Rain-fed rice (34)					Vegetable (15)
AEZ-21	50–66		Fallow		Irrigated rice (38)				Fallow					
AEZ-22	82–90		Irrigated rice (20)					Rain-fed rice (20)						Irrigated rice (20)
AEZ-23	23–33			Irrigated rice (15)						Rain-fed rice (23)				
AEZ-24	7–13							Rain-fed rice (10)						
AEZ-25	59–66	1	Potato/ mastered (14)	Irrigated rice (20)									Potato/mastered (14)	

TABLE 3 Characteristics of farmers' status, weeding strategies, and sources of irrigated water.

Factor	Categories	Number (Frequency)
Gender	Male	832 (96.2%)
	Female	33 (3.8%)
Age (Years)	16–35	266 (30.74%)
	36–70	578 (66.82%)
	Above 70	21 (2.44%)
Education	No literacy	320 (36.99%)
	Primary (Class 1–5)	200 (23.06%)
	Secondary (Class 6–10)	238 (27.5%)
	Higher Secondary (Class 11–12)	74 (8.63%)
	Graduate	33 (3.82%)
Rice cultivation experience (years)	Less than 5	105(12.16%)
	5–10	112 (12.98%)
	11–15	108 (12.51%)
	16–20	158 (18.25%)
	21–25	88 (10.18%)
	26–30	88 (10.14%)
	Above 30	203 (23.51%)
Total cultivation area (acres)	0.33–0.79	251 (41.8%)
	0.80–1.29	219 (26.1%)
	1.30–1.99	171 (20.4%)
	2.00–2.64	40 (4.8%)
	2.65–3.65	34 (4.1%)
	3.66–10	24 (2.9%)
Weed management strategies	Herbicide-based weeding	(24) 2.7%
	Use herbicide and manual weeding	(686) 79.3%
	Manual weeding	(155) 17.8%
Sources of irrigation water	Groundwater	770 (89%)
	Surface water	69 (8%)
	Others	26 (3%)

3.2 Respondents' characteristics

3.2.1 Demographic profiles of participants

The respondents to the survey included 865 farmers from all 30 AEZs, with a significant majority (96.2%) being male and the remaining (3.8%) female. The age distribution revealed that none of the surveyed farmers were younger than 16 years. Among the participants, 30.74% were between 16 and 35, 66.82% were between 36 and 70, and just over two percent (2.44%) were 71 and older (Table 3).

3.2.2 Education levels

Virtually all participating farmers disclosed their educational backgrounds. Of these, 36.99% were illiterate, 23.06% had completed primary school (≤5 years of schooling), 27.50% had finished secondary education, and 8.63% had attained higher secondary education. In contrast, a smaller percentage (3.82%) had received tertiary education.

3.2.3 Experience in rice cultivation

A significant number of farmers (23.5%) reported having more than 30 years of experience in rice cultivation. Around 18.2% of farmers have 16–20 years of experience, while approximately 13% and 12.5% have 5–10 years and 11–15 years of experience, respectively. Only slightly more than 10% of farmers mentioned having 21–25 years and 26–30 years of experience in rice cultivation.

Approximately 12.2% of farmers are new to rice farming, with less than 5 years of experience.

3.2.4 Land ownership and distribution

The study included farms with a minimum of 0.33 acres of rice-growing land. The farmers surveyed cultivate rice on land ranging from 0.33 to more than 10 acres. Most (41.8%) farmers cultivate between 0.33 and 0.79 acres. Around 60.1% of all surveyed farmers own less than one acre of land for regular crop cultivation. Additionally, 26.1% and 20.4% of farmers cultivate land ranging from 0.8 to 1.29 acres and 1.3 to less than 2 acres, respectively. Furthermore, 4.8% and 4.1% of farmers cultivate land between 2 to 2.64 acres and 2.65–3.65 acres, respectively. Only 2.9% of farmers cultivate land spanning from 3.66 to 10 acres for rice production. The average land size based on the countrywide data is 1.2 acres.

3.2.5 Frequency of land utilization for crop production

Interesting trends were noted in the findings about land allocation for crop production. According to the data, 45.18% of farmers utilize their land for two crop cycles annually, while 35.08% allocate their land for three crop cycles. Surprisingly, 19.74% of farmers focus on a single crop cycle. Within the three-crop-cycle areas, it was observed that the cultivation of two rice crops is predominant (80%), while the practice of three rice crops in succession is rare. All participating farmers grow transplanted rice. The survey focused on rice cultivation in Bangladesh during two main seasons: *boro*, which runs from January to May and involves irrigation, and *aman*, which typically takes place from July to November and relies on rain, with occasional irrigation if there is not enough rain. In some AEZs (e.g., AEZ-19), rice cultivation is not feasible in the *aman* season in most places due to submergence (low-lying land).

3.2.6 Rice varieties

Most rice growers cultivate high-yielding varieties (90%). The shares of low-yielding local varieties and landraces are relatively small. The cultivation of local varieties is confined mainly to the *aman* season. Among high-yield, open-pollinated varieties dominate the field. F1 hybrids have only a small share.

3.3 Weed management practices and water use

3.3.1 Herbicide application and manual weeding

As a traditional practice, all surveyed farmers believe that manual hand weeding is the best method of weed control, if possible. However, only 17.8% did not apply herbicides and solely depended on manual hand weeding. The main reason is the high cost and availability of labor. Our data suggest that 79.3% of the respondents use a combination of manual weeding and herbicides. Only a small proportion of farmers, 2.7%, used herbicides solely. The average weed control strategies by zone are illustrated in Figure 1. The frequency of people using herbicide increases, and the frequency of people using hand weeding tends to decrease slightly, and *vice versa*, as there is a small significant relationship between the variables (Supplementary Table S1).

AEZ-18 and AEZ-22 possess the highest percentage of solely herbicide adopters, and AEZ-12 had the smallest proportion of herbicide users, except for AEZ-24, where no farmers reported using herbicides. They showed no interest in herbicides even after learning about the benefits. Due to its small agricultural area and traditional farming methods deeply ingrained in the community, farmers may favor manual weed control techniques over chemical inputs. In addition to the high labor cost and availability, the reasons for not using herbicides differed across AEZs (Figure 2). Approximately 14.2% of farmers do not have sufficient knowledge of the efficacy of herbicides. Approximately 81.3% of the non-adopters believed that herbicide use causes harm to crops or soil, and manual weeding is more beneficial to yield. Very few farmers (1.3%) think herbicides may harm their health. Approximately 3.2% of farmers know about the efficacy of the method but do not use it as they have a sufficient workforce to conduct manual weeding. Most non-herbicide users (80%) expressed their willingness to accept herbicides, provided that they will not damage crops or soil. Interestingly, in AEZ-18 and AEZ-12, although farmers know about herbicides, they do not use them. They use collected weed as fodder. Weeds are a kind of minor crop to them.

Thus, inadequate knowledge of efficacy appears to be the main reason for not adopting herbicides. At the same time, marginal farmers with an adequate workforce in their families also feel that herbicide is not the most profitable option. Statistical analysis showed a non-significant negative correlation (small relationship) between land size and herbicide adoption. This indicates that herbicide adoption is random (Supplementary Table S2).

3.3.2 Timing and frequency of herbicide application

Respondents from all AEZs apply herbicides within 1–7 days of transplantation. This practice effectively reduces the emergence of weeds in muddy fields. However, in some cases, all weeds are not entirely suppressed. Thus, weeds need to be removed again within 3–6 weeks. Rice is not tolerant to most of the post-emergence systemic herbicides. Hence, manual hand weeding is essential. Approximately 44.3% of farmers must conduct one manual weeding. On the other hand, two hand-weeding sessions is the most common requirement, and around 47.4% of farmers have used this practice in their fields.

According to farmers, the presence of high amounts of weed propagules (8%), lack of standing water after transplantation (80%), and improper doses of herbicides (10%) are the primary cause of the weed emergence and subsequent requirements of manual weeding even after using herbicides.

