frontiers | Research Topics

Speed breeding systems for food

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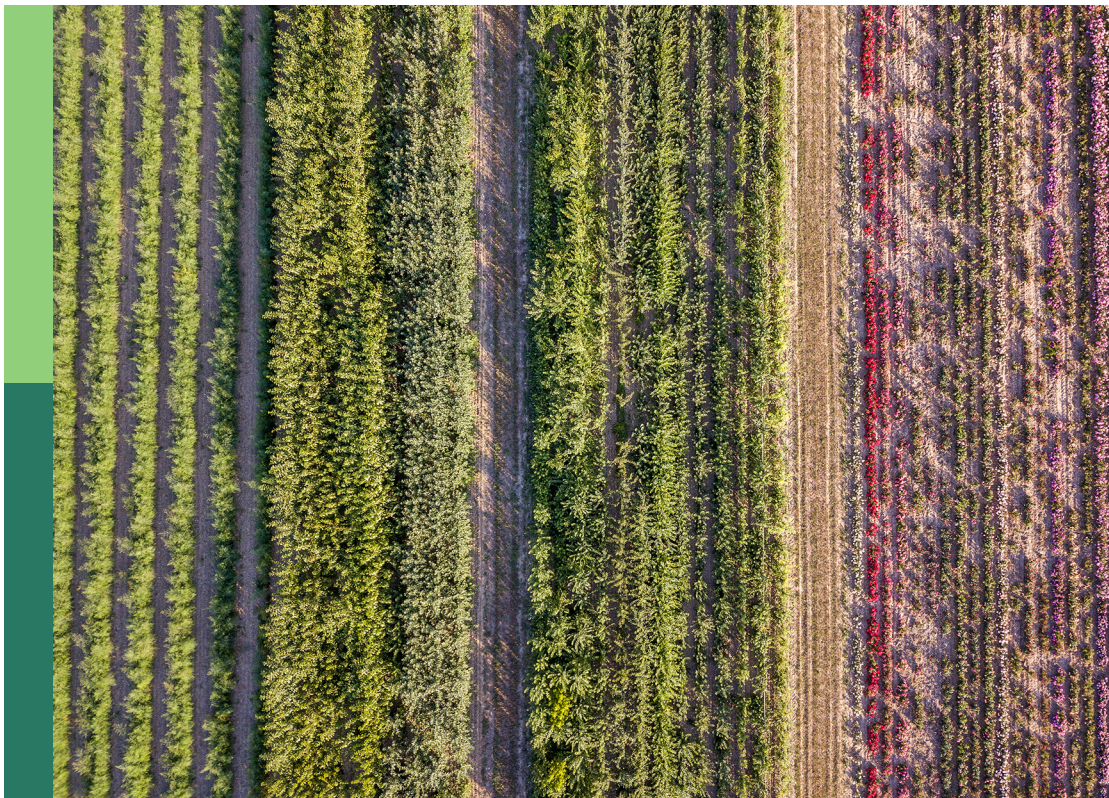
Published in
Frontiers in Sustainable Food Systems



ISSN 1664-8714
ISBN 978-2-8325-5565-1
DOI 10.3389/978-2-8325-5565-1

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Speed breeding systems for food

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Citation
Paul, K., Kancharla, N., Gautam, K., Ceran, M., eds. (2024). *Speed breeding systems for food*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5565-1

Table of contents

frontiers | Frontiers in Sustainable Food Systems

TYPE Editorial
PUBLISHED 04 October 2024
DOI 10.3389/fsufs.2024.1493864

OPEN ACCESS

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RECEIVED 09 September 2024
ACCEPTED 13 September 2024
PUBLISHED 04 October 2024

CITATION
Paul K and Ćeran M (2024) Editorial: Speed breeding systems for food.
Front. Sustain. Food Syst. 8:1493864.
doi: 10.3389/fsufs.2024.1493864

Editorial: Speed breeding systems for food

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KEYWORDS

speed breeding, vertical hydroponics, data-driven agriculture, molecular markers, marker-assisted selection (MAS), genomic selection (GS)

Editorial on the Research Topic
Speed breeding systems for food

Sustainable food production systems hold great promise for addressing the global challenges of food security and environmental sustainability. This Research Topic centers around food systems that incorporate speed breeding technologies, vertical hydroponics, and data-driven smart sensor applications.

Speed breeding technologies enable the rapid generation of new plant varieties, accelerating crop development with desired traits, such as pest resistance, drought tolerance, high nutritional value, and high productivity. Achieved through advanced genetics, artificial lighting, and controlled environments, these techniques allow multiple generations of plants to be grown and harvested in a single year, surpassing the typical one to two generations of traditional field-based breeding. By analyzing specific crop genomes using molecular markers, breeders can identify and characterize genetic variation. This knowledge helps select desirable traits, such as pest or disease resistance and improved yield. Marker-assisted selection (MAS) and genomic selection (GS) are groundbreaking methods that enhance the efficiency and accuracy of trait selection. MAS identifies desirable traits early in the breeding process, while GS predicts plant performance before growth, accelerating breeding. These techniques have significantly improved breeding efficiency, allowing for the development of new varieties and breeds in less time.

Vertical hydroponics, which uses nutrient-rich water instead of soil, enables higher yields per unit of land, efficient resource utilization, and year-round production, while data-driven smart sensors optimize growing conditions and automate processes like nutrient delivery and harvest.

Eleven articles were published in this Research Topic, authored by experts from different disciplines. The first study was by Choi et al. on the development of a speed breeding protocol for pepper (*Capsicum annuum*) by controlling the photoperiod and light quality. The authors revealed that the combined influence of Epp and FR light affects flowering gene expression in pepper plants, providing valuable insight into the potential of the speed breeding system to expedite genetic research by reducing generation time. The submission by Tetreault et al. was a hypothesis and theory article that defined the integration of recirculating aquaculture systems (RAS) with hydroponic cropping systems (HCS) into a single system with shared water treatment units.

A review on "Genomics-assisted speed breeding for crop improvement: present and future" by Ćeran et al. provided an overview of current research on speed breeding in crops. They covered various aspects of the manipulation of different environmental factors in speed breeding. The authors provided a summary of developed speed breeding protocols for main crops and discussed the possibilities of integrating speed breeding with genomic approaches. The current status and challenges of speed breeding and future perspectives in breeding were also reviewed. In the article "Efficient regeneration of *in vitro* derived plants and genetic fidelity assessment of *Phalaenopsis* orchid" by Sarmah et al. the authors successfully developed a simple and rapid regeneration protocol for *Phalaenopsis* by using flower stalk nodes as explants, which resulted in a high frequency of plantlets in a short period of time. The integrity of the micropropagated clones created *in vitro* and the genetic maintenance of the mother plant were confirmed by DNA fingerprinting, contributing to the advancement of reliable and efficient micropropagation methods. The study "*Genomic analysis and identification of potential duplicate accessions in Burkina Faso cassava germplasm based on single nucleotide polymorphism*" by Soro et al. showed high genetic diversity and complex genetic structure within cassava accessions grown in Burkina Faso using molecular markers. The study "*Development of portfolio management tools in crop breeding programs: a case study of cassava in sub-Saharan Africa*" by Egesi et al. presented pilot work on cassava to improve varietal development that is efficient, effective, and delivers higher genetic gain. The authors proposed the transformation of cassava breeding programs by developing guiding, flexible, and adaptive tools for portfolio management. A deeper understanding of the genetic diversity within the cassava population, transformation of breeding programs, and speed breeding could provide resources to advance its breeding by improving disease resistance, climate change adaptation, and other critical factors necessary to ensure food security in sub-Saharan Africa. Anshori et al. developed an innovative and effective approach for selecting F3 transgressive segregants in cayenne pepper populations using a semi-objective-based selection index that includes canopy width, fruit weight, and yield.

Nhamo et al. addressed resource-poor farmers residing in many marginal areas by promoting the adoption of multi-cropping, rainwater harvesting, and soil conservation techniques using underutilized indigenous crops like Bambara groundnut. Cepková et al. reviewed current information on quinoa's genetic resources, focusing on the variability of economically important traits like yield and bioactive compound content, including protein and amino acid composition, under different growing conditions. They also discussed how variety and environmental factors, such as elevated temperatures, high salinity, and extreme weather, can negatively impact the growth, productivity, and nutritional content of quinoa. Luo et al. assessed the impact of soybean breeding on phosphorus (P) and nitrogen (N) utilization efficiency by increasing their partitioning to pods. They concluded that soybean breeding improved the agronomic efficiency of P fertilizer and P and N utilization efficiency. Increased partitioning to pods and decreased partitioning to stems contributed to these improvements by reducing the demand for P and N. However, while P supply increased nutrient accumulation, it reduced P utilization efficiency. These findings highlight the importance of appropriate resource allocation among plant organs and efficient P management to improve nutrient utilization and reduce fertilizer requirements. Armengot et al. evaluated the yield performance of four locally-selected cacao clones, four widely-used international clones, and four full-sib families in a long-term trial in Bolivia. The cacao trees in monocultures had higher yields than those in agroforestry systems, with no differences observed between conventional and organic management. On average, the local clones had two and five times higher yields than the international clones and full-sib families, respectively.

This special edition Research Topic sheds light on sustainable food production systems from plants, which offer promising alternatives to conventional agriculture, delivering high yields, efficient resource utilization, and reduced environmental impact. Speed breeding has the potential to revolutionize plant breeding, accelerating the development of new crop varieties and contributing to food security by increasing productivity and resilience. The integration of innovative technologies and data-driven approaches will further advance these systems, and make them more accessible for widespread adoption.

Author contributions

KP: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Formal analysis, Conceptualization. MC: Writing – review & editing, Writing – original draft, Resources.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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

frontiers | Frontiers in Sustainable Food Systems

TYPE Original Research
PUBLISHED 06 October 2022
DOI 10.3389/fsufs.2022.990213

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Land, Livelihoods and Food Security, a section of the journal Frontiers in Sustainable Food Systems

RECEIVED 12 July 2022
ACCEPTED 20 September 2022
PUBLISHED 06 October 2022

CITATION
Nhamo L, Paterson G, van der Walt M, Moeletsi M, Modi A, Kunz R, Chimonyo V, Masupha T, Mpandeli S, Liphadzi S, Molwantwa J and Mabhaudhi T (2022) Optimal production areas of underutilized indigenous crops and their role under climate change: Focus on Bambara groundnut.
Front. Sustain. Food Syst. 6:990213.
doi: 10.3389/fsufs.2022.990213

Optimal production areas of underutilized indigenous crops and their role under climate change: Focus on Bambara groundnut

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Food demand in Africa continues to outstrip local supply, and the continent currently spends over US$35 billion annually on food imports to supplement local deficits. With the advances in agronomy and breeding, commercial crops like maize (*Zea mays*) and soybean (*Glycine max*) in the region are under threat from climate change, decreasing rainfall and degraded lands. Unlike commercial crops that are generally adapted from other regions, underutilized indigenous crops are uniquely suited to local environments and are more resilient to climatic variations and tolerant to local pests and diseases. This study, done in Limpopo Province, South Africa, identifies optimal areas for cultivating Bambara groundnuts (*Vigna subterannea*), an indigenous crop suitable for arid and semi-arid regions. The aim is to promote the production of underutilized indigenous crops at a large scale with fewer resources, while still meeting local demand and reducing the food import budget. Suitability maps are delineated using a multicriteria decision method in a Geographic Information System (GIS). The procedure is important for diversifying farming systems, making them more resilient (to biotic and abiotic stresses and climate change) and more successful at enhancing water, food and nutritional security. With the province's limited water and land resources for agriculture expansion, promoting indigenous underutilized crops is a pathway to reduce water allocated to agriculture, thereby enhancing drought resilience and ensuring water, food and nutritional security. Large tracts of degraded agricultural land deemed unsuitable for adapted crops, and which may require costly land reclamation practices, can be used to cultivate underutilized crops that are adapted to extreme local conditions.

KEYWORDS

climate change, resilience, geographic information system, food and water security, dryland agriculture, adaptation

Introduction

Underutilized indigenous crops are locally produced crop species primarily grown in particular native communities but have been losing their popularity as they have not been mainstreamed into the main food system (Mabhaudhi et al., 2019a; Jahanshiri et al., 2020). They cover a limited area that is neglected in terms of research and are consequently underfunded due to their limited importance in the global food market (Chivenge et al., 2015; Akinola et al., 2020). However, they are often characterized by their resilience and adaptation to extreme climatic and edaphic conditions and have local significance (Padulosi et al., 2011; Stamp et al., 2012; Akinola et al., 2020). Indigenous crop varieties are best-suited to local environmental conditions, and farmers' needs in marginal agricultural situations (Mabhaudhi et al., 2016). Their low input requirements give them an economic advantage over commercial crops like maize (*Zea mays*), soybean (*Glycine max*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Chibarabada et al., 2017). Current changing environments in southern Africa, characterized by extreme droughts and a shortening rain season, favor indigenous crops (Mabhaudhi et al., 2019b; Nhamo et al., 2019). Historically, they have always helped ensure food and nutrition security as part of a balanced diet, when adapted crops fail, or in between harvests (Tadele and Assefa, 2012; Mabhaudhi et al., 2019b). They provide important vitamins, proteins, and micronutrients and contribute to alleviating the challenges of growth stunting in children in developing countries (Chivenge et al., 2015; Akinola et al., 2020). Examples of priority indigenous underutilized crops suitable for southern Africa are shown in Table 1 (Mabhaudhi et al., 2017).

Unlike commercial crops that require costly agronomic practices and a lot of water for their cultivation, underutilized indigenous crops are uniquely suited to local environments, are generally more resistant to certain climatic variations and more tolerant to local pests (Chivenge et al., 2015; Keleman Saxena et al., 2016). Therefore, focusing on the cultivating of indigenous crops is an alternative climate change adaptation strategy (Mabhaudhi et al., 2019b). Furthermore, the consumption of indigenous crops provides nutritional diversity for communities, provides crop rotation options for farmers, creates niche markets in local economies, harnesses and enhances local knowledge (Chivenge et al., 2015; Massawe et al., 2015; Lin Tan et al., 2020). They further provide opportunities to enhance agro-biodiversity at the field level, promote nutritional diversity, disrupt pest and disease cycles (Kahane et al., 2013; Kimani-Murage et al., 2021), and reduce water share allocated to agriculture (Mabhaudhi et al., 2018a). Thus, harnessing and mainstreaming local knowledge and traditional crop species and developing underutilized crop breeds have enormous potential to improve water, food and nutrition security (Padulosi et al., 2013; Adhikari et al., 2017; Gerrano et al., 2021).

Regional and national policies in developing countries aim to increase the area under irrigation as a remedy to meet the food requirements of a growing population and ensure food and water security (CAADP, 2009; NDP, 2011). The increase in the irrigated area mainly targets commercial crops as they require more water than indigenous crops (Shelef et al., 2017; Fernández García et al., 2020). Although such initiatives of promoting commercial crops through the expansion of irrigation sound noble, the main challenges are water scarcity and land unavailability for an expanded irrigated area (Mancosu et al., 2015; Chibarabada et al., 2017). In South Africa, it is estimated that 98% of available water resources are already allocated, with over 60% of available water resources allocated to agriculture (Blignaut and Van Heerden, 2009; Von Bormann and Gulati, 2014). In southern Africa, more than 70% of available freshwater resources are used for agriculture, yet the region is regularly devastated by recurring food insecurity challenges (Nhamo et al., 2018; Ngcamu and Chari, 2020). Under such a changing environment, more emphasis should be on promoting the production of indigenous crops as they are suited to harsh local conditions (Stamp et al., 2012; Chivenge et al., 2015; Mabhaudhi et al., 2016). Indigenous crops are generally acceptable and appealing to local communities as they promote the preservation of culture and improve food selection and preparation concerning local people's needs and cultural values (Baldermann et al., 2016; Mabhaudhi et al., 2019b). Identifying suitable areas for cultivating underutilized crops is the initial step for their promotion, commercialization and mainstreaming into the main food system.

Cropland suitability mapping is an assessment of land performance when used to produce specific crops (Jahanshiri et al., 2020). It is a prerequisite to achieving optimum utilization

TABLE 1 Examples of underutilized crops suitable for arid and semi-arid areas.

Crop type	Common name	Scientific name
Cereals	Sorghum	*Sorghum bicolor*
	Tef	*Eragrostis tef*
Legumes	Bambara groundnut	*Vigna subterannea* (L.)
	Lablab	*Lablab purpureus* (L.) Sweet
	Cowpea	*Vigna unguiculata* (L.) Walp
	Marama bean	*Tylosema esculentum*
Root and tubers	Taro	*Colocasia esculenta*
	Sweet-potato	*Ipomoea batatas*
Leafy vegetables	Jews mallow	*Corchorus olitorius*
	Spider plant	*Cleome gynandra*
	Amaranth	*Amaranthus* spp.
	Nightshade	*Solanum nigrum*
	Wild watermelon	*Citrullus lanatus* L.

Source: Mabhaudhi et al., 2017.

of the available land resources for sustainable agriculture production (FAO, 1976; Li et al., 2010; Hagos et al., 2022). Adaptation of crop growth to local agro-ecological conditions' capabilities and constraints is a key principle of sustainable land management (Pretty and Bharucha, 2014; Viana et al., 2022). Identifying optimum land for cultivating indigenous crops is critical for the conservation of environmental resources while at the same time achieving maximum yields (Eastman, 1999; Mabhaudhi et al., 2019b). Thus, cropland suitability mapping provides information for growing potential crops and deriving maximum economic benefits with lower production costs (Kihoro et al., 2013). It also facilitates better water and land management. As indigenous underutilized crops are the 'future food' (Baldermann et al., 2016), this study used relevant agro-ecological factors in a Geographic Information System (GIS) to delineate optimum areas for cultivating Bambara groundnut [*Vigna subterannea* (L.)] in Limpopo Province, South Africa. The objective is to provide a procedure to delineate optimum areas for cultivating indigenous crops as a first step to promoting and mainstreaming underutilized crops into the main food system, focusing on the Bambara groundnut.

An overview of the Bambara groundnut

Bambara groundnut (Figure 1) is an indeterminate annual crop that grows close to the ground with seeds being produced underground (DALRRD, 2011; Gerrano et al., 2021; Khan et al., 2021). Being a highly adaptable legume, Bambara groundnut is grown in diverse agroecosystems and generally grows well under drought conditions (Mabhaudhi et al., 2013; Khan et al., 2021). Bambara groundnut has a low water requirement (Mabhaudhi and Modi, 2014), and has been identified as exhibiting all three categories of drought adaptation strategies which are escape, avoidance and tolerance (Mayes et al., 2019; Khan et al., 2021). However, the crop has a low tolerance for waterlogged soils and grows best in well-drained soils (Khan et al., 2021). Some varieties of Bambara groundnut exhibit tolerance to salinity (Mayes et al., 2019). Also, the presence of variation in photoperiod and temperature sensitivity among Bambara groundnut genotypes is a good indicator that there is room for improvement in varieties for adaptation in broad agroecological farming zones (Kendabie et al., 2020). Bambara groundnut has been identified as a potential future crop under climate change (Mabhaudhi et al., 2019b; Khan et al., 2021). In South Africa, Bambara groundnut yield and water productivity are projected to increase by ~ 37.5% and 33% under climate change (DALRRD, 2011; Mabhaudhi et al., 2018a).

The Bambara groundnut seeds are rich in carbohydrates, proteins, fiber, ash, fat, and micronutrients, making them a valuable source of nutrition for resource-poor farmers (Lin Tan et al., 2020; Khan et al., 2021). However, limited genetic improvement, poor milling characteristics, long cooking time, and anti-nutritional factors have resulted in the crop being underutilized (Lin Tan et al., 2020; Gerrano et al., 2021). Even though the crop is underutilized, the Bambara groundnut plays a key role in both food, and cultural practices of farmers in Africa and Asia and has been integrated into intercropping systems (Mayes et al., 2019; Lin Tan et al., 2020; Khan et al., 2021).

FIGURE 1
Bambara groundnut in leaf and the seeds.

Materials and methods

Description of the study area

The Limpopo Province of South Africa is the northernmost province, sharing international boundaries with Botswana, Mozambique, and Zimbabwe. The province (Figure 2) covers 125,755 km^2, comprising 10.4% of the total national area. It has a population of about 6 million people (StatsSA, 2021). It has a varied topography, ranging from lowlands dominated by bushveld vegetation to imposing mountains rich in native forests and unspoilt savanna wilderness (Cai et al., 2017). The topography is divided into three distinct regions that define the climate and vegetation of the province. These include (a) Lowveld region (arid and semi-arid), (b) Middle-veld region (semi-arid region) and (c) Escarpment region (sub-humid climate with rainfall above 700 mm per annum) (Cai et al., 2017).

Rainfall is received in the summer (October to March), averaging 500 mm per annum, whilst the other three seasons are generally dry. The eastern and northern parts are subtropical, with humid and hot summers. Average summer temperatures are around 27°C. In winter (May to September), the nights are cold and mostly frost-free, with chilly mornings and dry, sunny days (Vincent et al., 2010). However, the Lowveld is very hot with temperatures often exceeding 45°C. The province has abundant fruit and vegetable production as it is endowed with abundant agricultural resources. However, the most limiting resource for agriculture in the province is water, and most of the smallholder farms are under rain-fed agriculture (Oni et al., 2012). Both commercial and smallholder farmers contribute significantly

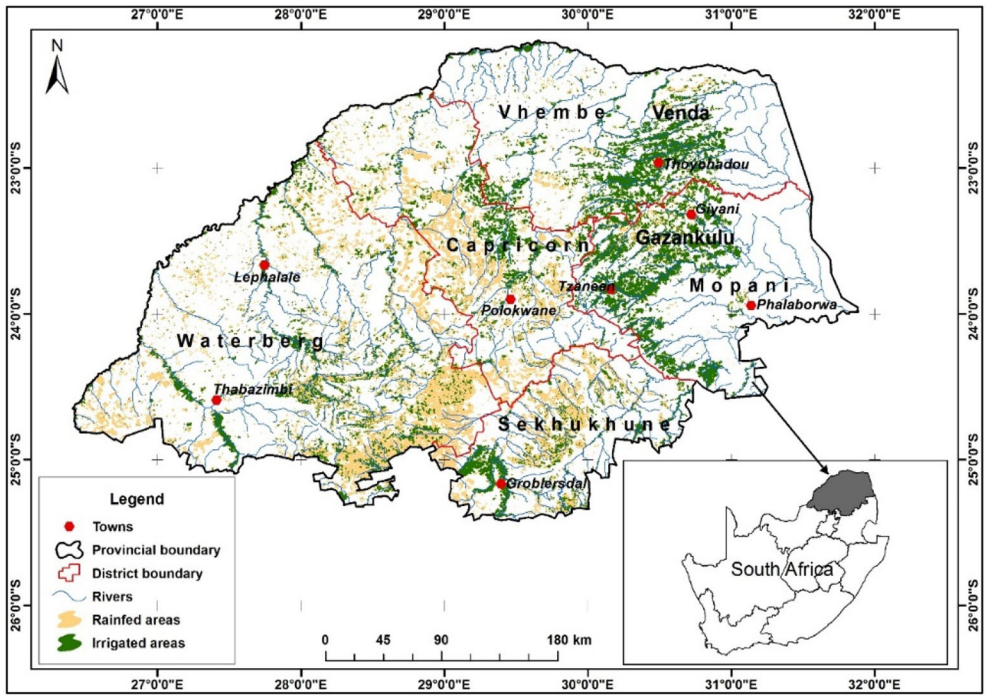

FIGURE 2
Location map of Limpopo Province showing the distribution of agricultural systems.

to crop production, although many rural people still practice subsistence farming and are highly vulnerable to climate change.

Background of the land suitability mapping algorithm in South Africa

As knowledge of South African soils grew in the late 1960's and early 1970's, there was also a growing realization that, while the soils in many areas were wellknown and had been comprehensively sampled and studied, there was a real need for a comprehensive reconnaissance survey of the natural resources of the whole country (ARC, 2014). This would include the recording of ***soils, terrain*** and ***macroclimate*** information so that these three pillars of agricultural production would be addressed simultaneously. This survey, which became known as the Land Type Survey (Land Type Survey Staff 1972–2002), was systematically carried out by the Soil and Irrigation Research Institute (SIRI) of the Department of Agriculture, Land Reform and Rural Development (DALRARD), which in 1992 became the Agricultural Research Council - Institute for Soil, Climate and Water (ARC-ISCW) (ARC, 2014).

The survey's fundamental mapping unit is the land type, which is a unique combination of broad soil pattern, terrain type, and macroclimate. Where any one of these three factors changes, a new land type unit would then be identified (ARC, 2014). The survey was carried out using 1:50,000 scale topo-cadastral maps as background, but it was always intended that the final product would be a series of maps at 1:250,000 scale. After the survey in 2002, a total of 7,071 unique land types had been identified, some of which had more than one occurrence. Therefore, over 15,000 distinct polygons were eventually defined and mapped (ARC, 2014). It is estimated that around 400,000 soil observations (mainly using a soil auger, but also including road cuttings, quarries, riverbanks, etc.) were made, which equates to approximately one observation per 400 ha across the whole country. This is more intense in the more productive areas, such as the Highveld and other eastern zones.

In contrast, the drier areas and the more inaccessible mountainous zones were investigated at a noticeably lower detail level. However, every effort was made to physically cover as much of the landscape as possible (ARC, 2014). Therefore, it is important to note the following:

- The Land Type Survey was carried out across the country, making South Africa probably the only country in Africa, and one of a select few in the world, that has systematic soil information of the whole surface area, backed by comprehensive analytical data.
- The fact that specific principles and guidelines were established and written down (MacVicar et al., 1974; Laker, 2004) means that the survey was carried out to a

TABLE 2 Bambara groundnuts – crop suitability algorithm.

Parameter	Highly suitable (S1)	Moderately suitable (S2)	Marginally suitable (S3)	Not suitable (N)
Slope (%)	0–4	4–8	8–20	>20
Rainfall (mm), Nov-Apr	550–750	450–550	350–450	>750, <350
Av temp (°C) (Nov-Apr)	20–28	20–32	32–35	>35
Soil depth (mm)	≥500	≥400	≥300	<300
Soil type	1, 2*, 3	4, 8, 9, 13*	6, 7, 12, 14	Other
Topsoil clay %	15–25	25–30	30–40	>40

*Calcareous series one suitability class lower (Cv40-48, Hu40-48, Sd30-32, Oa20-27 & 40-47, Gs20-29, Ms20-24).

consistently high, uniform standard throughout the almost 30 years of its completion.

- There was a high degree of staff continuity, with several SIRI/ARC-ISCW employees being involved with the survey for almost the entire 30 years.

The above factors mean that the Land Type Survey can be regarded as a high-quality, comprehensive product that has massively contributed to natural resource characterization, and agricultural productivity in South Africa, and continues to do so. As the only source of continuous natural resource data for South Africa, it is an important national asset.

Digitization of land type data

The knowledge that the land type data, comprising the boundary lines of each mapping unit (vector data) as well as the soil, climate and terrain data contained therein (raster data), could be digitized, and captured on a GIS was the most significant development since the start of the survey. Although this involved a significant amount of trial and error and a great degree of checking and correcting data, it had several advantages. Firstly, the data was available in a digital format, which meant it could be stored, manipulated, and interpreted. Secondly, each land type had a measured area, both in total extent and for the various component terrain types (crest, scarp, mid-slope, foot-slope, valley bottom), one or more of which occurred in varying proportions in every mapping unit. This meant that the estimated occurrence of the soil forms and series (derived from the ground-truthing exercise that comprised the land type field investigation phase), could be empirically linked to each terrain type and consequently to each full land type. This allowed for the determination of soil occurrence within a land type, or combination of land types, to be made.

Algorithm development

Even in the pre-digitisation era, there were various attempts to use land type data for a broad assessment of agricultural potential (Schoeman, 1978; Scheepers, 1984). However, with the advent of GIS and big data management platforms, it has become much easier to store, process and analyse large amounts of data. The first major initiative to use computerized data involved the concept of land suitability. The original concept derived from the USDA (Klingebiel and Montgomery, 1961) considered the allocation of land into one of eight general classes of suitability for rainfed agriculture, depending on the limitations in terms of a combination of soil (depth, structure, rockiness, etc.), climate (rainfall or temperature restrictions) or terrain (slope). The availability of soil data from the Land Type Survey and interpolated climate data from the Agrometeorology database (Laker, 2004) allowed the development of such a classification for South Africa.

The land capability determination was done primarily through an algorithm, a computer-based, step-by-step procedure for calculations, data processing, and automated reasoning. An algorithm's advantage is that instructions can readily be adjusted, then rerun very quickly and efficiently, thus facilitating comparison of previous and new results. In this way, specific algorithms can be created for various crops and adjusted to differing production areas or scenarios (such as winter rainfall in the Western Cape vs. summer rainfall on the Highveld, rain-fed vs. irrigated conditions, and so on) to obtain a set of comparable results.

The algorithm is determined as follows: Using a combination of local knowledge and yield results from a specific area, the various production parameters are determined. These include climatic factors (mainly rainfall and temperature), soil factors (such as soil form, effective depth and texture) and terrain (slope class). The algorithm for Bambara groundnuts, using information derived from all available sources, is shown in Table 2.

Using local expert knowledge from various sources, such as the ARC, the maximum practical, sustainable yield for any crop is established and the type of yield reduction that would make a crop uneconomic and thus not sustainable (DALRRD, 2011). This allows the initial suitability classes to be established. In this case, four suitability classes (S1, S2, S3 and N) are shown (Table 2). Still, it may be logical to determine either a higher or lower number of classes per crop, depending on what is sensible

TABLE 3 A generalized description of the soil categories together with the soil forms (symbols in brackets) used in the crop suitability algorithm.

No.	Generalized description of soil category with a listing of the soil forms*
1	Soils with humic topsoil horizons (*Soil forms* ***Ia, Ma, Kp, No***)
2	Freely drained, structureless soils (*Soil forms* ***Hu, Cv, Gf, Sd, Oa***)
3	Red or yellow structureless soils with a plinthic horizon (*Soil forms* ***Av, Gc, Bv, Pn***)
4	Imperfectly drained sandy soils *(Soil forms/series* ***Sp, Ct, Vf, Fw 10 & 20, Du***)
5	Swelling clay soils (*Soil form* ***Ar***)
6	Dark clay soils that are not strongly swelling (*Soil forms* ***Bo, Ik, Tk***)
7	Soils with a pedocutanic (blocky structured) horizon (*Soil forms* ***Va, Sw***)
8	Imperfectly drained soils, often shallow and often with a plinthic horizon (*Soil forms/series* ***We, Cf, Lo, Wa, Kd 10-15, Kd 20-22***)
9	Podzols (*Soil forms* ***Lt, Hh***)
10	Poorly drained dark clay soils that are not strongly swelling (*Soil form* ***Wo***)
11	Poorly drained swelling clay soils (*Soil form* ***Rg***)
12	Dark clay soils, often shallow, on hard or weathering rock (*Soil forms* ***My, Mw***)
13	Shallow soils on hard or weathering rock (Lithosols) (*Soil forms* ***Ms, Gs***)
14	Texture contrast soils (sandy topsoils abruptly overlie clayey, structured subsoils), often poorly drained (*Soil forms/series* ***Es, Ss, Kd 16-19***)
15	Wetland soils (*Soil forms/series* ***Ch, Fw 30, Ka***)
16	Non-soil land classes (pans, streambeds, erosion etc.)
17	Rock (surface outcrops)

*Soil form abbreviations as defined in MacVicar et al. (1974).

according to the various factors involved. Climatic parameters can then be established, in this case, rainfall over the summer period and different temperature characteristics in the most important part of the growing season. However, other variables, such as evaporation or frost period, may also be used as required (and if available). The assumption is that the lower the rainfall and the more extreme the other parameters, the less suitable the area is for crop production.