3.3.3 Frequency and timing of manual hand weeding

Apart from herbicide treatment, most farmers (97.3%) used hand weeding alone or in combination with herbicide application. This method included either a single session of hand weeding or a combination of hand weeding plus herbicide application. Farmers

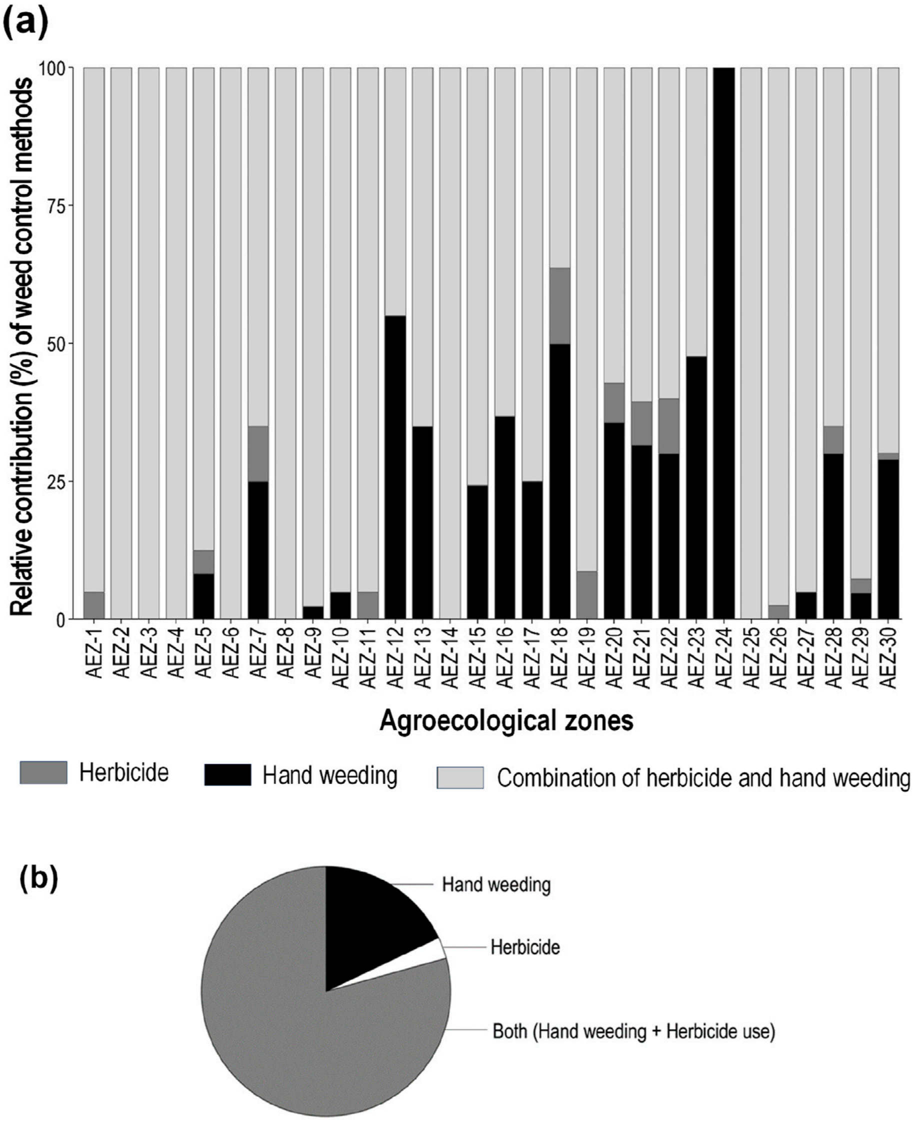

FIGURE 1
(A) Zone-wise relative contribution of various weed control methods: hand weeding, herbicide-based weeding, and their combinations. Each bar represents a specified agroecological zone of Bangladesh. **(B)** Overall contribution of major weed management practices. Data represent the average of all AEZs in Bangladesh.

who rely solely on manual weeding typically conduct one to three sessions of manual weeding to remove unwanted plants by hand, ensuring the optimal growth of their crops. However, the weeding frequency varied by AEZ.

For those who do not adopt herbicides, 32% opt for a single manual weeding session, 57% for two sessions, and 10.9% for three sessions. On the other hand, herbicide adopters reported that manual weeding continues to be an essential part of the process despite using herbicides. In those cases, the frequency of hand weeding was reasonably balanced, with 45.2% of farmers doing one session and 48.4% doing two sessions, while the requirement of three sessions is rare (6.4%).

The farmers expressed that the frequency of manual weeding was contingent upon the prevalence of weeds in the rice field. A significant number (49.3%) of farmers weeded by hand 15 days after planting, followed by a second session after 30 days. Various timeframes for hand weeding were observed within and across AEZs, with no indication of hand weeding being necessary beyond 7 weeks.

3.3.4 Types-pre-emergence and post-emergence

In some AEZs, rice fields are overrun by weeds. Therefore, pre-planting weed clearing is necessary. In those cases, non-selective post-emergence herbicides are used. Glyphosate and paraquat are the primary herbicides, contributing 33% and 67%, respectively. Such pre-cleaning also varies by season and AEZs. It may be required in *aman* season in some AEZs, while necessary in *boro* season elsewhere.

Farmers' perceptions of herbicide effectiveness were focused on the pre-emerging stage, with 81.9% reporting herbicide application

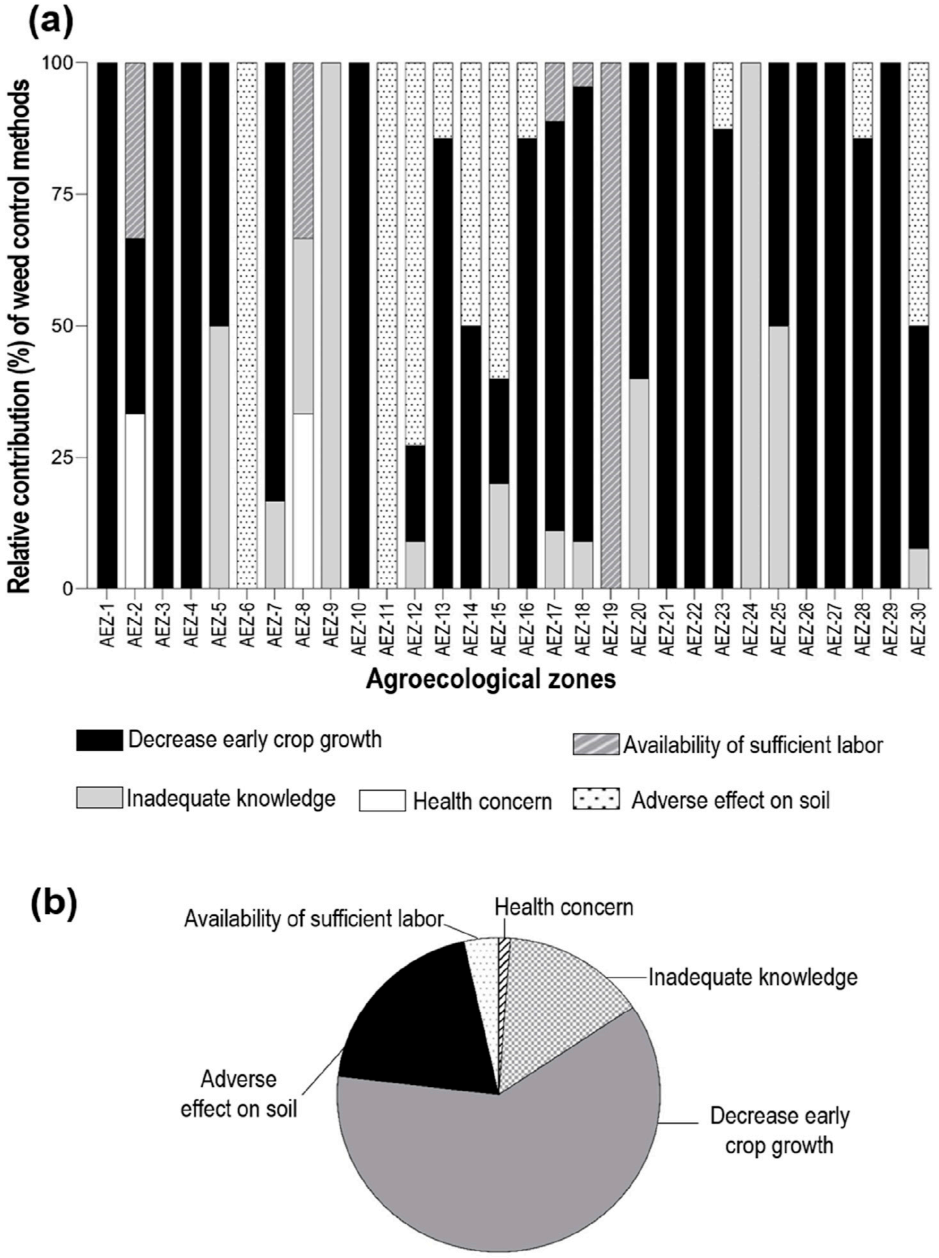

FIGURE 2
Reasons for not adopting herbicides (zero-herbicide cultivation). **(A)** Response variation in 30 AEZs and **(B)** the country's average data. Only the perceptions of farmers relying solely on manual weeding without any herbicides are considered.

at this time. In contrast, only 6.6% of farmers used herbicides during the post-emerging period. Among the herbicide users, 92.7% of respondents used pre-emergence-type herbicides. This class of herbicides is mainly applied within 1–7 days of the transplantation of rice seedlings. A total of 86% of farmers apply herbicide by this time. Such an application prevents the emergence of weeds. By contrast, herbicides are used after weed emergence to a much lesser extent (7.3%) in some cases. We observed such herbicide application (after weed emergence) exclusively in AEZ-19 and AEZ-29.