The ideal slope class is generally determined as being below 4%, with steeper slopes being both more problematic to cultivate and potentially posing an increased hazard for soil erosion. The various soils occurring within any land type are allocated into a specific soil category (as defined in Table 3). The soils listed in Table 3 comprise all of the soil forms occurring in the Binomial System (MacVicar et al., 1974), which was the system in use at the commencement of the Land Type Survey and was used for the whole project.

The soils are logically grouped into seventeen categories, which are more or less sequential in terms of decreasing arable agricultural potential, due to a combination of factors such as texture, structure, drainage, and overall ease of cultivation. Generally, soils in categories 1–4 are those where limitations would be expected to be the least, categories 5–9 are more limiting but can still be used for production if other criteria are met, while categories 10–17 usually pose significant problems and are often not recommended for cultivation.

When this soil category delineation is combined with ***effective soil depth*** (defined as the depth from the surface to any layer in the profile that is significantly limiting for root and/or water penetration), soil suitability can then be established. The hypothetical assumption is that the deeper the soil, the higher the suitability class. However, it is wellknown that in certain areas with low to marginal rainfall, coupled with light-textured soils, it is often advantageous to have a limiting layer (such as hard plinthite) at a specific depth range in the profile, to help with water retention in the root zone, especially in times of below-average rainfall. Such a scenario can also be built into the algorithm as needed.

Results and discussion

Algorithm results

For an area to have the highest possible degree of suitability, all the relevant parameters (climate, terrain, and soil) of the algorithm must be met. If one or more of these parameters are not met, the computer uses the algorithm to determine which class the site must be placed. Suppose all parameters are met, except one, then the class will need to be adjusted downwards, and so the principle of the lowest common denominator will apply.

Each land type inventory table is a list of all the soils occurring in that land type and their properties, along with the percentage occurrence of each one. Once the soil from a particular area is assessed in terms of the soil category group and the texture and depth information has been added, the algorithm uses the relevant slope and climate data and combines all these numerical combinations into one assessment per land type to determine relative occurrence percentages. The legend for the various crop suitability maps was designed so that firstly, all the high potential areas are determined and land types with >50%, 30–50% and 10–30% high potential land are identified (shown in shades of green in the map legend). If a land type contains <10% high potential land, the moderate potential areas are then assessed for the same percentages (shown in shades of orange/brown on the map) and so on.

The main benefit of the crop-specific algorithms is that, once the user is satisfied with the results, the respective areas of each suitability class can quickly be calculated (this can also be done

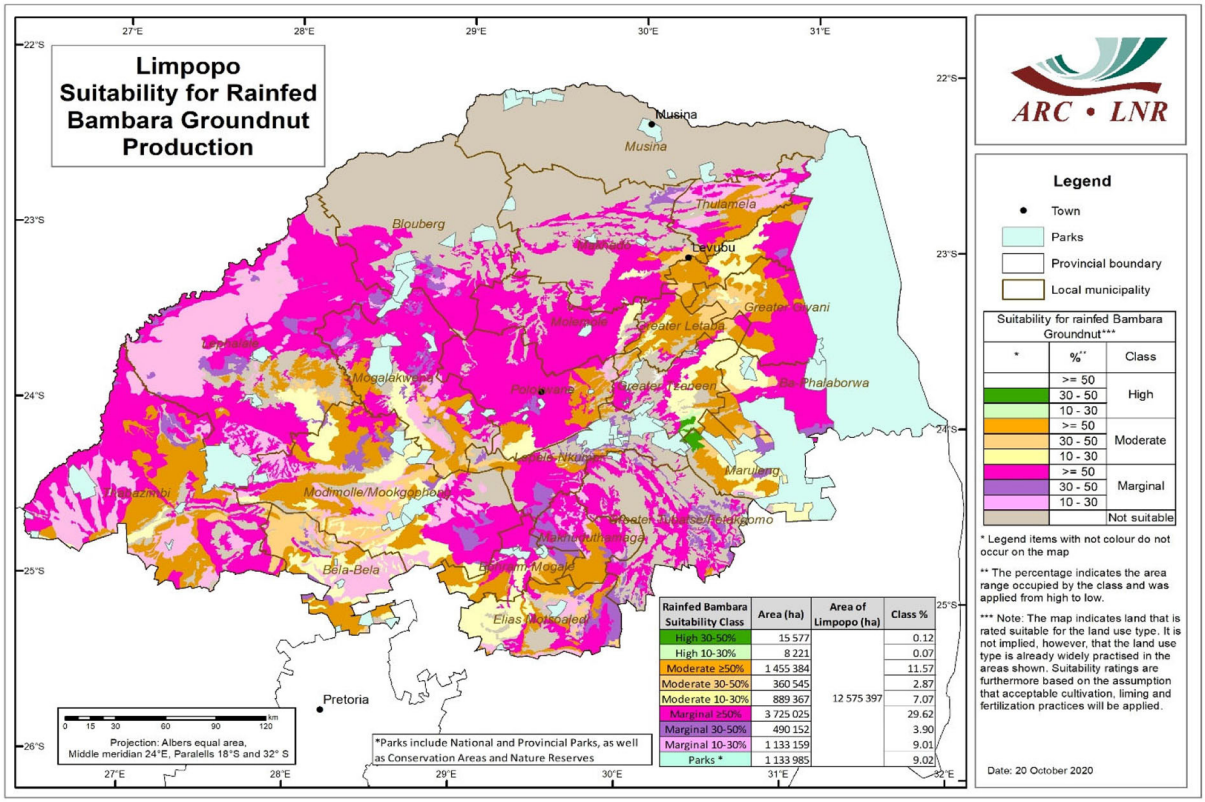

FIGURE 3
Suitability map of Bambara groundnuts under dryland cultivation for Limpopo Province.

for a province, local or district municipality, drainage basin or other study area as desired) and comparisons can be made. This can be used by other specialists, such as agricultural economists, to determine potential profit margins for a specific area.

Identifying areas suitable for growing Bambara groundnut

The suitability algorithm results can then be shown on a map to obtain a graphic representation of the distribution of the various classes. To illustrate this, the results of the dryland (rainfed) Bambara groundnuts algorithm for the province are shown in Figure 3.

According to the procedure used to develop the map shown in Figure 3, much of Limpopo Province has marginal or no suitability for Bambara groundnut production, mainly due to climatic restrictions. However, if the algorithm is run without the rainfall requirement (using soil and terrain only), the results are significantly different (Figure 4). Under irrigated conditions (Figure 4) there is a much larger area with suitable soils, but where the rainfall is to a greater or lesser degree, insufficient.

As shown in Table 4, only around 22% of the province has moderate or high rainfed suitability and around 37% either has no suitability or falls within a national park. If rainfall is removed as a parameter (so that all moisture requirements need to be supplied by irrigation, the soil suitability situation is very different. In this scenario, around 30% of the province has some or other degree of high potential, with another 29% with moderate potential.

However, the scale of the Land Type Survey (1:250,000) means that the maps show areas of dominance only and that significant variation will exist within many of the map units. Such variation needs to be addressed by more detailed field investigations, as and when required.

Regarding degraded and/or eroded areas, land degradation is a challenging aspect to quantify. It is not easy to do from remote sensing (satellite) data since it takes many forms. Gullies (dongas) may be reasonably easy to delineate, but other aspects also occur, such as loss of topsoil, bush encroachment, soil acidification, and compaction. These problems usually need a detailed field investigation to even start to get reliable results.

The National Land Cover Database, which has gone through at least three versions since the first one in the 1990's, has had various methods to determine degraded land. Due to differences

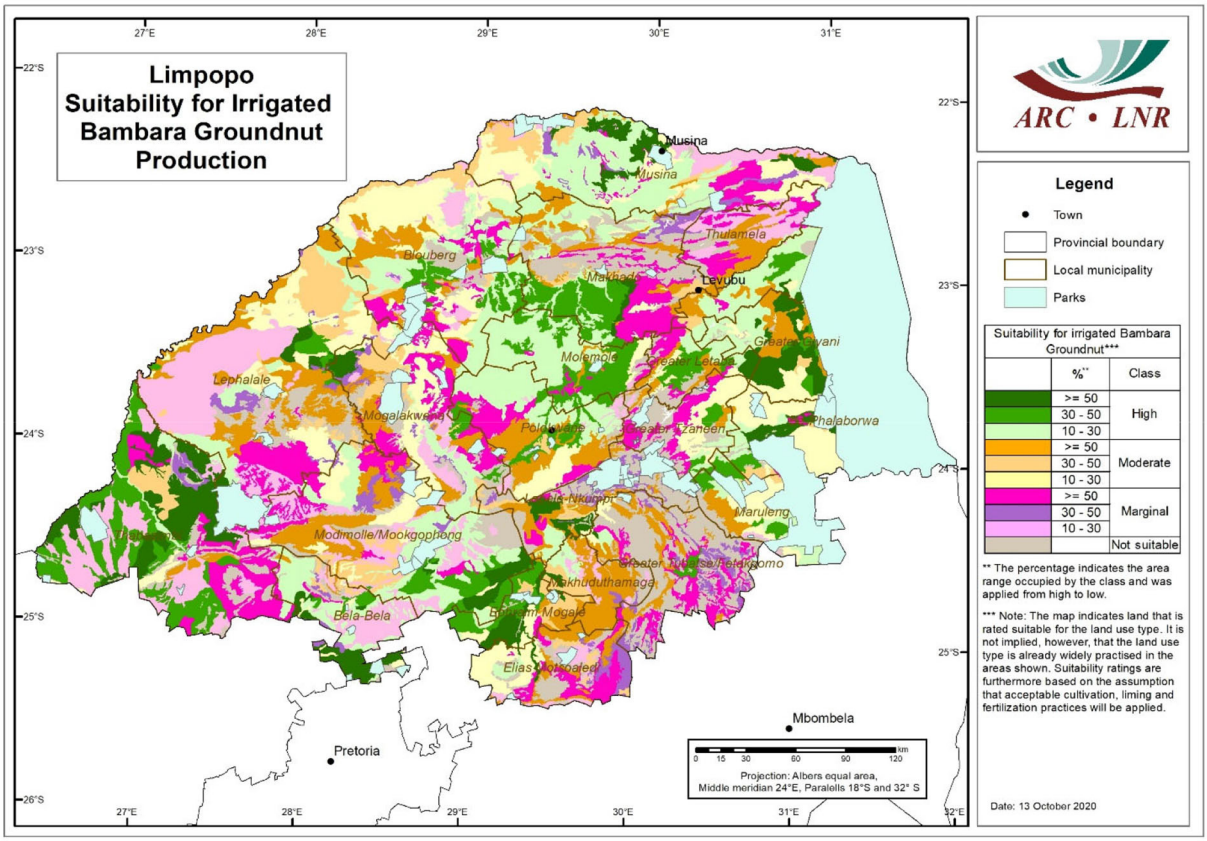

FIGURE 4
Suitability for irrigated Bambara groundnut production for Limpopo Province.

in methodologies between versions 1 and 2, there was a 36% decrease in degraded land in Limpopo (GTI, 2015). In a recent update of the dataset, the category "Degraded Land" has been removed, so there are no up-to-date figures. One source of information is a project carried out by ARC in conjunction with the Department of Agriculture, Forestry and Fisheries (Mararakanye and Le Roux, 2011) where every observable gully in South Africa was physically mapped. When the distribution of gullies in Limpopo (shown in black in Figure 5) is overlaid on the suitability map, it can be seen that there are significant areas in the province where this is a problem for cultivation as well as long-term environmental stability.

There is a need for further research in many areas to quantify the various forms of erosion and soil degradation that will reduce agricultural potential. In this way, some quantification of these areas might be obtained.

Delineating accurate suitability areas for the cultivation of crops is essential under climate change as it improves decisions on crop production and irrigation development to promote food and water security (Magidi et al., 2021b; Hagos et al., 2022). However, the main drawback in achieving accurate statistics on cropped area and the most suitable areas for specific crops has been the lack of reliable input data at reasonably high spatial resolution. There is more that still needs to be done on developing input datasets at acceptable spatial resolution as the current ones are too coarse to derive accurate statistics on spatial distribution and extent of irrigation (Magidi et al., 2021a). As the datasets used in this study may not be readily available in other regions, other crop suitability areas mapping approaches such as the multi-criteria decision method (MCDM) can be used and to achieve reliable results (Jahanshiri et al., 2020; Hagos et al., 2022).

Considerations for climate change adaptation

Our results showed that Limpopo province has some marginal or no unsuitable areas for Bambara groundnut production, mainly due to climatic restrictions. As the climate continues to change, suitable and moderately suitable areas may become marginally or unsuitable for Bambara groundnut production. The main risks associated with climate change

TABLE 4 Bambara groundnut soil and land suitability classes.

Suitability class	Land suitability (includes climate), ha	Class %	Soil suitability only, ha	Class %
High, >50%	-	-	737,366	5.86
High, 30–50%	15,577	0.12	1,024,573	8.15
High, 10–30%	8,221	0.07	2,015,410	16.03
Moderate, >50%	1,455,384	11.57	1,387,117	11.03
Moderate, 30–50%	360,545	2.87	636,509	5.06
Moderate, 10–30%	889,367	7.07	1,531,525	12.18
Marginal, >50%	3,725,025	29.62	1,169,549	9.30
Marginal, 30–50%	490,152	3.90	309,900	2.46
Marginal, 10–30%	1,133,159	9.01	1,182,883	9.41
Not suitable	3,363,982	26.75	1,446,580	10.79
Parks*	1,133,985	9.02	1,133,985	9.02
Total area	12,575,397	100.00	12,575,397	100.00

*Parks include National and Provincial Parks, as well as Conservation Areas and Nature Reserves.

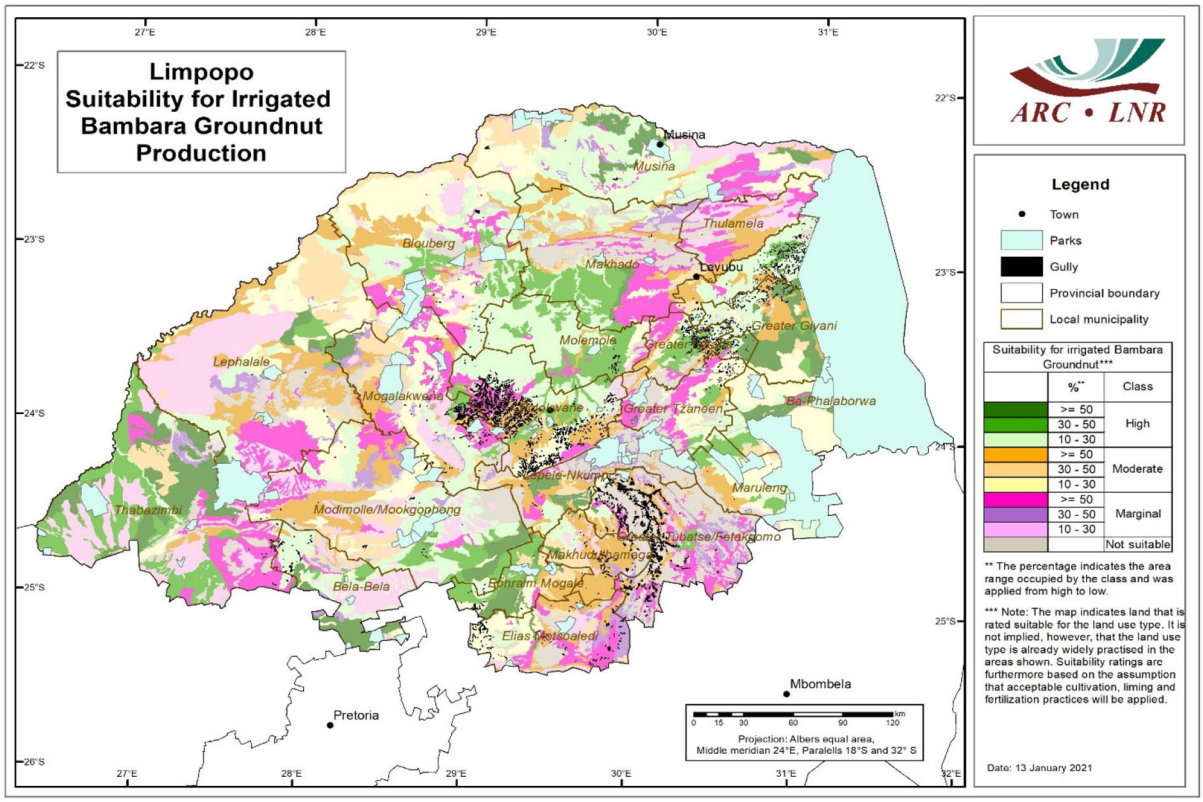

FIGURE 5
Incidence of gullies mapped in Limpopo Province.

include shifts in rainy season and increasing extreme weather conditions (heat stress, drought and floods) (DEEF, 2013) (Table 5). In the shorter term and regardless of land class, farmers, more so those under rainfed systems, are already vulnerable to current weather variability and associated shocks (Kruger and Nxumalo, 2017). Within the delineated production zones, a key element of climate adaptation is building resilience, and this entails redirecting and/or absorbing the disturbance without system collapse. To better represent adaptation, there is a need to expand the research to consider all factors, including social, economic and environmental domains. Also, more systems (agroecosystem, landscape, catchment) and

TABLE 5 Assessing the impacts of climate change for Bambara groundnut suitability [Adapted from Mpandeli et al. (2019) and Chimonyo et al. (2020)].

Observed/ possible climate change	Risk	Class	Mitigation strategy	Adaptation impact*
Shortened rain season (Onset, duration and cessation)	• Shifting agroecological zones. • Change in crop suitability due to the shortened growing duration • Low yields and/or crop failure	High, moderate and marginal	• Asynchronous intercropping with other drought-tolerant crop species • Staggering planting • Adopting rainwater harvesting and soil conservation techniques • Diversifying Bambara groundnut varieties • Increasing access to climate information and extension services	• Short • Short to medium term • Long term
Increase in the length and severity of midseason dry spells	• Intermittent water stress • Low yield due to a reduction in flower and pod set • Temperature stress • Increase incidence in pest and disease	Moderate and marginal	• Use of water conservation techniques such as mulching and minimum tillage to conserve soil water • Diversifying Bambara groundnut varieties • Adoption of integrated pest and disease management strategies • Adopting rainwater harvesting and soil conservation techniques • Deficit irrigation scheduling • Increasing access to climate information and extension services	• Short to medium term • Long term
Increase in day and night temperature from the norm	• Low yield due to a reduction in flower and pod set • Temperature stress • Increase incidence in pest and disease	High, moderate and marginal	• Ensuring good crop establishment (optimum management options to be employed) • Mulching to lower soil temperatures and conserve water by minimizing bare soil evaporation • Diversifying Bambara groundnut varieties • Relay or intercropping with taller heat stress-tolerant crops like sorghum and millet • Irrigating crops	• Short term • Short to medium term • Medium to long term
Increased frequency and intensity of drought	• Reduction in in-season rainfall • Reduced availability of freshwater resources for irrigation	High, moderate and marginal	• Diversifying Bambara groundnut varieties • Use of water conservation techniques such as mulching and minimum tillage to conserve soil water • Adopting rainwater harvesting and soil conservation techniques • Deficit irrigation scheduling • Increased access to drought early warming information, before and during the season • Consider the use of groundwater for irrigation purposes and water markets	• Short to medium term • Long term
Increased risk of floods	• Waterlogging in the field (especially in areas with shallow soils) • Soil erosion (on steep slopes) • Low yield and/or crop failure	High, moderate and marginal	• Ridging • Intercropping, hedgerows and agroforestry for increased water capture • Increasing field drainage through deep plowing • Contour tillage, cross slope plowing • Wetland restoration • Water retention through catchment storage schemes • Increased access to flood early warming information, before and during the season • Flood risk maps	• Short to medium term • Medium to long term

*Short term - adaptation strategies that allow farmers to cope better with current weather-induced risk; medium-term - adaptation strategies that would enable farmers to cope better with seasonal and/or annual weather risk; long term–adaptation strategies that allow farmers to adapt agriculture to future climate change.

place-based approaches representing local context, knowledge and aspects of food and nutrition security production other than land type, rainfall and temperature may be required (Beveridge et al., 2018).

By comparing the Bambara groundnut crop requirements with the availability of resources, an assessment can be made of the possible levels of investments made in the system. For instance, it would be most favorable to look at areas where water requirements for Bambara groundnut and season rainfall show small differences to invest in an irrigation system. Also, in areas with slopes prone to soil erosion, short- and long-term strategies such as contour plowing and agroforestry, respectively, may be adopted (Table 5). The adoption of multi-cropping coupled with asynchronous or sequential planting can be viewed as a low-cost, short- to medium-term option to improve productivity and resource use efficiency under increasing temperature and water scarcity (Chimonyo et al., 2020) (Table 5). The inclusion of other traditional crops known as drought and heat tolerant into Bambara groundnut cropping systems should also be considered a complementary strategy to increasing medium- and long-term climate resilience in identified marginal areas (Chimonyo et al., 2020).

Estimating the potential land resources suitable for irrigation and evaluating the possible impact of climate change on land suitability are essential for planning a sustainable agricultural system. However, South Africa is already challenged with water scarcity. Groundwater use and water marketing are considered options to alleviate medium- to long-term water scarcity challenges (Matchaya et al., 2019). Still, the extent to which they can bring relief to the stressed water resources is yet unknown (Mabhaudhi et al., 2018b). Tapping into groundwater also requires reliable energy resources, which brings to the fore the need for a water-energy nexus planning to enhance agricultural and water productivity (Magidi et al., 2021b).

Conclusions

By using available natural resource information and matching it to specific crop production requirements, the basic principles of "matching" used in the land evaluation process are followed. The example used here of Bambara groundnuts can be compared with other similar crops, in association with yield estimates and enterprise budgets, to empirically evaluate the suitability for a range of underutilized crops in any specific area. Further to this, and due to the minimum data set required, the method can be replicated across South Africa to assess underutilized crops' suitability. Growing Bambara groundnut with the appropriate management options can be used as an adaptation strategy in areas classified as moderately suitable and marginal. It is imperative to accompany the information regarding land suitability with transformative and autonomous adaptation strategies to mitigate climate risks. In cases where resources are limited, the adoption of multi-cropping and rainwater harvesting, and soil conservation techniques can be used. This is particularly important for resource-poor farmers who reside in many of the marginal areas identified. To smallholder farmers, underutilized crops can address several socio-economic related challenges; therefore, future studies should consider factors such as access to markets, proximity to roads and population density.

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/s.

Author contributions

Conceptualization and writing—original draft preparation: LN and TM. Methodology: LN, GP, MW, and TM. Software, formal analysis, and data curation: LN, GP, and MW. Validation: TM, AM, JM, SL, and VC. Resources and project administration: LN, SM, and TM. Visualization: JM, SL, GP, and MW. Supervision: LN and TM. Funding acquisition: AM and TM. Investigation and writing—review and editing: LN, GP, MW, MM, AM, RK, VC, TM, SM, SL, JM, and TM. All authors have read and agreed to the published version of the manuscript.

Funding

The Water Research Commission (WRC) of South Africa was acknowledged for initiating, funding and directing the study through WRC Project No. K5/2717//4 Developing guidelines for estimating green water use of indigenous crops in South Africa and estimating water use using available crop models for selected bio-climatic regions of South Africa. This research was funded, in part, by the Wellcome [Grant number: 205200/Z/16/Z]. For the purpose of Open Access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission.

Acknowledgments

The authors would like to thank Harold Weepener of the Agricultural Research Council for his role in developing high-resolution maps for the suitability of Bambara groundnuts.

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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frontiers | Frontiers in Sustainable Food Systems

TYPE Review
PUBLISHED 26 October 2022
DOI 10.3389/fsufs.2022.960159

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Climate-Smart Food Systems, a section of the journal Frontiers in Sustainable Food Systems

RECEIVED 02 June 2022
ACCEPTED 10 October 2022
PUBLISHED 26 October 2022

CITATION
Hlásná Cepková P, Dostalíková L, Viehmannová I, Jágr M and Janovská D (2022) Diversity of quinoa genetic resources for sustainable production: A survey on nutritive characteristics as influenced by environmental conditions. *Front. Sustain. Food Syst.* 6:960159. doi: 10.3389/fsufs.2022.960159

Diversity of quinoa genetic resources for sustainable production: A survey on nutritive characteristics as influenced by environmental conditions

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Environmental extremes and climatic variability have enhanced the changes in numerous plant stressors. Researchers have been working to improve "major" crops for several decades to make them more adaptable and tolerant to environmental stresses. However, neglected and underutilized crop species that have the potential to ensure food and nutritional security for the ever-growing global population have received little or no research attention. Quinoa is one of these crops. It is a pseudocereal, considered a rich and balanced food resource due to its protein content and protein quality, high mineral content, and health benefits. This review provides currently available information on the genetic resources of quinoa and their quality in terms of variability of economically important traits such as yield, and the content of bioactive compounds, such as protein and amino acid composition. The influence of variety and environmental conditions on selected traits is also discussed. The various types of nutrients present in the different varieties form the basis and are key for future breeding efforts and for efficient, healthy, and sustainable food production.

KEYWORDS

amino acids, genetic resources, nutritive value, protein, quinoa, environmental conditions

Introduction

Most staple foods comprise grain crops; therefore, feeding the ever-increasing global population means increasing the production of these crops (Bvenura and Kambizi, 2022). But it is well known that climate change is rapidly degrading the conditions of crop production. Salinization and aridity are forecasted to increase in most parts of the world (Choukr-Allah et al., 2016). Moreover, globally, the food crisis is mainly triggered by shocks such as drought and escalated by trade restrictions leading to price rises as an impact of the Covid-19 pandemic and as a consequence of the current war in Ukraine (Rahut et al., 2022).

As a consequence of this reality, new stress-tolerant or new alternative crops or species must be identified and used for future food security (Choukr-Allah et al., 2016). The present situation is that common wheat, rice, and maize as major crops seem to be near 80% of their potential. This shows the potential of many small-scale and marginal crops and wild plants that can be used as high-quality food sources. Since many of these species are well adapted to extreme environments, their role in the current scenario of climate change has become extremely important (Chrungoo and Chettry, 2021).

These crops have the potential to complement the major cereals and play a greater role in a safe household diet. A better understanding of these crops that feed the world and their potential role in nutrition will help secure their future and ensure food and nutrition security. *Chenopodium quinoa* Willd. was selected as one of the crops that will contribute to food security in the twentifirst century, because of its high resilience to extreme environmental conditions and its qualities as a functional food (Bvenura and Kambizi, 2022; Singh et al., 2022) and a potentially strategic crop that plays a vital role in food security and sovereignty (Rojas et al., 2015). In addition, quinoa has gained importance in international consumer markets in the last decade, which provides economic opportunities for Andean producers (Anaya et al., 2022). On the other hand, quinoa could be used for crop diversification in Europe and other parts of the world, outside of its genetic origin, as an alternative for marginal agricultural land (Jacobsen, 2017).

In the present work, we attempt to summarize the available information about quinoa genetic resources for the whole world by highlighting the situation in the Czech Republic. We also explore the results of current research focused on nutraceutical properties, including carbohydrates, lipids, proteins, amino acids, vitamins, and minerals. This overview provides an insight into the enormous variability of morpho-phenological traits and nutritive components which are possessed by quinoa germplasm cultivated in different global conditions and shows us how important it is to conserve and protect this richness.

Conservation of global quinoa genetic resources and history of research on quinoa in the Czech Republic

Quinoa plant genetic resources are essential for food and nutrition security and sovereignty of peoples, and they make a significant contribution to meeting the basic needs of humanity. They are part of ancestral and cultural heritage, especially for the countries of the Andean region. Their conservation and sustainable use are therefore the responsibility of society as a whole (Rojas et al., 2015). Quinoa is one of the underutilized crops with public breeding or evaluation programmes in South American countries such as Peru, Ecuador, and Bolivia (Galluzzi and Noriega, 2014). Quinoa seeds of different accessions are currently being conserved in several gene banks around the world (*ex situ* conservation). However, conservation of agrobiodiversity means conservation of the associated culture, that of indigenous farmers living in the Andean region (Bazile et al., 2016a; Jacobsen, 2017). Thus, although the importance of gene banks for biodiversity conservation is well known, the success of future conservation and breeding programmes depends on the transfer of knowledge and associated practices that can help to adapt quinoa to new regions (Ruiz et al., 2014).

Quinoa germplasm and its wild relatives are estimated at 16,422 accessions worldwide; it is held in 59 institutions (universities, gene banks, research, and agricultural institutions) in 30 countries around the world. 88% of accessions are conserved within the Andean region. The largest collections of quinoa and its wild relatives are held by institutions in Bolivia and Peru, with more than 6,000 accessions (Rojas et al., 2015). Compared to published data from many years ago about quinoa accessions conserved in gene banks (Jacobsen and Mujica, 2002), the collection, characterization, and evaluation of quinoa genetic resources have greatly improved in recent years.

According to available data, the genetic resources of quinoa conserved in collections outside the Andean region comprise a total of 2,137 accessions (Table 1). In the database, the biological status of 1,329 accessions is indicated as traditional cultivar/landrace, 552 accessions are listed as wild, while 1,007 accessions are shown as advanced/improved cultivar, and 100 accessions as others (Genesys, 2022). The provenance of accessions is mostly Peru, followed by the USA and Bolivia. In 1,329 accessions the type of germplasm storage is not identified, 543 genetic resources are kept in as long-term seed collection, 193 are conserved in seed collection, and 45 accessions in the short-term collection. In total, 478 accessions have safety duplication in Svalbard Global Seed Vault in Norway and 143 accessions in National Seed Storage Laboratory, USDA-ARS in the USA. Most of the accessions (1,306) are conserved in the International Center for Biosaline Agriculture in the United Arab Emirates. In Europe, the largest collection (528 accessions) is held by the Genebank of Leibniz Institute of Plant Genetics and Crop Plant Research in Germany (Eurisco, 2022).