3.3.5 Active ingredient

We noticed farmers across the country use 10 formulations to control weeds. Use of glyphosate and paraquat is limited to pre-cultivation weed control. Various formulations are used after seedling transplantation. Among the various herbicides enlisted in this survey, pyrazosulfuron ethyl emerged as the most frequently used herbicidal compound, with approximately 35% of farmers using it. The combination of bensulfuron methyl (4%) and acetochlor (14%) ranked next at around 27%, followed by pretilachlor, which accounted for 15%. Some other chemicals,

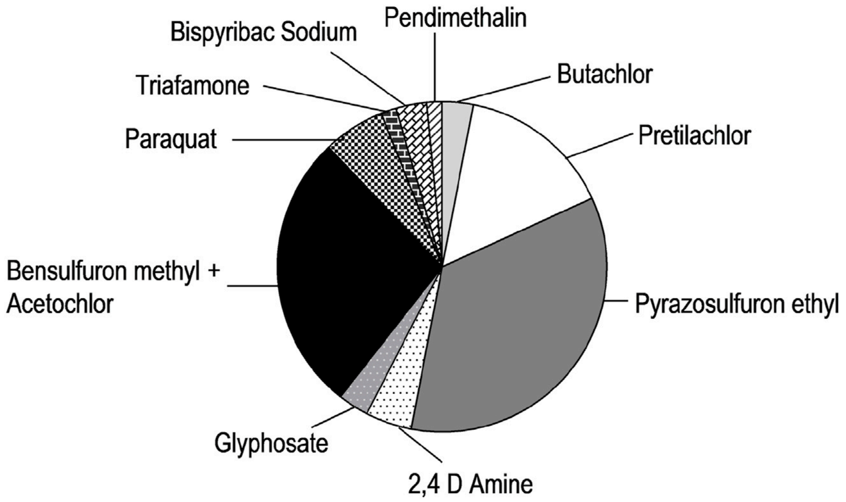

FIGURE 3
Pie chart showing various active ingredients of herbicides used by the farmers in all 30 AEZs.

such as paraquat, glyphosate, 2,4-D amine, butachlor, triafamone, and bispyribac sodium, are used by farmers all across the AEZs. The relative contribution of these chemicals is shown in Figure 3. The utilization of different herbicides in 30 AEZs is shown in Figure 4. Only 0.5% of farmers are conscious of the active ingredients. Most know herbicides and their effectiveness by trade name.

3.4 Availability of herbicides in the marketplaces

We collected data from agrochemical shops in all 30 AEZs during the survey. A total of 49 trade-named herbicides were listed. These products represent ten different herbicide chemicals (Table 4). Among them, eight products contained single-active

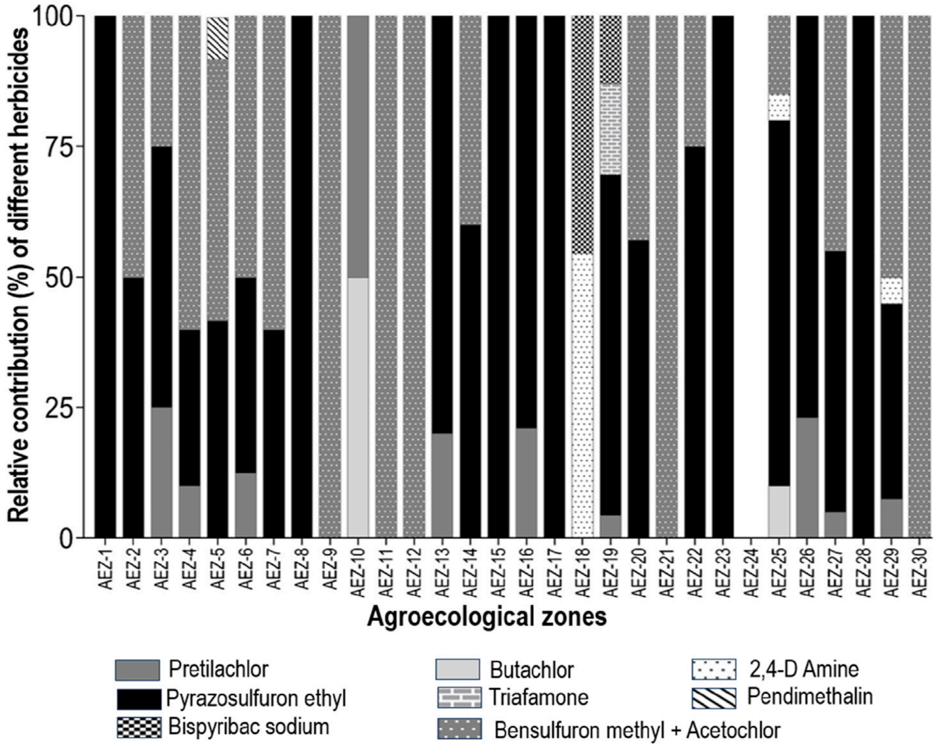

FIGURE 4
Percentage of various herbicides' active ingredients used in the 30 different AEZs. Each bar represents a specified AEZ.

TABLE 4 List of herbicides used by farmers in studied locations classified using the WHO Hazard Class and Health Effects, 2019 (World Health Organization, 2020).

Number of products	Active ingredients	Mode of action	WHO Class	Agroecological zone
2	Butachlor	Pre-emergence	Class III	AEZ-25, 10
10	Pretilachlor	Pre-emergence	Class III	AEZ-19, 29, 13, 10, 3, 27, 6, 4, 16, 26
8	Pyrazosulfuron ethyl	Pre-emergence	U	AEZ-25, 19, 14, 29, 23, 5, 28, 22, 20, 13, 17, 2, 1, 3, 27, 6, 8, 7, 4, 15, 16, 26
3	2,4 D Amine	Pre-emergence	Class II	AEZ-25, 29, 18
4	Glyphosate	Post-emergence	Class III	AEZ-14, 29
4	Paraquat	Post-emergence	Class II	AEZ-14, 29, 28, 6
12	Bensulfuron methyl+ Acetochlor	Pre-emergence	U Class III	AEZ-25, 14, 29, 5, 22, 20, 11, 2, 3, 27, 6, 12, 7, 4, 9, 21, 30
2	Triafamone	Pre-emergence	Not listed	AEZ-19
2	Bispyribac sodium 40%	Pre-emergence	Class III	AEZ-19, 18
2	Pendimethalin	Pre-emergence	Class II	AEZ-05

ingredients, while two were combined formulations. In all cases, shopkeepers report that most farmers seek their advice on selecting appropriate herbicides. Shopkeepers received training on herbicide usage from their manufacturer/supplier companies.

Glyphosate and paraquat were the most common herbicides used when pre-cultivation weed cleaning is required. These two herbicides were sold under eight different trade names and contributed 3% and 6%, respectively. Compared to pre-emergence rice herbicides, glyphosate and paraquat were sold in much higher quantities in AEZ-29. Shops in AEZ-24 did not sell any herbicides.

Among the pre-emergence herbicides, bensulfuron methyl with acetochlor is sold under 12 different trade names. Pyrazosulfuron ethyl was sold under eight trade names, pretilachlor under 10 trade names, and 2,4 D amine under three trade names. The price of herbicides is not influenced by AEZs but rather by the brand of the herbicide. Paraquat is the cheapest at $1 per acre among post-emergence types. In contrast, triafamone, pyrazosulfuron ethyl, and bensulfuron methyl with acetochlor were sold at $2.08 per acre, $0.80 per acre, and $1 per acre, respectively. According to the labels, all product is registered.

3.5 Cost, yield, and profitability

3.5.1 Cost of weed control

We gathered data on weed control expenditures and categorized them as herbicide costs and manual weeding expenses. Total cost varied by AEZs, ranging from $12–$116 during the entire crop life. Where herbicides are used solely, the expenditure is the least. Herbicide costs at retail shops ranged from $1–$3 for each acre of land, depending on brand and active ingredients. In most regions, using a combination of herbicide application and manual weeding costs less than relying on manual weeding alone (Figure 5). Such a difference in expenditure is associated with high labor wages. During a narrow 3-week window in a region, labor shortages for weed control often lead to price increases. Labor wages ranged from $3.2 to $7 per day during the survey period. In addition to herbicides, labor cost for weeding is an average of $43.7 for each acre. A significant difference ($p < 0.05$) in total weed management costs was observed among the 30 AEZs, as determined by a one-way ANOVA test. During the survey, we aimed to assess the role of herbicides in weed control expenditure and profitability of rice cultivation. We requested farmers to estimate the cost of weed control through manual labor if herbicides were eliminated, compared to the current method of using both herbicides and manual labor. Farmers projected that if no herbicides are used, the average cost for weed management would be $98.5 per acre (Supplementary Table S5). Responses suggested that in AEZ-17, the weed management cost would be as high as $307 per acre. The savings due to herbicide application would be as high as 65%. In conclusion, rice cultivation would not yield significant profits in most AEZs if manual hand weeding was the sole method employed. A paired sample *t*-test was performed to determine the effect size of herbicide adoption in reducing the cost, and significant differences were observed with a large effect size (Supplementary Table S3).

3.5.2 Negative effects of herbicides and mitigation measure

Regarding the perceived effects of herbicide use, 44.5% of farmers reported that herbicides harmed the growth of rice seedlings. These include reduced growth and sluggish tiller formation. Association between herbicide application and consequent adverse effects are found to be statistically significant with a large relationship ($p < 0.001$). Approximately 35% of farmers apply additional fertilizers to compensate for the early growth cease of seedlings. Most use urea and sulfur, while a few percent use their combinations. Some farmers (10%) were willing to apply additional fertilizer but were held back by the hefty cost.

3.5.3 Yield observation

Most farmers (64.1%) agreed that using herbicides significantly increased their yield. They immediately attributed the increase in production to their agronomic practices, which included herbicides.

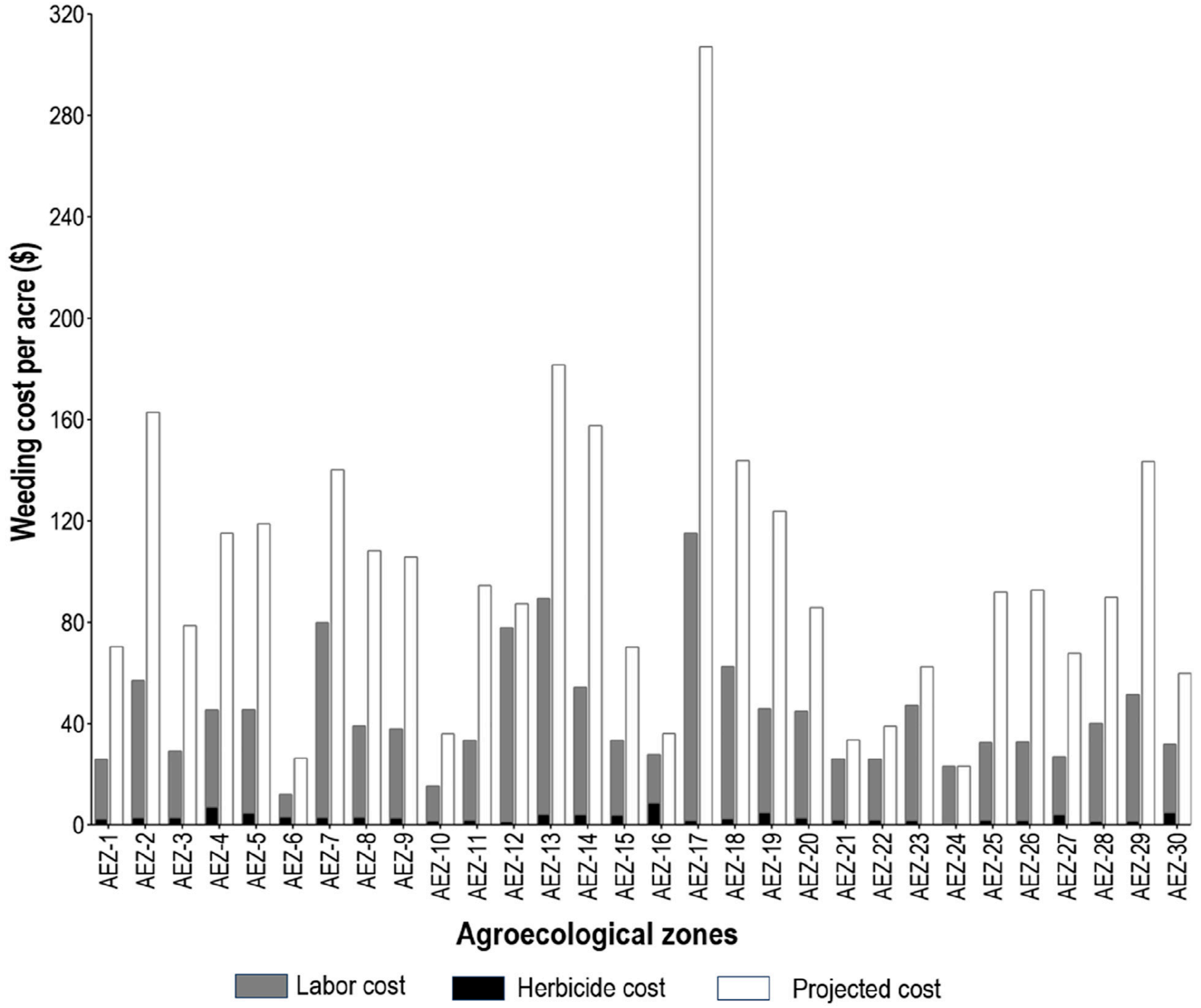

FIGURE 5
Differences in weeding cost. The cost of the currently used combination of herbicide and manual weeding method is compared to the no-herbicide scenario (projected cost based on a full hand weeding session).

They asserted that higher yields do not directly result from the herbicide application; however, they can correlate. This subset of farmers noticed that manual weeding techniques could yield results similar to those obtained with herbicides, eliminating the need to use them to increase yields directly. However, timely weeding is not often possible when it depends on labor forces, negatively affecting yield. Herbicide application, by contrast, requires little labor and is easily manageable. Notably, most farmers agreed that herbicide-based weed control was the least costly. Most respondents consented that this cost-saving feature was a positive consequence. Approximately 34.9% of the farmers disagreed with herbicide-mediated yield increase. They mainly reasoned the adverse effects of herbicides on crop growth. Only 1% of the farmers who participated in the survey had no opinion about the impact of herbicide treatment on crop growth. These farmers inferred that there was no apparent connection between the usage of herbicides and increased crop yields. Statistical analysis, however, suggests a significant association with a medium positive relationship has been found in the chi-square test (Supplementary Table S1). Thus, herbicides indirectly contribute to yield increase and benefit overall rice production in the country.

3.6 Knowledge of farmers, health, and environment

3.6.1 Knowledge and attitude

Our questionnaires also assessed the farmers' knowledge of herbicide ingredients, dose, and safety considerations. We found that only 5.1% of the farmers read labels to consider application dosage, appropriateness, and safety. The chi-square test suggested that the number of people aware of these issues is not concentrated to any AEZ but is somewhat randomly distributed.

We observe a moderate degree of positive association between herbicide adoption and the education qualification of the participants (Supplementary Table S1). Most farmers (69.3%) depend on pesticide shops to select herbicides and their dosages. Approximately 15.5% of farmers learned appropriate herbicide formulations as trade names from the experience and suggestions from their friends and neighbors. According to farmers, suggestions from government extension field workers are rare (8%). Almost no farmers are aware of the active ingredients of the herbicides.

Approximately 72.7% of farmers reported that applying herbicides did not cause any harmful effects on their health. The association of the variables is found to be non-significant (Supplementary Table S1). They observe this in contrast to insecticide applications. A small proportion of farmers reported minor health issues such as headaches and eye irritation. Interestingly, approximately 15.4% of farmers think that herbicides may harm the ecosystem, although this percentage is statistically not significant (Supplementary Table S1). No farmers see any immediate adverse effects on the animals, including frogs, earthworms, fish, or other beneficial animals inhabiting the rice field.