In the Czech Republic, research on quinoa genetic resources began in 1999 with Dr. Anna Michalová, who obtained 22 quinoa genotypes from South America. Subsequently, a working collection of quinoa genotypes was established in the gene bank of the Crop Research Institute in Prague. The quinoa accessions were evaluated under field conditions for selected agro-morphological traits (days to flowering, days to harvest, 1,000-seed weight, etc.), and selected nutritional components in the seeds (crude protein content) were also analyzed in the laboratory. Evaluation of the quinoa working collection was stopped until 2016 when Dr. Dagmar Janovská and Dr. Petra Hlásná Cepková resumed work on quinoa

TABLE 1 Quinoa genetic resources in collections outside the South American region (Genesys, 2022).

Country	Holding Institute	Institute code	No. of accessions
United Arab Emirates	International Center for Biosaline Agriculture	ARE003	1,306
Germany	Genebank, Leibniz Institute of Plant Genetics and Crop Plant Research	DEU146	528
United States	North Central Regional Plant Introduction Station, USDA-ARS, NCRPIS	USA020	162
United Kingdom	Genetic Resources Unit, Institute of Biological, Environmental and Rural Sciences, Aberystwyth University	GBR016	23
Hungary	Centre for Plant Diversity	HUN003	19
Slovakia	NAFC-Research Institute of Plant Production	SVK001	14
Australia	Australian Grains Genebank, Agriculture Victoria	AUS165	13
Ethiopia	International Livestock Research Institute	ETH013	11
Slovenia	Crops and Seed Production Department, Agricultural Institute of Slovenia	SVN019	5
Australia	Australian Pastures Genebank	AUS167	4
Others			20
Total			**2,105**

genetic resources cultivated under the conditions of the Czech Republic. Currently, the working collection of quinoa includes 70 genotypes. They are being tested under field conditions using descriptors for quinoa and its wild relatives (Bioversity International et al., 2013) while analyses are being conducted in the laboratory to determine the nutritional quality of the seeds of each genotype. The promising material will be used for future breeding purposes.

Global production of quinoa

At present, quinoa is grown throughout North and South America, Europe, Asia, Africa, and Oceania (Hinojosa et al., 2021). Alongside South American countries, China, India, and some European countries cultivate quinoa (Bazile and Baudron, 2015; Mosyakin and Schwartau, 2015; Yang et al., 2019). However, the biggest world producers remain countries of the traditional region of quinoa cultivation: Peru, with the production of 100,115 t; Bolivia, with 70,170 t (Faostat, 2022); and Ecuador, with more than 4,500 t (Hinojosa et al., 2021), while the United States is the top importer (Bvenura and Kambizi, 2022). The global harvested area of quinoa almost doubled last decade from 95,979 ha in 2010 to 188,878 ha in 2020. Annual production in China was 20,000 t in 2018 and harvested area reached nearly 12,000 ha (Yang et al., 2019). Globally, the average yield slightly increased from 0.83 $t.ha^{-1}$ in 2010 to 0.93 $t.ha^{-1}$ in 2020 (Faostat, 2022). For example, in Ecuador, the obtained yield in the variety comprises 66% of the total quinoa area which is 1.30 $t.ha^{-1}$ (Hinojosa et al., 2021).

In the last decade, quinoa has evolved from being a neglected traditional food to an important export crop, promoted as a "superfood" throughout the Western world (Bazile and Baudron, 2015; Nuñez De Arco, 2015). Rising demand among Western consumers has created new economic opportunities for quinoa farmers in Bolivia's southern Altiplano. The negative aspect of the high interest in quinoa and the extreme increase in demand for quinoa seeds is that it has caused a spectacular increase in market price (Tschopp et al., 2018). However, this quinoa boom has brought environmental disaster in the traditional regions of quinoa cultivation in Bolivia (Jacobsen, 2011). Similarly, in Peru, the area under quinoa cultivation has been expanded by 264% and its cultivation has spread to all regions of Peru (Bedoya-Perales et al., 2018) which had a strong negative impact on the environment – soil degradation, pest, and diseases occurrence; likewise on socio-economic links and relations in local communities (Jacobsen, 2011). In the context of the above-mentioned facts, countries of the Andean region have tried to make a great effort to establish a harmonious interaction between socio-economic and environmental demands (Bedoya-Perales et al., 2018) and apply strategies for saving quinoa diversity, established breeding and research priorities, built more transparent commercial chain policy, and ensure more efficient cooperation with local farmers and cooperatives to decrease the negative impact of quinoa growth expansion (Ruiz et al., 2014; Bazile and Baudron, 2015; Bazile et al., 2016a; Bedoya-Perales et al., 2018; Hinojosa et al., 2021).

Quinoa's adaptability to a diverse environment

In different countries around the world, farmers and researchers have been trying to find, test and introduce nutritionally valuable seed crops that would be suitable for diverse growing conditions, achieve satisfactory yields, and

TABLE 2 Different quinoa genotypes performance in different environments.

Plant Material	Growing condition	Locality, country	Agro-morphological evaluation	Biochemical markers	Seed yields	References
468 genotypes	Drip irrigation	Dubai, the United Arab Emirates	11 morphological traits	400 seed metabolites	n.d.	Tabatabaei et al. (2022)
two cultivar ICBA-Q5, Titicata	Field, supplemental irrigation	Rehamna region, Morocco	Physiological and morphological traits in plants, yield, and its components	n. d.	0.08–0.84 t.ha^{-1}	Taaime et al. (2022)
nine novel quinoa genotypes and 1 commercial cultivar Regalona Baer	Field with full and reduced irrigation	Atacama Desert	Physiological and morphological traits in plants, thermal infrared and hyperspectral imaging	n. d.	2.45–3.24 t.ha^{-1}	Dumschott et al. (2022)
15 quinoa varieties and five breeding lines	Eastern lowland region and Highland region, exp. fields	the Eastern lowland and northern highlands, Rwanda	Emergency, Days to flowering, Days to maturity, Plant height, Grain yield	n. d.	Min: 0.14 t.ha^{-1} QuF9P1-20 Max: 3.00 t.ha^{-1} NL-6	Habiyaremye et al. (2022)
30 quinoa accessions	Greenhouse	Tunja, Columbia	12 qualitative and 9 qualitative traits	n. d.	n.d.	Manjarres-Hernandez et al. (2021)
13 quinoa commercial or selected varieties	Field experiments	North-West European, Melle, Belgium	Seed characteristics	Chemical composition of seeds	Min: 0.47 t.ha^{-1} Atlas, Pasto Max: 3.42 t.ha^{-1} Vikinga, Titicaca	De Bock et al. (2021b)
Cultivars Regalona, Puno, titicaca, Vikinga, Q3, Q5	Field experiments under irrigation	Zamadueñas, Spain	Seed weight, area, viability, color, and germination rate, grain yield	Saponin content, protein content, AA profile, mineral content, FRAP assay, TPC, TFC	Min: 0.70 t.ha^{-1} Vikinga Max: 3.25 t.ha^{-1} Q3 cultivar	Granado-Rodriguez et al. (2021a)
Jessie, Marisma, Roja, Duquesa, Pasto	Field experiments under irrigation	Southwestern Spain	Above-ground biomass, HI, seed yield, 1000-SW, nutrient uptake	Moisture, fat, total dietary fiber, protein, carbohydrate, mineral, and ash contents	Min: 1.58 t.ha^{-1} Roja Max: 3.04 t.ha^{-1} Marisma	Matías et al. (2021)
Regalona, AG 2010, Cauhil, Morado	Field experiments under 5 irrigation treatments	Diguillín Province, Ñuble Region, Chile	Seed yield, seed yield efficiency	Total protein content, globulin and albumin yield, and technical efficiency	Min: 0.41 t.ha^{-1} Morado Max: 3.35 t.ha^{-1} Cahuil	Pinto et al. (2021)
KVL-SRA2, Chipaya, Q-37	Field experiment	Cairo, Egypt	Plant growth performance, leaf pigment	Protein, ash, fat, dietary fiber, total carbohydrate content, total saponin, and tannin content, TPC, TFC	Min: 1.20 t.ha^{-1} KVL-SRAZ Max: 2.40 t.ha^{-1} Q-37	El-Serafy et al. (2021)

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TABLE 2 (Continued)

Plant Material	Growing condition	Locality, country	Agro-morphological evaluation	Biochemical markers	Seed yields	References
14 genotypes	Field experiments	Rabat, El Kbab, Meknes, Berrechid, Tinejdad	Germination rate, seed size and yield, plant height, stem diameter, dry matter, HI, 1000-SW, *Peronospora farinosa* sensitivity	n. d.	Min: 0.00 t.ha^{-1} Amarilla de Marangani Max: 7.83 t.ha^{-1} SW2	Thiam et al. (2021)
Q5 variety	Three levels of salinity, greenhause	Karakalpakstan, Uzbekistan	Plant height, shoot lengths, panicle weight, seed yield, 1,000-seed weight	Protein content, AA content, oil content, FA content, Element content	n.d.	Toderich et al. (2020)
six quinoa accessions	Field experiments	Northern Israel	Biomass and seed characterization	Chemical composition of seeds and biomass	Min: 1.54 t.ha^{-1} accession 5 Max: 6.36 t.ha^{-1} accession 4E	Asher et al. (2020)
six quinoa genotypes - Q18, Q21, Q22, Q29, AMES 13761, NSL 106398	Field experiments with three salinity treatments, drip irrigation	Dubai, United Arab Emirates	Various morphological traits of plants, seed yield, yield stability, HI	Protein content	Min: 1.27 t.ha^{-1} Q21 genotype Max: 2.30 t.ha^{-1} Q18 genotype	Hussain et al. (2020)
Q5 variety	The circular drainable lysimeters	Semi-arid area with a warm climate, Bahgar, Iran	Crop evapotranspiration, grain yield, biomass, water productivity	n. d.	n.d.	Ahmadi et al. (2019)
Different varieties	Field	24 provinces of China	Grain yield	Protein content	Min: 1.48 t.ha^{-1} Longli Max: 5.27 t.ha^{-1} Qingli-1	Yang et al. (2019)
Cultivar Regalona, Salcedo-INIA, Titicaca	Rainfed field experiments	El Pobo, Teruel, Spain; Arequipa, Peru; Río Hurtado, Chile	Grain yield, seed weight per plant, HI, plant height, Stem diameter, panicle length and diameter, plant weight, days to flowering, and maturity	Mineral composition, phytate content, protein content, AA content, FRAP assay, fiber, and saponin content	Min: 1.53 t.ha^{-1} Titicaca Max: 5.17 t.ha^{-1} Salcedo	Reguera et al. (2018)
Jessie, Titicaca, Puno, Zeno	Field experiments	Southwestern Germany	Soil mineral content, grain yield, 1000-SW,	Total protein, lipid content, FA and AA profile, saponin content	1.73–2.43 t.ha^{-1}	Prager et al. (2018)
Commercial genotype Regalona and one quinoa accessions	Three thermal treatments (increased night temperatures), exp. fields	Valvidia, Chile	Physiological and morphological traits of biomass, grain yields, chlorophyll content, water-soluble carbohydrates, grain protein content	n. d.	Min: 2.93 t.ha^{-1} Accession Max: 6.00 t.ha^{-1} Regalona	Lesjak and Calderini (2017)

(Continued)

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TABLE 2 (Continued)

Plant Material	Growing condition	Locality, country	Agro-morphological evaluation	Biochemical markers	Seed yields	References
$F_{2:5}$ population	Field experiments	Coastal environment, Rabat, Morocco	16 qualitative and seven qualitative traits	n. d.	n. d.	Benlhabib et al. (2016)
10 landraces, eight varieties under development,three registered varieties	2–5 sites in nine countries	North Africa, the Near East, Asia	19 morphological and phenological traits	n.d.	Min: 0.20 t.ha^{-1} Sajama Iranshar Max: 2.05 t.ha^{-1} Titicaca	Bazile et al. (2016b)
10 quinoa cultivars	Field experiments under irrigation	Encalilla, North Western Argentina; Altiplano, Bolivia	Root, aerial, and seed biomass, plant height, grain yield	AA composition, total protein content	Min: 0.38 t.ha^{-1} Samaranti Max: 3.86 t.ha^{-1} Sayana	Gonzalez et al. (2012)
Titicaca	Field experiment under three salinity treatments and three irrigation regimes	Southern Italy	Seed yield, climatic conditions, soil water content, electrical conductivity	Saponin content, carbohydrate, protein, oil, ash, and dietary fiber content	Max: 2.3 t.ha^{-1} Min: 2.7 t.ha^{-1}	Pulvento et al. (2012)

HI, harvest index; TPC, total phenolic content; TFC, total flavonoid content; 1000-SW, weight of thousand seeds; n. d., not defined.

offer versatile applications in food production and consumption (Gardner et al., 2019; Toderich et al., 2020; Habiyaremye et al., 2022).

To fully exploit the potential of the crop for marginal environments, identification of new and high-yielding quinoa genotypes with good local adaptation and high nutritional quality are crucial, which requires intensified screening and adaptation research (Choukr-Allah et al., 2016).

Recently, the performance of different quinoa genotypes in different global environments with an emphasis on their adaptability and seed nutritional quality has been studied in many countries and regions (Table 2). The considerable variability in yield for different quinoa genotypes in the different environments was confirmed outside of the Andean region. The lower yields were observed at 0.08 t.ha^{-1} in Morocco (Taaime et al., 2022) and the highest at 7.86 t.ha^{-1} (Thiam et al., 2021) also in Morocco. The range of yield in experimental fields of the Czech Republic in 2018–2021 was estimated between 0.12 and 3.99 t.ha^{-1} (unpublished data). Observed yield levels in Northern Europe were 1–3 t.ha^{-1} (Pulvento et al., 2012; Jacobsen, 2017; Prager et al., 2018; De Bock et al., 2021b; Granado-Rodriguez et al., 2021b; Matías et al., 2021). However, quinoa yields over the years have remained unpredictable and very low, averaging between 1.2 and 1.4 t.ha^{-1} while the maximum attainable yield can be up to 8–10 t.ha^{-1} in Morocco. A range of factors was suggested as affecting production, such as the choice of cultivars, optimal sowing date, and nutrient management was suggested affecting the production (Choukr-Allah et al., 2016). In the same way, high salinity can reduce the yield significantly (Hussain et al., 2020). Grain yield was more influenced by the environment and the genotype-environment interactions. The results of (Thiam et al., 2021) confirmed the significance and challenge of evaluating the varietal grain yield stability across contrasting environments.

The marginal effect of salt stress on nutritional composition was presented by (Choukr-Allah et al., 2016), whereas (Hussain et al., 2020) reported a significant impact of salt stress on grain protein contents dependent on genotype. However, the salinity common in these regions promotes growth but up to a certain threshold, beyond which growth and productivity start to be negatively affected (Choukr-Allah et al., 2016). In testing 20 quinoa genotypes in two different environments in Rwanda, it was confirmed that low water availability affected the growth and yield of quinoa and there is a need to identify the best genotypes adapted to specific agro-ecological zones and even growing seasons (Habiyaremye et al., 2022).

Rising temperatures are challenge for quinoa as well as for other crops. High temperatures during flowering and heat stress during the vegetative stage in certain quinoa varieties considerably lowered yield and changed protein and fiber content (Matías et al., 2021). In the growing conditions of Chile, the influence of increased night temperature on quinoa plants

was evaluated (Lesjak and Calderini, 2017). Grain yields were reduced in the range of 12–31% by increased night temperatures. Similarly, the aboveground biomass was affected negatively in contrast with values for harvest index, individual grain weight, grain protein content, and water-soluble carbohydrates, which have changed only slightly.