Only a small number of respondents (12.4%) have linked the use of herbicides to adverse health effects. Wearing personal protection equipment is, therefore, rare. Nevertheless, they admit that using PPE would be better. The use of personal protective equipment (PPE) during herbicide application was cited as a safety practice by 32.2% of respondents. Despite the potential risks they assume, 67.8% of respondents admit to not using essential personal protective equipment (PPE). Notably, a considerable segment (78%) recognized the necessity of PPE but acknowledged their non-adherence to this safety measure. As mentioned earlier, most farmers depend on shopkeepers' recommendations regarding efficacy and safety.

3.6.2 Knowledge of herbicide toxicity

Within the herbicide-using group, 34.6% of farmers reported adopting herbicides for more than a decade. A significant proportion (63.2%) of this subgroup cited the adverse effects on the development of rice seedlings as the main reason for their unwillingness to use herbicides. On the other hand, 36% did not use herbicides because they knew enough about alternatives. Only 1.3% of respondents said health issues prevented them from using herbicides. Around 18.1% of farmers mentioned that applying herbicides may harm soils. Regarding the method, 60.1% of farmers chose to combine herbicides with fertilizers, while 24.5% preferred direct spraying. A small number of farmers used both methods.

3.6.3 Inefficacy of herbicides

In almost all AEZs, farmers complained about the efficacy of herbicides on several weed species. Many weeds can skip the pre-emergence treatment during rice cultivation. A list of such weeds is provided in the Supplementary Figure S1. These weeds necessitate manual hand weeding, adding extra financial burden on farmers. Thus, effective post-emergence herbicides would be helpful.

3.7 Farmer needs and response to future intervention

Farmers urge for appropriate technology to control some weeds that are not easily controlled by currently used pre-emergence herbicides. Notably, post-emergence herbicides are even more harmful to rice crops. Therefore, their relative efficacy to weed species and stages should be studied to determine the effective use of herbicides and minimize their effects. Most current herbicides are ineffective during the post-emergent phase, which poses a significant problem. Application is, therefore, not flexible. Rice can be genetically engineered to resist herbicides, including those of post-emergence types. Among the surveyed farms, only 1% of the farmers know about GMOs or genetically engineered crops. At this point, they have yet to learn about the safety or efficacy of GE traits. However, 99% are willing to cultivate GE rice varieties if they better tolerate herbicides, are profitable, and contribute to a higher yield.

As mentioned earlier, the efficacy of pre-emergence herbicides requires standing water after their application. We asked farmers about possibly introducing new rice varieties that are tolerant to post-emergence herbicides. It appears that farmers often practice AWD methods for rice cultivation. They observe that the AWD method benefits from water savings and cost reduction. However, it is not practiced much as a consequent emergence of numerous weeds. If post-emergence herbicide-tolerant varieties are available, they want to apply AWD and save water.

3.8 Response pattern on survey questions

The latent structure of the weed management strategy (WMS) was examined using principal component analysis with varimax rotation. The scree plot selects the number of components based on the eigenvalues. It arranges the eigenvalues from the largest to the smallest. In our data, the scree plot determines the optimal number of components to be considered in the model (Figure 6A). The initial analysis revealed five factors with eigenvalues larger than 1, explaining 55.12% of the variance (Supplementary Table S6). The eigenvalue of each component in the initial analysis is plotted. The components on the shallow slope contribute little to the solution. Our data exhibit a steep curve pattern, followed by a bend at the fifth component (elbow threshold), and then nearly form a straight line. Such a pattern suggests a potential five-factor solution for the WMS.

Figure 6B illustrates the loading of the five factors for the individual items in the questionnaire. Questionnaire items that have high loadings on factor 1 (only) are, for example, "frequency of herbicide application in the field," "time to use herbicide," and "herbicides cost in the field;" items that have high loadings on factor 2 (only) include "cultivated farming areas" and "hand weeding cost." The other nine items are allocated as follows: "negative impact on the environment," "gender," "number of crops grown in a field around the year," and "negative impact on health" have a high impact on factor 3, "herbicide on the yield increase," and "hand weeding number" have an impact on factor 4, and "experiences in rice cultivation," "participants' education qualification," and "negative impact of herbicide" have an impact on factor 5. Items have high loadings

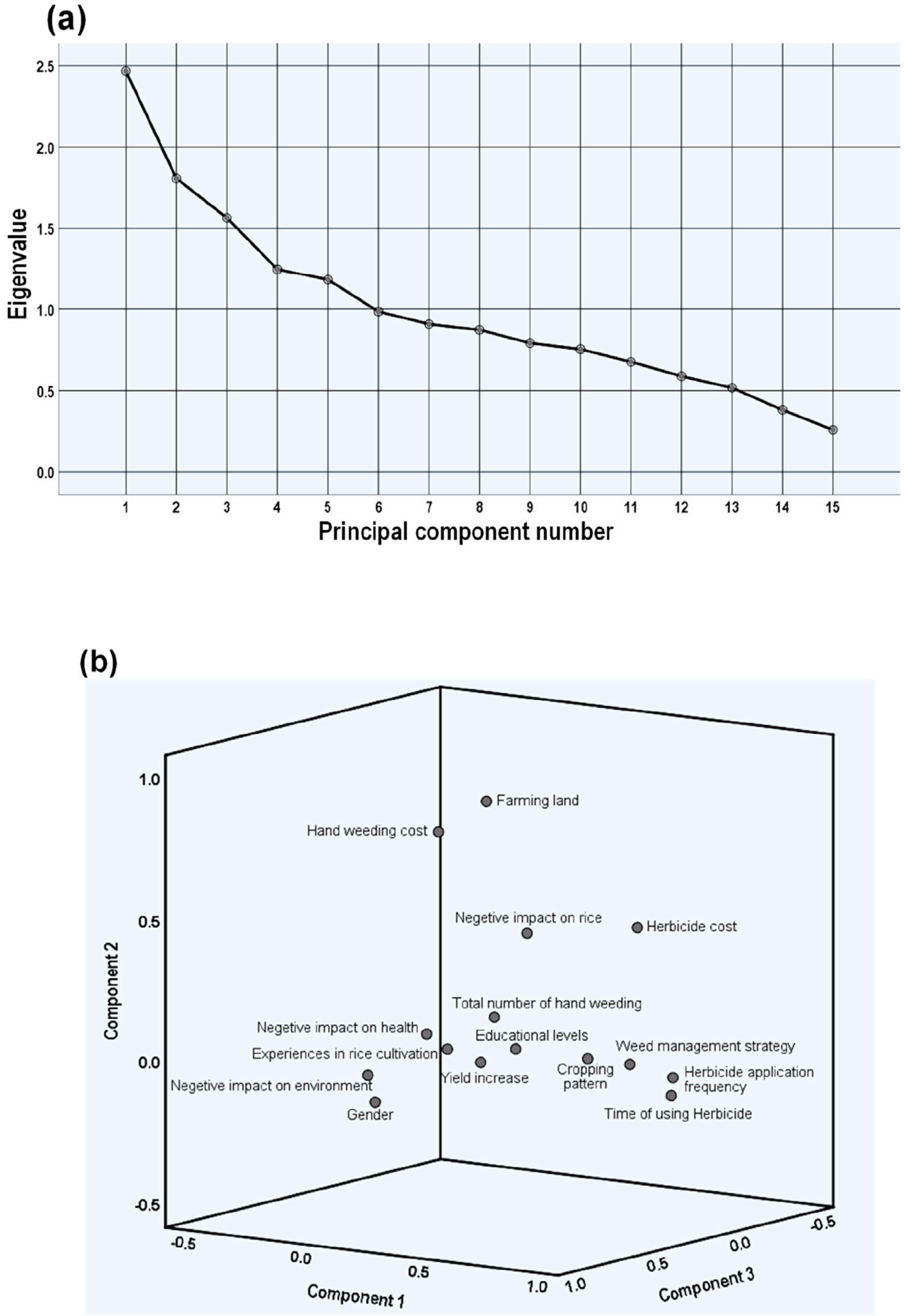

FIGURE 6
Factor loadings of farmers' questionnaire items on herbicide use. **(A)** A scree plot representing the eigenvalues and the proportion of variance accounted for by the principal components and **(B)** a component plot shows the factor loading of the 3D model after varimax rotation.

on other factors. Therefore, this indicated that factor 1 largely summarized the properties of the WMS.

3.9 Relationships among various factors

The multinomial logistic regression analysis was conducted to examine the effects of cost and herbicide impact on farmers' weed management practices. The key results are summarized in the Supplementary Table S4. The overall fit of the model was evaluated using the likelihood ratio chi-square test, Pearson and deviance chi-square tests, Cox and Snell pseudo-R-square, and Nagelkerke pseudo-R-square. The likelihood ratio chi-square test was statistically significant ($p < 0.0001$). This suggests that our model containing the full set of predictors fits the data significantly better than the intercept-only model. The Pearson chi-square and deviance chi-square tests were both statistically non-significant ($p > 0.05$), suggesting the robustness of the model. High pseudo-R-square values of Cox and Snell (0.665) and Nagelkerke (0.958) indicate that the predictors in the model explain a substantial portion of the variance in the dependent variable.

From the multinomial regression table (Supplementary Table S4), we see that higher herbicide costs do not influence the probability of choosing hand-weeding alone (O.R. = 0.993) or a combination of hand-weeding and herbicide (O.R. = 0.993) compared to herbicide use only. More frequent herbicide use decreases the probability of choosing hand-weeding (O.R. = 3.209E-07) and a hand weeding–herbicide combination (O.R. = 0.014). This shows that farmers perceive more frequent applications of herbicides as saving costs. On the other hand, the current tendency of hand-weeding increases the likelihood of choosing hand-weeding alone (O.R. = 1102.775) and a combination of hand-weeding and herbicide use (O.R. = 2542.574). This indicates that farmers would preferably depend on only manual labor if it were available, and the cost was competitive to herbicides.

In addition to costs, potential negative effects on early seedling growth are important determinants of farmers' choice of weeding method. Farmers who perceive herbicides as negative for rice are less likely to support herbicides alone (O.R. = 0.003). The impact of herbicide use on the environment and whether there is a perceived yield benefit to using herbicides influence the model. However, they are not statistically significant. Overall, the results suggest that economic factors and seedling health are key factors that influence the adoption of weed management strategies.

4 Discussion

Farmer surveys that evaluate the current status and determine their knowledge and perception of weed problems, their limitations in dealing with the situation, and their attitudes in the weed management system are one of the most vital approaches for data assembly. Therefore, we focused on the current knowledge level of the farmers regarding weed control, as well as their perceptions and attitudes toward the efficacy and safety of herbicide usage. Our survey covers the whole of Bangladesh, which is represented by 30 AEZs. It provides valuable insights into farmers' variable weed management practices, their characteristics, attitudes, the challenges they face, and possible interventions.

Due to the edaphic and climatic diversity of cultivation areas (AEZs), weeds are also diverse. Their control measures require different practices. Our study mainly found and focused on transplanted rice in both dry and rainy seasons. The demographic profile of surveyed farmers indicates a male-dominated workforce, with a significant portion (66.82%) of respondents between 36 and 70 years old, indicating a mature farming population. Most farmers have substantial experience in rice cultivation (over 50% with more than 15 years).

Our research indicates that a significant percentage of farmers (37%) does not have formal education, underscoring a potential need for educational interventions and assistance to improve agricultural practices and productivity. A noticeable percentage of newcomers to rice farming was observed. Age and experience distribution potentially highlights knowledge transfer to younger generations, ensuring sustainable rice production in the country. This also translates into well-established weed management approaches.

Most farmers cultivate relatively small landholdings, with less than one acre owned by most respondents. This highlights the prevalence of small-scale farming. Despite variations in land size, the average acreage remains modest, suggesting limited land consolidation. According to World Bank data, the current average arable land size is 0.12 acres *per capita*. Thus, our survey data are consistent with the national average. The frequency of land utilization for crop production varies, with a significant proportion of farmers opting for two or three crop cycles annually. Transplanted rice is the primary crop, predominantly cultivated during the *boro* and *aman* seasons.

Weed control methods varied by AEZs as regional factors, such as the availability of labor, farmers' economic status, etc., were diverse. Sole use of herbicides or manual labor is not the ideal case. A significant proportion of farmers deploy only manual weeding. Such a limited adoption of herbicides among farmers, especially smallholders, is due to various factors, including high costs, lack of knowledge about herbicide application, readily available workforce from their own family, and unwillingness to use toxic agrochemicals. Market surveys suggest that access to modern agricultural technologies and herbicides is not the predominant factor. This is in contrast to earlier findings (Rahman et al., 2019).

A significant number of this group perceived that herbicides negatively affect early crop growth. Farmers' observations align with scientific investigations. A study from Zhang (2015) found that although bensulfuron methyl and pyrazosulfuron ethyl are good herbicides for controlling weeds, they can stunt the growth of rice seedlings and reduce overall crop vigor. It has also been found that these herbicides can cause phytotoxic effects on rice plants, resulting in delayed development and stunted growth. (Chauhan, 2013; Zhang et al., 2015).

Labor-intensive manual methods may be effective on a small scale but can be time-consuming and costly, posing challenges to farmers during peak seasons. Farmers' unwillingness to use herbicides in AEZ-24, attributed to factors like limited access to herbicides and traditional farming methods, highlights the need for tailored interventions to address specific regional challenges.

The sole use of herbicides is limited in certain places where the weed predominance is the lowest. At the same time, many such farmers admit that they cannot afford the manual labor that is needed. A combination of herbicide use and manual labor is the most common scenario. This indicates a growing acceptance of herbicides alongside traditional practices. At the same time, it is also clear that many weeds are not eliminated by herbicide treatments. The requirement for manual labor following herbicide treatment varied. This is due to several factors, such as the high presence of weeds, lack of knowledge on proper dosing, and unavailability of standing water after transplanting. This highlights the adverse effects of herbicides and potential herbicide-resistant weeds that need further attention.

Our data also revealed a preference for pre-emergence herbicide application. This aligns with the survey findings on weed control practices, highlighting the focus on preventing the initial emergence of weeds. Most farmers (86%) apply herbicides within 1–7 days of transplanting rice. Manual weeding alone or in combination is needed within 3–6 weeks of transplantation. This suggests a very high demand for a manual workforce within a limited period in a region. This eventually makes weed removal too tricky for many farmers, impacting productivity.

Glyphosate and paraquat, two post-emergence herbicides, are used by farmers to prepare their land before rice planting in certain zones, such as AEZ-19, where land is left fallow for a few months. Consequently, weed prevalence is high, and pre-planting land cleaning is essential. Therefore, these chemicals are not directly used for weed management during rice cultivation but are crucial for pre-cultivation weed control. Various formulations of chemicals are employed during cultivation for weeding purposes. Pyrazosulfuron ethyl emerges as the most frequently used herbicidal compound, followed by other chemicals such as bensulfuron methyl with acetochlor and pretilachlor. Interestingly, the data indicate limited awareness among farmers regarding the active ingredients of herbicides, with most farmers identifying herbicides by trade names rather than active ingredients.

The availability of herbicides in agricultural marketplaces plays a crucial role in determining farmers' weed management practices and overall crop productivity. We surveyed the herbicide market by interviewing shopkeepers. The survey reveals various herbicide products in agrochemical shops across all 30 AEZs. With a total of 49 trade-named herbicides listed, farmers have access to a variety of options for weed control. These products encompass ten different herbicide formulations, with a notable presence of both single-active ingredients and combined-formulation herbicides. The availability of diverse formulations allows farmers to choose products tailored to their specific weed management needs and preferences.

Our data suggest regional variations in the popularity and availability of certain herbicides. Interestingly, this is not linked to farmers' knowledge but to sellers' promotions. Glyphosate and paraquat emerge as the most common herbicides used for pre-cultivation weed cleaning, with a significant presence in the market. However, there are notable differences in herbicide sales across AEZs, with higher quantities of glyphosate and paraquat sold in AEZ-29 than in other regions. Conversely, the absence of herbicide sales in AEZ-24 highlights their unsuitability among farmers. AEZs do not significantly influence the cost of herbicides; the price varies depending on the specific herbicide formulation and its brand. Triafamone, pyrazosulfuron ethyl, and bensulfuron methyl with acetochlor had higher prices, ranging from $0.80 to $2.08 per acre. These price differentials impact farmers' purchasing decisions, making cost-effective options more appealing, especially for resource-constrained farmers. Our study highlights that herbicides are available throughout the country and season. Their accessibility to farmers depends on the farmer's economic status and decision to apply herbicides. All herbicide products sold in the marketplaces are registered, indicating compliance with regulatory requirements. This ensures farmers can access approved and regulated herbicides undergoing safety and efficacy evaluations.

Our data highlight the cost-effectiveness of combining herbicides with manual weeding compared to relying solely on manual labor. This is primarily attributed to the herbicide efficiency and high labor cost associated with manual weeding and the narrow window of time for weed control, leading to labor shortages and increased wages. In addition, the affordability of herbicides makes them a cost-effective solution for initial weed suppression. Farmers' responses suggested that manual weeding alone would be financially unsustainable in most regions due to high labor costs. This implies that some form of weed control, potentially including herbicides, is crucial for profitable rice cultivation.