In Chile, the local landrace genotype Cahuil had the best performance regarding seed yield under water stress (Pinto et al., 2021). Further, the genotype Titicaca (originating from the Andes) showed a good adaptation to the Mediterranean environment with tolerance to salinity and drought (Pulvento et al., 2012). On the other side, in some regions of southeast China, the combination of the high temperatures and heavy rainfalls had negative effects on the growth of quinoa. Fortunately, quinoa germplasm collected from Taiwan showed resistance to high temperatures and heavy rainfalls (Yang et al., 2019). In quinoa growing in the conditions of Morocco, optimal temperatures (10–25°C), high and well-distributed precipitation, and short photoperiods contributed to better growth and the highest yield (Taaime et al., 2022). The susceptibility of quinoa to temperatures above 32°C was confirmed due to the flower closing during the day and limited pollination caused a reduction of the yield by up to 86% (Tovar et al., 2020).

The high degree of variability in the performance of nutritional profiles of quinoa seeds under various salinity stress was assessed while the nutritional value of seeds remained unchanged, especially the high protein content, all essential amino acids, high mineral content, and flavonoids (Pulvento et al., 2012; Toderich et al., 2020). On the other hand, high temperatures increased protein and fiber content (Matías et al., 2021).

However, the establishment of this crop in many agronomical areas outside South America is still limited. It could be considered that the quinoa cultivar selection process remains unfinished for new cultivation areas, including those located in southern Europe which are characterized by having intense precipitations at early growth stages and high temperatures at later stages of crop development (Granado-Rodriguez et al., 2021b). There is still very limited information regarding the stability of seed nutritional characteristics under changing environments (Granado-Rodriguez et al., 2021b).

As with any other new crop, one of the key factors for the successful introduction and establishment of quinoa under new climatic conditions will be the identification of appropriate planting material. Therefore, it is important to study the adaptation and yield of several potential quinoa genotypes from different provenances to select the most promising ones suitable for the local agro-climatic conditions (Choukr-Allah et al., 2016). Not only should adaptation of quinoa be discussed, but also sustainable establishment in a new environment.

Nutritional characteristics of quinoa seeds and plants

Quinoa has outstanding nutritional value in all its edible parts – seeds and leaves, which were recognized even by ancient populations that considered quinoa a sacred food (Jacobsen et al., 2003). Quinoa seeds are a superior source of vitamins, minerals, dietary fiber, and lipids with the presence of health-beneficial polyunsaturated fatty acids (Repo-Carrasco et al., 2003). As reported by Schlick and Bubenheim (1996), quinoa is one of the single food sources that can supply all essential macro and micronutrients required for balanced human nutrition.

Carbohydrates, starch, and total dietary fiber

Quinoa seeds contained a relatively high amount of carbohydrates, with the content ranging from about 42% reported in the variety "Roja" up to 83% found in accessions cultivated in Peru (Encina-Zelada et al., 2017). As summarized in Table 3, there are significant differences in carbohydrate content in various genotypes. For example, Miranda et al. (2012) detected higher carbohydrate content in Chilean highland ecotypes as opposed to southern ecotypes. Pereira et al. (2019) reported slightly higher mean carbohydrate content in black and white varieties but lower in red varieties. In spite of that, many other variables modify total carbohydrate content, such as environmental conditions and sowing date. For example, in sea level genotypes and one cross genotype cultivated in Argentina, winter sowing at 18°C resulted in expanded seed weight, and therefore higher carbohydrate content in seeds (Curti et al., 2018). On the other hand, high carbohydrate content negatively affects total protein content (Craine and Murphy, 2020; De Bock et al., 2021a,b).

In terms of environmental influence, increased carbohydrate content was reported for lowland/coastal quinoa genotypes "Regalona Baer" and "Villarrica" in arid conditions with lower soil organic matter content and a mean temperature of approximately 18°C during the growing season (Miranda et al., 2013). Experiments conducted with genotypes cultivated in Spain resulted in decreased carbohydrate content in a growing season with a mean temperature of approximately 25°C, in contrast to a growing season with a mean temperature lowered by 5°C (Matías et al., 2021). This was also supported by Garcia-Parra et al. (2022), indicating the highest carbohydrate content (65.5%) in cultivars grown in a cold climate.

The most prevailing component of quinoa carbohydrates is starch, situated primarily in the perisperm, in contrast to the cereals (Burrieza et al., 2014). The minimal value for starch content was 44%, found in genotype "Cica" (Jimenez et al.,

TABLE 3 Variability of the carbohydrate content in quinoa seeds divided according to the genotype name and seed color.

	Sample Genotype	Seed color	Production area	Carbohydrate content	References
Genotype name	Highland ecotypes: Ancovinto, Cancosa Central ecotypes: Cahuil, Faro Southern ecotypes: Regalona, Villarrica	n. d.	Chile	Min: 56.54 [1] Villarrica Max: 68.12 [1] Ancovinto	Miranda et al. (2012)
	$n = 78$ accessions	n. d.	Bolivia Brazil Peru	Min: 43.64 [1] Max: 76.37 [1]	Ferreira et al. (2015)
	$n = 77$ accessions	Beige Black Orange Yellow	Peru	Min: 78.48 [1] Max: 82.89 [1]	Encina-Zelada et al. (2017)
	Real	n. d.	Colombia	68.30 [1]	Contreras-Jimenez et al. (2019)
	Cica Kamiri Inga Pirca		Argentina	Min: 72.81 [2] Inga Pirca Max: 74.74 [2] Kamiri	Contreras-Jimenez et al. (2019)
	F5:F6 advanced breeding lines Cherry Vanilla CO407 Dave Kaslaea	n. d.	USA	Min: 69.56 [2] Max: 74.00 [2]	Craine and Murphy (2020)
	Atlas Jessie Marisma Pasto Pot_4 Roja	n. d.	Spain	Min: 41.52 [3] Roja Max: 52.62 [3] Pasto	Gomez et al. (2021)
	Blanca real Nariño Pasankalla Soracá Puno Titicaca	n. d.	Colombia	Min: 56.00 [1] Puno Max: 70.66 [1] Pasankalla	Garcia-Parra et al. (2022)
	Iniap Tunkahuan	n. d.	Ecuador	60.37 [1]	Villacres et al. (2022)
Seed color	Commercial – unknown ($n = 29$) Blanca Kancolla Blanca Hualhuas Negra Collana Negra Pasankalla Pasankalla Roja Pasankalla Rosada de Huancayo Salcedo INIA	Black Red White	Peru Spain	Min: 75.3 [2] Red quinoa Max: 77.0 [2] White quinoa	Pereira et al. (2019)

[1]The results are expressed as %. [2]The results are expressed as g.100 g^{-1} of dry weight. [3]The results are expressed as g.100 g^{-1} of fresh weight. Max, maximum value; Min, minimum value; n. d., not defined.

2019) cultivated in Argentina, whereas the most abundant starch content of 72.5% was described by (De Bock et al., 2021b) in genotype "Titicaca" grown under North-West European field conditions. Nonetheless, the values for total carbohydrate content in this study varied between different years of field experiments. Similarly, (Grimberg et al., 2022) characterized the genotype "Titicaca" as one with the most prominent starch content. (Aluwi et al., 2017) evaluated maximal starch content

in genotype "CO 407D" [64% in dry weight (dw)] and the lowest for "UDEC-1" (55%), both cultivated in the USA.

Quinoa starch is rich in polysaccharide amylopectin, which represents 54–85% of dw (Dong et al., 2021; Kheto et al., 2022). Amylose content is, on the other hand, relatively low. It ranges from approximately 6% in "Tianjing Tibet Quinoa" (Li and Zhu, 2017) up to 20% in the Argentinian variety "Jujuy" (Nascimento et al., 2014). Specific starch and amylopectin structure give quinoa starch various functional properties that can be used in a wide range of food products (Li et al., 2016; Aluwi et al., 2017; Li and Zhu, 2017). Nevertheless, climatic conditions during the growing season may alter final functionality, even though starch biosynthesis is determined primarily by genetics (Garcia-Parra et al., 2021, 2022). Additionally, seed color seems to correlate with starch physiochemical properties, as reported by Peng et al. (2022), in opposition to Li et al. (2016), describing no correlation between the seed color and starch characteristics.

Total dietary fiber (TDF) content in quinoa is also highly heterogenous, ranging from approximately 7% (De Bock et al., 2021a) up to 23% (Granado-Rodriguez et al., 2021b). The variation can be explained by the genotype effect (Curti et al., 2018), but also by growing conditions since fiber content can be enhanced under saline conditions (Pulvento et al., 2012) and high temperatures during the grain filling period (Matías et al., 2021). Negative correlations were found between TDF, carbohydrate, and fat content (Vidueiros et al., 2015). Overall, high amounts of TDF (over 18% TDF) were found in genotypes "Rainbow", "Faro", "Baer", "Colorado 407D" cultivated in Poland (Sobota et al., 2020), "Titicaca" grown in Italy (Pulvento et al., 2012), and "Roja" and "Duquesa" grown in Spain (Matías et al., 2021). Less prominent amounts (below 14% TDF) were presented in "Faro Red", "Puno" (Sobota et al., 2020), "Pasto" (Matías et al., 2021), white Bolivian and Peruvian quinoas (Pellegrini et al., 2018), "Cica", "Kamiri" and "Inga Pirca" (Jimenez et al., 2019).

Protein content and amino acid composition

Quinoa seeds are often considered high in protein; yet overall protein content is quite variable (Table 4) and sometimes comparable to or higher than in most cereals such as wheat (12%), oat (13%), rice (7%), and corn (6%) (USDA, 2020). Variations in protein content were significant in several genotypes cultivated in distinctive agro-ecological conditions. For example, the cultivar "Jessie" originating in France was cultivated in Belgium and reached almost 19% protein content (De Bock et al., 2021b), whereas the same genotype cultivated in Germany reached a protein content of approximately 12% (Prager et al., 2018). Nevertheless, "Jessie" cultivated for two years in southwest Spain showed a steady mean protein content of 16.7% (Matías et al., 2021).

The Danish-bred cultivar "Titicaca" was analyzed in at least 10 studies under distinctive environmental conditions. Despite that, this genotype reached analogous values (13–15%) in the cultivation conditions of Ethiopia (Agza et al., 2018), Morocco (Mhada et al., 2020), Belgium (De Bock et al., 2021b), USA (Aluwi et al., 2017), and Germany (Prager et al., 2018). Besides this, slightly higher protein content (above 15%) was observed under cultivation in Poland (Sobota et al., 2020) and Colombia (Garcia-Parra et al., 2022). In addition, Reguera et al. (2018) reported higher protein content for "Titicaca" cultivated in Chile compared to Spain, which follows the results of Granado-Rodriguez et al. (2021a), reaching comparable values in mean protein content averaged for three cultivation years.

Genotype "Regalona", originating in southern regions of Chile, was described in at least eight studies. The values for protein content were quite inconsistent. Miranda et al. (2012), Graf et al. (2016), and Granado-Rodriguez et al. (2021a) detected protein content reaching approximately 13–15% for "Regalona" cultivated in Chile and Spain, whereas other authors achieved higher values of approximately 17% under field experiments in Chile and Egypt (Lesjak and Calderini, 2017; Reguera et al., 2018; Saad-Allah and Youssef, 2018). Even higher values were achieved by Gargiulo et al. (2019) (18.30%); however, the authors did not define the cultivation location.

The protein content of the Danish cultivar "Puno" was described in at least seven studies. The majority of the results were quite consistent in diverse environments (USA, Germany, Poland, Belgium, Colombia), ranging between 13 and 15% (Aluwi et al., 2017; Sobota et al., 2020; De Bock et al., 2021b; Garcia-Parra et al., 2022). On the other hand, (Garcia-Parra et al., 2021) evaluated slightly reduced protein content, reaching almost 12% in "Puno" cultivated in Colombia.

Although the Peruvian genotype "Pasankalla" was tested in at least 4 studies, the referred values of protein content are quite distant. Apaza et al. (2015) and Gargiulo et al. (2019) discovered protein content of 18.73–20.60%, while Garcia-Parra et al. (2021) and Garcia-Parra et al. (2022) achieved lower values (14.5–15.5%, respectively) during experiments conducted in Colombia. Genotype "Cahuil" originating in central Chile was investigated in a total of three studies. Miranda et al. (2012) reported protein content of 11.13%, whereas Graf et al. (2016) presented a lower concentration of nearly 9%. Aluwi et al. (2017) recognized a much higher protein content of 14.4% under cultivation in the USA.

Nonetheless, there are many factors affecting the resulting protein content. Besides the influence of genotype, the importance of soil matric potential (SMP) and nitrogen fertilization was indicated (Wang et al., 2020). High SMP values (over −55 kPa) cause significant water stress and may also limit nitrogen uptake, which concurs with other studies (Sun et al., 2014; Walters et al., 2016). Therefore, to reach optimal protein content, irrigation is crucial for some genotypes cultivated in adverse soil-water conditions, although slight water stress

TABLE 4 Variability of protein content in quinoa seeds divided according to the genotype and seed color.

	Genotype name	Seed color	Production area	Protein content	Reference
Genotype	Highland ecotypes: Ancovinto, Cancosa Central ecotypes: Cahuil, Faro Southern ecotypes: Regalona, Villarrica	n. d.	Chile	Min: 11.13 [1] Cahuil Max: 16.18 [1] Villarrica	Miranda et al. (2012)
	Breeding line AG2010 B080 Regalona	n. d.	Chile	Min: 17.40 [2] Max: 18.90 [2]	Escuredo et al. (2014)
	Jujuy Salta	n. d.	Portugal	Min: 12.20 [5] Jujuy Max: 16.30 [5]Salta	Mota et al. (2016)
	$n = 12$ accessions	Cream Gray Orange Yellow	Peru	Min: 13.58 [1] Quillahuaman INIA, cream Max: 17.83 [1] Pasankalla, gray	Apaza et al. (2015)
	$n = 9$ commercial varieties Ancovinto Blanco Ancovinto Roja Cancosa Socaire Cáhuil Faro Regalona Villarrica	Black Red White	Bolivia Chile Ecuador USA	Min: 7.47 [2] Kalustyan's Black, Peru Max: 15.73 [2] Wegman's Red, Bolivia/Peru	Graf et al. (2016)
	$n = 28$ accessions	n. d.	USA	Min: 13.00 [1] CO 407D WMF Max: 15.8 [1] QuF9P39-64	Aluwi et al. (2017)
	$n = 77$ accessions	Beige Black Orange Yellow	Peru	Min: 8.33 [1] Max: 11.38 [1]	Encina-Zelada et al. (2017)
	Kvl-sra2 Kvl-sra3 Regalona Q37 Q52	n. d.	Egypt	Min: 12.03 [2] Kvl-sra3 Max: 19.03 [2] Kvl-sra2	Saad-Allah and Youssef (2018)
	Titicaca	n. d.	Ethiopia	13.57 [2]	Agza et al. (2018)
	Jessie Puno Titicaca Zeno	n. d.	Germany	Min: 16.10 [1] Zeno Max: ≈ 12 [1] Jessie	Prager et al. (2018)
	Regalona Salcedo-INIA Titicaca	n. d.	Chile Peru Spain	Min: ≈ 14 [1] Salcedo, Peru Max: ≈ 17 [1] Regalona, Chile	Reguera et al. (2018)
	Altiplano Pasankalla Regalona Titicaca	n. d.	n. d.	Min: 15.40 [1] Titicaca Max: 20.80 [1] Altiplano	Gargiulo et al. (2019)
	$n = 25$ accessions	n. d.	Poland	Min: 12.40 [2] Q629, USA Max: 15.98 [2] Faro, Argentina	Sobota et al. (2020)