Most farmers believe that herbicides have a positive role in increasing their rice harvest. They attribute this increase to their overall agricultural strategies, including herbicide application that enables timely and efficient weeding, significantly reducing competition between weeds and rice plants. On the other hand, farmers who disagree with herbicides increasing yield usually do not think of herbicides as having a positive impact on yield or growing plants. Rather than seeing herbicides as a tool to increase crop productivity, they might see them only as a way to control weeds. However, it is worth noting that some farmers perceived that manual weeding techniques could achieve comparable yields, albeit with labor availability and timely weeding challenges. The convenience and cost-saving benefits associated with herbicide application, particularly in reducing weeding costs, are widely recognized among respondents. Thus, the importance of timely weeding in getting high yields, for which herbicides have a positive and cost-effective role, is highlighted. However, nearly half of the farmers observed that herbicides negatively impact rice seedling growth. Herbicides inhibit the emergence of weed species from their propagules. However, they can also be toxic to rice plants, resulting in poor crop emergence, tillering, root damage, and potentially whiteheads. These issues typically arise when the herbicides are not used as recommended, such as when they are applied at the wrong rate or during the wrong stage of crop growth. The susceptibility of plants to damage varies depending on their variety, growth stage, and environmental conditions (Martini et al., 2022). Damage typically occurs shortly after the application of the herbicide. Toxic effects were noticed at an early stage of rice growth under treatment of various pre-emergence herbicides in a wide range of climatic conditions (Thapa et al., 2012; Tansay et al., 2021; Zhang et al., 2023). Farmers used additional fertilizers to alleviate such toxicity, imposing additional costs. Thus, research on mitigating toxicity would help develop effective plant protection strategies and maintain ecological balance (Barbaś et al., 2024). These include possibly integrating more post-emergence active substances (Juhász et al., 2024).

Only a small percentage of farmers follow product labels to obtain dosing and safety information. Although roughly 63% of farmers have completed at least elementary school, most of them are nevertheless unwilling to read herbicide labels that contain information about recommended dosages and possible side effects. Instead, most farmers rely on pesticide shops for guidance. However, this heavy reliance on shops can result in misinformation or overuse of herbicides, increasing costs and posing risks to health and the environment. A significant portion of the population learns from neighbors and friends, indicating the potential for community training.

Additionally, the low involvement of government extension workers suggests a lack of effective communication channels for disseminating accurate information and promoting best practices in herbicide use. Therefore, training programs for farmers are needed to create awareness. This strategy can reduce the possibility of overuse and its detrimental effects on the environment and crop health, ensuring the safe and efficient use of herbicides.

Most farmers do not report immediate health issues due to herbicide applications. However, a substantial number consider these to be potentially harmful. Despite recognizing the benefits of personal protective equipment (PPE), only a few farmers use it. Only a tiny proportion express concerns about soil health and environmental risks.

Identifying difficult-to-eradicate weeds from all AEZs would enhance our understanding of weed management issues, including herbicide dosage, resistance, and potential measures. During the survey, farmers also expressed their opinions on possible interventions. They reported the limited efficacy of pre-emergence herbicides on certain weeds, necessitating additional manual weeding that increases rice production costs. This highlights the need for more effective weed control strategies, including broader-spectrum herbicides. Considering the uncovered issues, farmers show a strong interest (99%) in genetically engineered rice varieties with tolerance to broad-spectrum herbicides, provided they are safe, profitable, and high yielding.

The survey also suggests that farmers are open to adopting the AWD irrigation method for water conservation. It has been reported that AWD could increase the farmers' income by up to 32% in Bangladesh (Lampayan et al., 2015). However, weed concerns deter them from implementing AWD. Multiple studies reported that farmers perceived yield increases from using AWD (Kürschner et al., 2010; Rahman, 2015). The availability of an appropriate weeding method was recognized as the primary limiting factor in adopting AWD. The introduction of rice varieties tolerant to broad-spectrum post-emergence herbicides could incentivize AWD adoption, leading to water savings and cost reduction.

5 Conclusion

Currently, high labor prices make manual hand weeding very expensive in Bangladesh. Our comprehensive survey reflects the varied patterns of weed management practices, specifically herbicide application, and the prevalent challenges farmers face across 30 AEZs in Bangladesh. Our study concludes that herbicide-free rice cultivation is not feasible due to intense weed competition and high labor costs. Commonly used herbicides, such as pyrazosulfuron ethyl and a combination of bensulfuron methyl with acetochlor, effectively control weeds but can negatively impact rice seedling growth. This necessitates additional fertilization, which increases costs for farmers. Several weeds are not affected by pre-emergence herbicides. Our data highlight the potential benefits of introducing herbicide-tolerant rice cultivars. In addition, cultivating such crops will reduce water and tillage requirements, which can help conserve soil moisture and improve soil health. They would also allow flexibility in herbicide application. Overall, herbicide-tolerant rice varieties offer multiple benefits for farmers and food security.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants or patients/participants legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

MMI: investigation, formal analysis, data curation, writing–original draft, writing–review and editing, methodology, visualization, validation. MMR: visualization, validation, software, methodology, writing–review and editing, writing–original draft, formal analysis, data curation, investigation. SSS: investigation, writing–review and editing, writing–original draft. MNI: writing–review and editing, writing–original draft, investigation. FHB: writing–review and editing, writing–original draft, investigation. MSK: validation, project administration, funding acquisition, writing–review and editing, writing–original draft. IA: writing–review and editing, writing–original draft, visualization, validation, supervision, resources, project administration, investigation, funding acquisition, conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2024.1410128/full#supplementary-material

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Opinion: Advancing science in support of sustainable bio-innovation: 16th ISBR Symposium – in memory of Professor Alan Raybould

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Introduction

Just months before the 16th Symposium of the International Society for Biosafety Research (ISBR) took place in St. Louis, MO (May 2023), the ISBR community suffered a shattering loss with the death of our dear friend and colleague Alan Raybould. Among Alan's innumerable contributions to the field of risk assessment and biosafety, he was an active contributor to ISBR over the course of his career, serving on the Board of Directors and as a member of the program committee, member and chair of the publication committee, contributing to symposium planning, chairing sessions and, as he was always known to do, delivering many notable and thought-provoking presentations at the symposia. The loss of Alan has left a hole in our community that simply cannot be filled. During the 16th ISBR Symposium, Professor Alan Gray, former President of ISBR, who was an advisor, colleague, and longtime close friend of Alan, shared a tribute. We honour the memory of Alan Raybould by sharing that tribute as part of this Research Topic produced after the Symposium.

The life and work of Professor Alan Raybould (1962–2022) - A tribute

> As presented by Professor Alan Gray at the 16th Symposium of the International Society for Biosafety Research, St. Louis, Missouri, 3 May 2023.

"First I would like to thank the Board of the ISBR for inviting me to pay this tribute to Alan – I am deeply honoured to do so. I will begin by recounting my personal experiences of Alan's life and work and will then review his contribution as a scientist and scholar. Finally, I will attempt to capture something of Alan's character and personality.

Alan Raybould–Colleague and friend

I first met Alan in 1985 when my friend Mike Lawrence of the University of Birmingham's Department of Genetics suggested that we share a PhD student to tackle a project on the topic of population genetics. He came to us with, to quote his professor, "the best first in Manchester in a decade". He worked on the evolutionary origins of the grass *Spartina anglica,* doing the lab work in Birmingham (in the dear old days of isoenzyme electrophoresis) and the field work from Furzebrook (then a research station of The Institute of Terrestrial Ecology). I had moved to Furzebrook, which is in Dorset in southern England, a few years before and had begun to collect some type material of this famous allopolyploid invasive species, the site of its origin being nearby in Southampton harbour; but other commitments had prevented me from working on it. A copy of Alan's simply brilliant thesis exploring genetic variation in the putative parental species, the F1 hybrid and the allopolyploid has pride of place on my bookshelves.

After a brief post-Doc in Birmingham Alan came to work in my group at ITE Furzebrook in 1990 and thus began more than 3 decades of exciting collaboration and wonderful friendship. We were researching genetic variation in natural populations of plants in relation to factors such as population size and isolation, breeding systems, life history traits and gene flow–mainly from a conservation genetics perspective. So when the GM crops debate took off we were well positioned to make a contribution to understanding the potential impacts of gene flow between GM crops and their wild relatives. In fact, one of our early papers in which we combined Alan's knowledge of genetic modification and my knowledge of the ecology of wild plants to look at the implications of hybridisation between modified crops and their United Kingdom relatives (Raybould and Gray, 1993) remains his second most cited publication.