(Continued)

TABLE 4 (Continued)

Genotype name	Seed color	Production area	Protein content	Reference
F5:F6 advanced breeding lines Cherry Vanilla CO407 Dave Kaslaea	n. d.	USA	Min: 10.04 [3] Max: 13.68 [3]	Craine and Murphy (2020)
Puno Titicaca	n. d.	Morocco	Min: 13.41 [3] Puno Max: 13.43 [3] Titicaca	Mhada et al. (2020)
Q5	n. d.	Uzbekistan	14.40 [3]	Toderich et al. (2020)
$n = 13$ accessions	Dark White	Belgium	Min: 12.10 [2,a] Oro de Valle Max: 18.80 [2,a] Jessie	De Bock et al. (2021b)
IC341709 IC329184 IC507733 IC107299 NIC22513 NIC22506	n. d.	India	Min: 14.10 [1] IC341709, IC507733 Max: 15.40 [1] IC329184, NIC22506	Ghumman et al. (2021)
Puno Q3 Q3 Regalona Titicaca Vikinga	n. d.	Spain	Min: 13.80 [1] Max: 19.10 [1]	Granado-Rodriguez et al. (2021a)
$n = 14$ accessions	Dark White	Spain	Min: ≈ 9 [1] A-SE-06, white Max: ≈ 16.50 [1] A-SE-15, dark	Granado-Rodriguez et al. (2021b)
Gannan Geermu Haili	n. d.	China	Min: 11.60 [1] Geermu Max: 12.60 [1] Haili	Jiang et al. (2021)
Duquesa Jessie Marisma Pasto Roja	n. d.	Spain	Min: 13.20 [1] Roja Max: 20.40 [1] Duquesa	Matías et al. (2021)
Atlas Jessie Marisma Pasto Pot_4 Roja	n. d.	Spain	Min: 15.59 [4] Pasto Max: 18.73 [4] Atlas	Gomez et al. (2021)
Blanca Real Nariño Pasankalla Soracá Puno Titicaca	n. d.	Colombia	Min: 12.36 [1] Soracá Max: 16.56 [1] Titicaca	Garcia-Parra et al. (2021)

(Continued)

TABLE 4 (Continued)

	Genotype name	Seed color	Production area	Protein content	Reference
Seed color	Bolivian quinoa (BQ)	Black	Bolivia	Min: 11.62 [4] SQ, white	Pellegrini et al. (2018)
	Peruvian quinoa (PQ)	Red	Peru	Max: 13.66 [4] BQ, white	
	Spanish quinoa (SQ)	White	Spain		
	Commercial – unknown (n=29)	Black	Peru	Min: 14.4 [2] White quinoa	Pereira et al. (2019)
	Blanca Kancolla	Red	Spain	Max: 15.6 [2] Red quinoa	
	Blanca Hualhuas	White			
	Negra Collana				
	Negra Pasankalla				
	Pasankalla Roja				
	Pasankalla				
	Rosada de Huancayo				
	Salcedo INIA				
	n. d.	Black	Peru	Min: 16.20 [1] Black quinoa	Sanchez-Resendiz et al. (2019)
		Yellow		Max: 18.70 [1] Yellow quinoa	

[1]The results are expressed as %. [2]The results are expressed as g.100 g^{-1} of dry weight. [3]The results are expressed as g.100 g^{-1} sample. [4]The results are expressed as g.100 g^{-1} of fresh weight. [5]The results are expressed as g.100 g^{-1} of the edible portion on a fresh weight basis. [a]The protein content per variety averaged over the different years of field trials. Max, maximum value; Min, minimum value; n. d., not defined.

may enhance protein content (Wang et al., 2020). The intense application of nitrogen from 80 to 240 kg/ha increased protein content by approximately 1.5%. The positive effect of nitrogen fertilization was also presented by Wu et al. (2016) and Jacobsen and Christiansen (2016).

In addition, protein content in quinoa rises under salinity treatment, which was reported for varieties "CO407D", "UDEC-1", "Baer", "QQ065" (Wu et al., 2016), and "NSL106398" (Hussain et al., 2020). In contrast, Ruiz et al. (2016) expressed a drop in protein content by 7–12% in coastal lowland Chilean landraces ("VI-1", "Villarrica") and genotype "R49" (salares ecotype). In terms of temperature influence, protein content under heat stress was outstanding in varieties "Pasto", "Marisma", "Jessie", "Roja", and "Duquesa" (Matías et al., 2021). Garcia-Parra et al. (2022) detected slightly higher mean protein values for cultivation in the cold climate of Colombia, compared to temperate and warm conditions; but, as reported by the authors, protein content was not rapidly affected by elevated temperatures. The exception in this paper was the cultivar "Pasankalla", showing a decline in protein content in hotter conditions.

Those results suggest the great potential of the selected quinoa genotypes for cultivation in adverse environments. A high correlation was detected between embryo weight ratio and protein content since proteins are mostly stored in the embryo (Gargiulo et al., 2019). Protein content negatively correlates with panicle height and panicle biomass, whereas positive correlations were determined for total phenolic content, antioxidant activity, and saponin content (Granado-Rodriguez et al., 2021b).

Probably even more important than overall protein content is the quality of protein, given by the composition of essential amino acids (EAA). Quinoa protein generally contains all EAAs and several authors throughout the literature have concluded that quinoa protein is complete due to the superior composition of amino acids (AA; Nowak et al., 2016; Maradini et al., 2017; Schmidt et al., 2021). Nonetheless, Craine and Murphy (2020) argue that many of those studies evaluated outdated daily requirements or considered AA requirement values only for adults, not for children, whose requirements for EAAs are greater. The authors further stated that the quinoa protein is only "nearly complete". Regarding this statement, Boye et al. (2012) labeled valine and lysine as limiting AA for children up to the age of 10 years. In comparison, Gonzalez et al. (2012) suggested lysine, tyrosine, and tryptophan as limiting AA for the age group of 2–5 years. Craine and Murphy (2020) identified low leucine content, which does not achieve the recommended daily requirements for infants and children, therefore considering it as limiting AA.

As expressed in Table 5, the content of each EAA shifted between authors. The most abundant EAA was leucine with the highest content in the variety "Atlas" (Gomez et al., 2021), whereas the least represented EAA was tryptophan, with the content reaching 0.58–1.9 in g.100 g^{-1} protein in genotypes "Chucapaca" and "Bastille", respectively (Escuredo et al., 2014; De Bock et al., 2021b). With regards to

TABLE 5 Minimum and maximum values of amino acid composition (g.100 g $^{-1}$ protein) in various quinoa genotypes and production areas.

	Miranda et al. (2012)	Gonzalez et al. (2012) [a]	Escuredo et al. (2014)[a]	Prager et al. (2018)	Wang et al. (2020)	De Bock et al. (2021b)[a]	Gomez et al. (2021)
N. of accessions	6	10	3	4	6	12	6
Production area	Chile	Bolivia NW Argentina (A) Encalilla, Argentina (E)	Chile	Germany	China	Belgium	Spain
Growing seasons	2011	2007–2009	2010–2011	2015–2016	n. d.	2017–2019	2017
Histidine	2.70 Ancovinto, Cahuil 3.50 Villarrica	1.36 Sajama (E) 3.79 CICA (A)	1.71 Regalona 2.17 AG2010	1.33 Zeno (2015) 2.48 Puno (2016)	3.16 QWQ 3.70 QBQ	2.50 Bastille 3.20 Zwarte	3.67 Atlas 8.31 Roja
Isoleucine	2.90 Cahuil 3.80 Ancovinto	1.65 Chucapaca (E) 3.40 CICA (A)	0.75 Regalona 0.82 AG2010, B080	2.00 Zeno (2015) 3.19 Puno (2016)	2.80 QWQ 3.58 QBQ	3.90 Zwarte 4.80 Rouge Marie	3.75 Pot_4 4.61 Roja
Leucine	6.40 Cahuil 7.20 Villarrica	3.75 Sajama (E) 7.46 Ratuqui (E)	2.27 B080 2.52 Regalona	3.67 Zeno (2015) 5.55 Puno (2016)	5.07 QGQ 6.5 QBQ	7.00 Pasto 7.60 Atlas, Jessie	4.55 Pot_4 5.67 Pasto
Lysine	4.10 Cancosa, Cahuil 4.80 Villarrica	2.44 Sajama (E) 6.72 CICA (A)	2.35 AG2010 2.42 B080	2.77 Zeno (2015) 4.99 Puno (2016)	5.07 QWQ 6.02 SWQ	4.60 Rouge Marie 5.90 Pasto	5.40 Atlas 13.55 Jessie
Methionine	1.40 Ancovinto 1.90 Villarrica	0.73 Sajama (E) 1.87 CICA (A)	0.31 AG2010 0.69 Regalona	1.10 Zeno (2015) 1.80 Jessie, Puno (2016)	1.67 [b] QGQ 2.09 [b] SGQ, QBQ	2.00 Atlas 2.60 Puno	1.37 Pasto 1.64 Atlas
Phenylalanine	3.90 Cancosa, Cahuil 4.50 Villarrica	2.26 Sajama (E) 4.55 CICA (A)	1.49 B080 1.54 AG2010	2.20 Zeno (2015) 3.55 Puno (2016)	2.62 [c] QGQ 3.70 [c] SWQ	3.60 Zwarte 4.50 Atlas	3.73 Atlas 4.81 Roja
Threonine	3.20 Cancosa 3.60 Faro	2.09 Sajama (E) 4.59 CICA (A)	5.53 B080 8.89 Regalona	2.13 Zeno (2015) 3.27 Puno (2015)	1.79 QGQ 2.15 SWQ	3.60 Atlas, Bastille, Rouge Marie 4.40 Zwarte	3.43 Atlas 7.82 Jessie
Tryptophan	n. d.	0.58 Chucapaca 1.05 Sajama	0.99 B080 1.07 Regalona	0.88 Zeno (2016) 1.11 Puno (2016)	n. d.	1.50 $n = 5$ accessions [b] 1.9 Bastille [b]	0.40 Pot_4 0.58 Atlas
Valilne	4.30 Regalona 4.90 Ancovinto	2.19 Chucapaca (E) 4.39 CICA (A)	1.83 AG2010 2.31 B080	3.80 Puno (2016) 5.67 Jessie (2016)	2.50 QWQ 3.58 QBQ	5.30 Bastille 6.40 Rouge Marie, Zwarte	3.76 Atlas 5.81 Roja

[a] Amino acid content per variety is averaged over the different years of field trials. [b] Values are expressed for Methionine + cysteine. [c] Values are expressed for Phenylalanine + tyrosine. n. d., not defined; NW, Northwestern; QBQ, Big black quinoa; QGQ, Sanjiang Gray, gray quinoa; QWQ, Qingli No.1, white quinoa; SGQ, Aihua No.1, gray quinoa; SWQ, Jiaqi Diamond No.1, white quinoa.

10.3389/fsufs.2022.960159

the previously mentioned limiting AAs, several genotypes accomplished the daily requirements for EAAs in infants and children (WHO/FAO/UNU, 2007). As such, sufficient lysine content (over 5.7 g.100 g^{-1} protein) was identified in genotypes "Jessie", "Pasto", and "CICA". Valine content (over 4.3 g.100 g^{-1} protein) was satisfactory in genotypes "Ancovito", "CICA", "Jessie", "Rouge Marie", "Zwarte", and "Roja". Suitable leucine content (over 6.6 g.100 g^{-1} protein) was found in genotypes "Villarrica", "Rataqui", "Atlas", and "Jessie". Tryptophan content (over 0.85 g.100 g^{-1} protein) was met in genotypes "Sajama", "B080", "Regalona", "Zeno", "Puno", and all genotypes analyzed by De Bock et al. (2021b) (Table 5).

Overall, the remarkable variations in EAA composition might be caused by genotype, environment, and their interactions. According to De Bock et al. (2021b), the content of EAAs varied between growing seasons, but not between varieties, in contrast to Prager et al. (2018), who noticed significant differences among cultivars and experimental years. In terms of cultivation area, Steffolani et al. (2016) pointed out that Bolivian varieties had higher essential AA content than Peruvian varieties. Gonzalez et al. (2012) indicated dissimilarities in AA content between two experimental sites with higher EAA content in the Bolivia/Argentina location, which authors then explain by adaptation of the genotypes to the conditions they were bred in. Reguera et al. (2018) noted that varieties grown in Chile did not exhibit inter-cultivar variations in AA content compared to the same varieties grown in Spain, except for cultivar "Titicaca" which had consistent AA content among varieties and locations. The highest EAA content in genotypes cultivated in the USA was recognized in samples from the Chimacum location, as opposed to Mount Vermont samples (Craine and Murphy, 2020).

Most of the EAAs were not negatively affected by salinity in "Q5", a new salt- and drought-tolerant line, except for tyrosine (Toderich et al., 2020). Aloisi et al. (2016) found variations in genotype response to saline conditions. EAAs remained constant or declined, except for increased methionine in genotype "R49", belonging to the group of salares ecotype; and leucine in genotype "Villarrica" (coastal-lowland ecotype). A strong decline in EAAs under salinity treatment was detected in genotype VI-1 (coastal-lowland ecotypes). Despite this, Ruiz et al. (2016) concluded better suitability of "VI-1" and "Villarrica" in saline environments in terms of growth, yield, phenolic content, and protein profiles compared to the "R49"; however, other nutritional characteristics were not studied in this paper. Therefore, the selection of saline-resistant genotypes and the analysis of nutritional modifications under stress are crucial.

An essential factor in protein quality evaluation is digestibility. The information about protein digestibility in available scientific literature is sparse and often outdated. For example, Ruales and Nair (1992) reported the true protein digestibility of raw and washed quinoa reaching almost 92%. In addition, the biological value of quinoa protein (above 80%) was considerably higher compared to common cereals or soybean. On the other hand, significantly lower protein biological values were reported by Paucar-Menacho et al. (2018). Recently, Shi et al. (2020) reported the *in vitro* protein digestibility (IVPD) in quinoa ranging from ∼73 to 79% with *in vitro* protein digestibility corrected amino acid scores (IV-PDCAAS) of 48–57%. Authors reported lower values in cultivar "NQ94PT", compared to the commercial blend of cultivars "Kankolla" and "Blanca Juli". Further, Jimenez et al. (2019) reported quinoa IVPD of ∼61–63% in varieties "Cica", "Kamiri", and "Inga Pirca" obtained from Argentina. In addition, Craine and Murphy (2020) evaluated the PDCAAS in varieties "Colorado D407" ranging from 0.74 to 0.90 and 0.78 to 0.95 for the 1–2 an10-year-old children, respectively.

Overall protein digestibility can be improved by various processing methods (Rizzello et al., 2016; Lorusso et al., 2017; Dong et al., 2021; He et al., 2022), as well as sprouting (Jimenez et al., 2019). On the other hand, digestibility is reduced by the presence of starch, fiber (Opazo-Navarrete et al., 2019), and various antinutritional compounds (Gilani et al., 2012).

Lipid content and composition

Lipid content is, among other factors, strongly affected by genotype (Curti et al., 2020; Garcia-Parra et al., 2022). Since the primary lipid storage is located in the embryo, embryo size may also correlate to overall seed lipid content (De Bock et al., 2021b). The highest lipid yield was described in the genotype "Yellow Marangí", cultivated in Peru, reaching almost 10% (Apaza et al., 2015), whereas the lowest lipid content reached nearly 3% in quinoa variety "QU5", cultivated in Belgium (De Bock et al., 2021a) and commercial variety "Gramolino" from Ecuador (Graf et al., 2016; Table 6). In addition, colored seed samples tend to exhibit higher lipid content than white seed samples (Pellegrini et al., 2018); yet Tang et al. (2015) and Shen et al. (2022) obtained the opposite findings. Overall oil content was negatively correlated to protein content (Matías et al., 2021).