These were exciting times for our group. We expanded our work on gene flow and population genetics to look at the wild relatives of some United Kingdom crops, most notably species of *Brassica* and *Beta* (and also some grasses). Among other areas, we studied hybridisation rates and effects and the prospects of ecological release by the transfer of ecologically relevant, fitness, traits to wild populations. Our models for this included virus resistance in wild *Brassica oleracea* and *Brassica rapa* and the role of the bolting gene in *Beta maritima*. To understand the role of virus resistance in wild Brassica populations required collaboration with the virologists in our own Institute's Oxford lab and with other specialist plant breeding institutes as well as University groups, as indeed did much of our research at that time.

And this was when we became aware of Alan's great gifts as a collaborator. His curiosity and fascination for science, the breadth of his intellect and his ability to forge friendships made him a natural collaborator (in my experience not necessarily a common trait in the intellectually gifted). He collaborated and published with a huge range of people in the broad field of Environmental Risk Assessment, in research institutes, crop breeding organisations and universities in the United Kingdom and abroad. Many of you are in this room. Such was his gift for understanding and making a contribution to a wide range of problems that, during this period, people in our lab and institute (which became the United Kingdom Centre for Ecology and Hydrology) working in other areas sought him out with their problems.

Alan's research with our molecular ecology group included a wide range of topics and collaborations. Working with the conservation genetics group he published "Conserving genetic variation in *Lobelia urens* populations" (a rare heathland plant) and "Status of *Rumex rupestris* populations, survival and genetic diversity" (a threatened shore dock). His work on gene flow included theoretical and empirical ways of defining and measuring gene flow in *B. maritima* (inferring patterns of dispersal from allele frequency data using molecular markers to estimate gene flow with distance and regional-scale estimates of transgene spread from oilseed rape (also using remote sensing).

His published work on viruses in *Brassica* species includes studies of spatial distribution of viruses in natural populations of *B. oleracea,* the effect of turnip mosaic virus and turnip yellow mosaic virus on survival growth and reproduction in *B. oleracea*, viruses in *B. rapa* and heritable variation for control of turnip mosaic virus and cauliflower mosaic virus in *B. oleracea*. Further work on fitness and selection in wild *B. oleracea* produced a series of papers on plant-herbivore interactions including the ecological genetics of aliphatic glucosinolates and host plant location and herbivory in the cabbage seed weevil *Ceuthorynchus assimilis.*

Alan Raybould–Scientist and scholar

I imagine that a lot of Alan's earlier work will be new to an ISBR audience. But its range and brilliance will, I'm sure, not surprise you. It foreshadows the pattern of his later work and contribution to risk analysis, risk assessment and the role of science in decision making. That work also involved collaboration with a wide range of actors, many of whom are in this room. First, however, I would like to trace an aspect of Alan's thinking and development whilst he was still with us.

By 1995 he had become Head of the Molecular Ecology group and, since my own responsibilities had increased, our interactions were less frequent and mainly consisted of chats in the lab, at meetings, coffee and in the pub. It was clear that he was becoming increasingly interested in the scientific method, in philosophy of science and in hypothesis-based approaches. He was reading the work of and fascinated by Karl Popper (as some will know, Alan and his wife Clare's beloved dog is called Popper). We began to see before he left us the thinking that prevailed upon us not to continue to accumulate data without first defining and clarifying the perceived risks. We began to hear in his talks about 'black swans' and 'buckets and searchlights' (Raybould, 2010a) and the various metaphors he employed to urge us all to stop accumulating the ecological data it is 'nice to know' but to focus on that which will help us answer the 'need to know'.

The titles of two of Alan's single author papers (in 2004 and 2010) reflect this viewpoint.

> "A decade of gene flow research: improved risk assessment or missed opportunities?" (Raybould, 2004)

> "Reducing uncertainty in regulatory decision -making for transgenic crops: more ecological research or clearer environmental risk assessment?"(Raybould, 2010b)

Papers with two rhetorical questions which his life's work went on to answer so effectively.

In a perverse way all the fascinating discoveries about ecology and selection in natural populations he made at the Centre for Ecology and Hydrology may have paved the way for the focus of his later highly influential contribution. I must conclude that my major influence on the thinking and development of this brilliant man was to show him how not to do an environmental risk assessment! Anyway by 1999 it was clear that his fascination with the issues of risk assessment and his love of applied and socially relevant problems would lead him to other things and specifically agricultural biotechnology, and in 2001 he joined Syngenta.

In 2019 Alan was appointed as the Chair of Innovation in the Life Sciences in the University of Edinburgh and a new phase of his life began in which he added "teacher" to his list of gifted skills. By all accounts he was exceptional at that too. In 2020 he was appointed to the Advisory Committee on Releases to the Environment (ACRE), the UK's independent body which provides statutory advice to the United Kingdom government on the risks to human health and the environment from the release of genetically modified organisms (GMOs). This is the same body to which I was appointed in 1994 and Chaired from 1999 to my retirement in 2003. I have recently learned that he was due to be made Vice Chair. I cannot tell you how delighted and proud I was on hearing that news.

Alan and I kept in touch, not only as friends but also meeting and working together in conferences, seminars and workshops around the world. In fact in 2007, after I had retired, Alan persuaded me to join him and others at a workshop in Washington on problem formulation (Raybould, 2006) which rekindled my interest in risk assessment and converted me to adopt his approach–the former student teaching the former teacher! I was privileged to be involved in several of the many workshops to which he made a key contribution and in which I learned so much from him.

There is absolutely no doubt that Alan's life and work were transformational. His contribution to biosafety research, environmental risk assessment, the application of problem formulation and policy-based decision-making, among other areas, has been immense, profound and game-changing. He was a prolific writer–of his more than 180 papers, articles and reports, more than 100 were concerned with this area. As when he was at the Centre for Ecology and Hydrology these publications demonstrate his skills as a collaborator (working with many of the ISBR family) but also his originality, clarity of thought, rigour and common-sense pragmatic approach to the issues he tackled. His work gained international recognition and influenced regulatory approaches around the world.

I hesitate, because I am probably not the most qualified person, to evaluate the impact of this later body of work. I quote below some of the things said about his influence in the various tributes paid to him.

> "a giant in the world of biodiversity research and risk assessment of new technologies in agriculture. . ..a leading force in improving the way new technologies are evaluated and regulated around the world"

> "a wonderful scientist who managed to bring clarity to any topic he approached. He fundamentally changed the way the risk assessment for genetically modified organisms was viewed by leading the discussion away from endless data generation to a targeted hypothesis driven approach informed by a policy context"

> "he left a legacy of scientific work that will not only inspire new generations of regulatory folk but also is adaptable enough to cover new technologies"

> "well recognised as a true leader and mentor in his field. His contributions have already had great impact and will continue to do so, as his published works will guide science and regulation for many years"

These are just a few of the many tributes to his work that say more powerfully than I could what a huge impact he has had. Hopefully these and countless other memories we have of a wonderful and gentle man, together with his magnificent legacy of words and ideas, will help to sustain us as we face a future without him."

Author contributions

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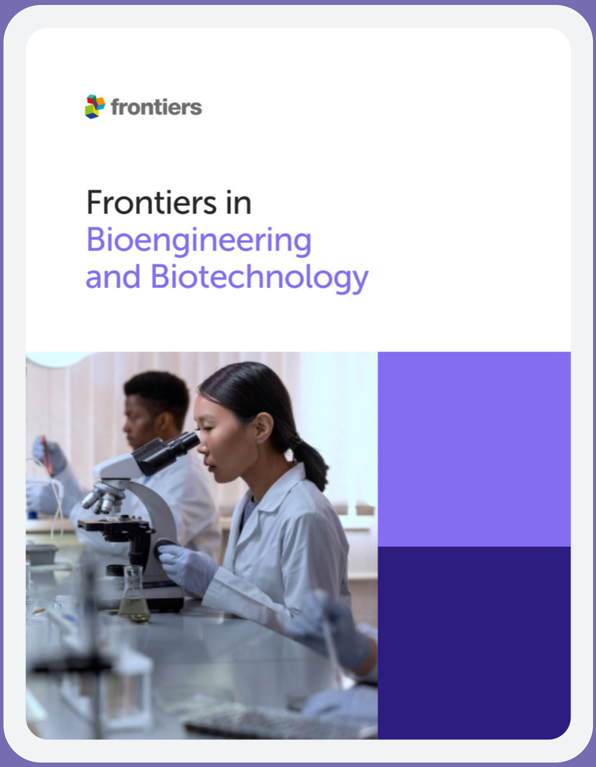

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