In terms of oil production, quinoa performed well in a temperate climate since heat stress reduced average oil content by almost 30% (Garcia-Parra et al., 2022). Curti et al. (2018) found strong interactions between cultivar and sowing date, related to the various photo-thermal conditions during sowing. In a two-year experiment with cultivars "Titicaca" and "Jessie", stable results were achieved with a mean crude fat content of 7.5 and 7.3%, respectively (Prager et al., 2018). Unfortunately, there are only a small number of studies on quinoa oil production with regard to meteorological conditions during the growing season and the adaptive response of the genotype.

Quinoa lipid profile is composed predominantly of essential polyunsaturated ω-6 linoleic acid (C18:2), with a minimum of 43% in accession "CHEN 414" originating in dry valleys of North

TABLE 6 Variability of lipid content in quinoa seeds divided according to genotype name and seed color.

	Genotype name	Seed color	Production area	Lipid content	References
Genotype	Highland ecotypes: Ancovinto, Cancosa Central ecotypes: Cahuil, Faro Southern ecotypes: Regalona, Villarrica	n. d.	Chile	Min: 5.57 [1] Villarrica Max: 7.06 [1] Cahuil	Miranda et al. (2012)
	$n = 12$ accessions	Cream Gray Orange Yellow	Peru	Min: 4.88 [1] Illpa Inia, cream Max: 9.78 [1] Yellow Maranganí, orange	Apaza et al. (2015)
	$n = 9$ commercial varieties Ancovinto Blanco Ancovinto Roja Cancosa Socaire Cáhuil Faro Regalona Villarrica	Black Red White	Bolivia Chile Ecuador USA	Min: 2.93 [2] Gramolino, white, Ecuador Max: 5.62 [2] Ancovinto Roja, white, Chile	Graf et al. (2016)
	Ecologicos Quinoa Mum's Original Heirloom Organic Quinoa Quinta Quinoa-BC12a Inca Gold Quinoa Vitabio Royal Quinoa Quinta Quinoa-BC12 Quinta Quinoa-BM12 Quinta Quinoa-Ch12 Quinta Quinoa-CVC12 GoGo Quinoa Red Organic Quinoa Organic Garage Organic Red Quinoa	Golden Red White	Bolivia Canada Unknown	Min: 6.03 [1] Mum's Original Heirloom Organic Quinoa Max: 6.74 [1] GoGo Quinoa Red Organic Quinoa	Tang et al. (2016)
	$n = 28$ accessions	n. d.	USA	Min: 5.08 [1] Blanca Max: 7.5 [1] Red Head	Aluwi et al. (2017)
	$n = 77$ accessions	Beige Black Orange Yellow	Peru	Min: 5.35 [1] Max: 7.78 [1]	Encina-Zelada et al. (2017)
	Kvl-sra2 Kvl-sra3 Regalona Q37 Q52	n. d.	Egypt	Min: 6.20 [2] Q37 Max: 8.04 [2] Kvl-sra2	Saad-Allah and Youssef (2018)
	Jessie Puno Titicaca Zeno	n. d.	Germany	Min: 5.50 [1] Zeno Max: 7.50 [1] Titicaca	Prager et al. (2018)
	Titicaca	n. d.	Ethiopia	6.30 [2]	Agza et al. (2018)

(Continued)

TABLE 6 (Continued)

Genotype name	Seed color	Production area	Lipid content	References
Cica Kamiri Inga Pirca	n. d.	Argentina	Min: 6.53 [2] Kamiri Max: 7.48 [2] Cica	Jimenez et al. (2019)
Amarilla de Maranganí Blanca de Juli Roja Pasankalla Negra Collana	White Red Black	Peru	Min: 4.97 [1] Amarilla de Maranganí Max: 6.46 [1] Roja Pasankalla	Vera et al. (2019)
F5:F6 advanced breeding lines Cherry Vanilla CO407 Dave Kaslaea	n. d.	USA	Min: 4.56 [2] Max: 7.19 [2]	Craine and Murphy, 2020
$n = 25$ accessions	n. d.	Argentina Chile Denmark Poland USA	Min: 4.22 [2] Faro Red Max: 6.82 [2] Titicaca Red	Sobota et al. (2020)
$n = 13$ accessions	Dark White	Belgium	Min: 5.42 [2,a] Pasto Max: 8.54 [2,a] Summer Red, dark	De Bock et al. (2021b)
$n = 7$ commercial varieties	n. d.	Belgium Netherlands	Min: 2.74 [2] QU5 Max: 7.34 [2] n. d.	De Bock et al. (2021a)
IC341709 IC329184 IC507733 IC107299 NIC22513 NIC22506 IC415403	n. d.	India	Min: 7.50 [1] IC341709 Max: 8.70 [1] IC507733, IC107299	Ghumman et al. (2021)
Gannan Geermu Haili	n. d.	China	Min: 4.00 [1] Haili Max: 5.21 [1] Gannan, Geermu	Jiang et al. (2021)
Duquesa Jessie Marisma Pasto Roja	n. d.	Spain	Min: 5.90 [1] Duquesa Max: 6.60 [1] Marisma	Matías et al. (2021)
Atlas Jessie Marisma Pasto Pot_4 Roja	n. d.	Spain	Min: 3.90 [3] Pot_4 Max: 5.21 [3] Marisma	Gomez et al. (2021)
Blanca real Nari no Pasankalla Soracá Puno Titicaca	n. d.	Colombia	Min: 5.77 Pasankalla Max: 7.50 Soracá	Garcia-Parra et al. (2022)

(Continued)

TABLE 6 (Continued)

	Genotype name	Seed color	Production area	Lipid content	References
Seed color	n. d.	Black Red White	South America	Min: 6.57 [1] Black quinoa Max: 7.17 [1] Red quinoa	Tang et al. (2015)
	Bolivian quinoa (BQ) Peruvian quinoa (PQ) Spanish quinoa (SQ)	Black Red White	Bolivia Peru Spain	Min: 4.87 [3] BQ, white Max: 6.48 [3] BQ, red	Pellegrini et al. (2018)
	$n = 29$ commercial varieties Blanca Kancolla Blanca Hualhuas Negra Collana Negra Pasankalla Pasankalla Roja Pasankalla Rosada de Huancayo Salcedo INIA	Black Red White	Peru Spain	Min: 6.00 [2] White quinoa Max: 6.80 [2] Black quinoa	Pereira et al. (2019)
	n. d.	Black Red White	China Peru	Min: 5.68 [2] Black quinoa Max: 6.19 [2] White quinoa	Shen et al. (2022)
Production area	n. d.	n. d.	Argentina	6.31 [2]	Nascimento et al. (2014)
	n. d.	n. d.	Egypt	6.79 [1]	El-Sohaimy and Mehany (2015)
	n. d.	n. d.	China	Min: 5.61 [1] Max: 5.68 [1]	Wu et al. (2020)

[1]The results are expressed as %. [2]The results are expressed as g.100 g^{-1} of dry weight. [3]The results are expressed as g.100 g^{-1} of fresh weight. [a]The lipid content per variety averaged over the different years of field trials. Max, maximum value; Min, minimum value; n. d., not defined.

Argentina (Vidueiros et al., 2015) and a maximum value of 63% in variety "Temuko" cultivated in the USA (Chen et al., 2019). Quinoa oil also contains a relatively high volume of monounsaturated oleic acid (C18:1), reaching minimum values of 16% in commercial variety "Quinta Quinoa-BC12" (Tang et al., 2016) and maximum values of 33% in accession "CHEN 465" originating in the transition zone of Northwest Argentina (Vidueiros et al., 2015). Saturated palmitic acid (C16:0) was presented in 3.4–13% in genotype "QuF9P39-73" (Chen et al., 2019) and white quinoa genotype (Tang et al., 2016; Shen et al., 2022), respectively. A negative correlation was found between palmitic acid (C16:0) and oleic acid (C18:1), as reported by (Chen et al., 2019).

Less abundant fatty acid in quinoa lipid profile is an essential ω-3 α-linolenic acid (C18:3), which reaches 4–8% (Tang et al., 2016; De Bock et al., 2021a,b; Shen et al., 2022); yet (Vera et al., 2019) found values reaching 11% in yellow quinoa cultivar. Vidueiros et al. (2015) determined the range for α-linolenic acid as 3.2–9.4% for accessions "CHEN 465" and "CHEN 60", respectively. Quinoa oil also has several minor fatty acids, such as myristic acid (C14:0), stearic acid (C18:0), behenic acid (C22:0), gadoleic acid (C20:1), arachidonic acid (C20:4), and erucic acid (C22:1); however, those are presented only in negligible amounts (below 2%; Tang et al., 2015; De Bock et al., 2021b; Shen et al., 2022).

Several authors noticed variations in fatty acid profiles between varieties (Tang et al., 2016; De Bock et al., 2021b; Shen et al., 2022), but Prager et al. (2018) did not report any significant alterations between varieties or years. Toderich et al. (2020) indicated changes in fatty acid composition in genotype "Q5" grown in saline soils. While the majority of fatty acids declined in medium salinity, the content of palmitoleic acid (C16:1) and arachidic acid (C20:0) was slightly raised. Besides that, the high mixed salinity of sodium chloride and sodium sulfate resulted in a significant increment of stearic acid (C:18:0). The authors also concluded that sulfate salinity affects the fatty acid composition more than sodium chloride type of salinity.

TABLE 7 Variability of lipid composition in quinoa seeds divided according to the genotype and seed color.

	Genotype name	Seed color	SFA (relative %)	MUFA (relative %)	PUFA (relative %)	ω-6/ω-3 (relative %)	References
Genotype	Ecologicos Quinoa Mum's Original Heirloom Organic Quinoa Quinta Quinoa-BC12a Inca Gold Quinoa Vitabio Royal Quinoa Quinta Quinoa-BC12 Quinta Quinoa-BM12 Quinta Quinoa-Ch12 Quinta Quinoa-CVC12 GoGo Red Organic Quinoa Organic Garage Red Quinoa	Golden Red White	Min: ≈ 10 Ecologicos Quinoa Max: ≈ 12 Quinta Quinoa-BC12	Min: ≈ 20 Quinta Quinoa-BC12 Max: ≈ 33 GoGo Quinoa Red Organic Quinoa	Min: ≈ 52 Organic Garage Red Quinoa Max: ≈ 63 Quinta Quinoa-BC12	Min: 5.30 Quinta Quinoa-BM12 Max: 10.60 Mum's Original Heirloom Organic Quinoa	Tang et al. (2016)
	n = 28 accessions	n. d.	Min: 3.30 CO 407 WMF Max: 9.10 QuF9P39-65	Min: 14.40 NL-7 Max: 28.30 UDEC2	Min: 36.70 NL-7 Max: 62.80 Temuko	n. d.	Chen et al. (2019)
	Amarilla de Maranganí Blanca de Juli Negra Collana Roja Pasankalla	Black Red White	n. d.	Min: ≈ 21 Amarilla de Maranganí Max: ≈ 34 Roja Pasankalla	Min: ≈ 55 Roja Pasankalla Max: ≈ 63 Amarilla de Maranganí	Min:4.68 Amarilla de Maranganí Max: 19.59 Negra Collana	Vera et al. (2019)
	n = 13 accessions	Dark White	Min: 10.20 Summer Red, dark Max: 13.40 Titicaca	Min: 18.10 Puno Max: 25.10 Vikinga	Min: 61.40 Vikinga Max: 70.60 Puno	Min: 6.70 Bastille Max: 12 Summer Red, dark	De Bock et al. (2021b)[a]
	Atlas Jessie Marisma Pasto Pot_4 Roja	n. d.	Min: 9.77 Jessie Max: 11.29 Pot_4	Min: 19.67 Marisma Max: 22.67 Roja	Min: 66.64 Pot_4 Max: 70.40 Jessie	Min: 7.03 Jessie Max: 8.92 Pasto	Gomez et al. (2021)
Seed color	n. d.	Black Red White	Min: 10.52 Black quinoa Max: 11.09 Red quinoa	Min: 29.88 Black quinoa Max: 33.29 Red quinoa	Min: 54.23 Red quinoa Max: 58.34 Black quinoa	Min: 5.62 White quinoa Max: 6.35 Red quinoa	Tang et al. (2015)
	Bolivian quinoa (BQ) Peruvian quinoa (PQ) Spanish quinoa (SQ)	Black Red White	Min: 10.66 BQ, black Max: 11.44 BQ, red	Min: 29.07 BQ, black Max: 33.28 BQ, red	Min: 55.28 BQ, red Max: 60.27 BQ, black	Min: 6.51 BQ, white Max: 11.42 PQ, white	Pellegrini et al. (2018)

(Continued)

10.3389/fsufs.2022.960159

TABLE 7 (Continued)

Genotype name	Seed color	SFA (relative %)	MUFA (relative %)	PUFA (relative %)	ω-6/ω-3 (relative %)	References
Unknown (n=29)	Black	Min: 27 Black, white quinoa	40 Black, red, white quinoa	Min: 31 Red quinoa	n. d.	Pereira et al. (2019)
Blanca Kancolla	Red	Max: 29 Red quinoa		Max: 33 Black, white quinoa		
Blanca Hualhuas	White					
Pasankalla						
Roja Pasankalla						
Rosada de Huancayo						
Salcedo INIA						
Negra Collana						
Negra Pasankalla						
n. d.	Black	Min: 14.48 Black quinoa	Min: 25.76 Red quinoa	Min: 52.53 White quinoa	n. d.	Shen et al. (2022)
	Red	Max: 18.87 White quinoa	Max: 27.76 White quinoa	Max: 56.87 Black quinoa		
	White					

[a]Fatty acid composition per variety averaged over the different years of field trials. Max, maximum value; Min, minimum value; n. d., not defined. MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; SFA, saturated fatty acids.

Elevated temperature, together with cultivar-specific response, resulted in lower content of some fatty acids, especially oleic acid (C18:1), stearic acid (C18:0), gadoleic acid (C20:1), and behenic acid (C22:0) (Matías et al., 2021). In contrast, the content of linoleic acid (C18:2) increased or remained unaffected in hot conditions in some cultivars (Curti et al., 2020; Matías et al., 2021). In terms of major fatty acid content, genotype "Jessie" with the shortest life cycle performed better in hot conditions compared to other genotypes. A very important role in quinoa oil quality is also played by optimal fertilization since correlations between some minerals and fatty acid content were observed by Matías et al. (2021).

Based on the available scientific literature, black genotypes tend to have higher polyunsaturated fatty acid (PUFA) content as opposed to red or white seed genotypes (Tang et al., 2015; Pellegrini et al., 2018; Pereira et al., 2019; Shen et al., 2022). Moreover, the highest monounsaturated fatty acid (MUFA) and saturated fatty acid (SFA) content were present in red genotypes (Tang et al., 2015; Pellegrini et al., 2018; Pereira et al., 2019; Vera et al., 2019), in contrast to Shen et al. (2022) who obtained opposed outcomes (Table 7). Nonetheless, as discussed in previous paragraphs, the content of fatty acids is strongly affected by genotype x environment interactions.

The overall nutritional quality of oils is characterized by the ω-6/ω-3 ratio, with an ideal composition of 1–4/1 in the human diet, as recommended by Simopoulos (2002). Nevertheless, the ω-6/ω-3 ratio of quinoa did not meet the required values since it ranged from 4.7% in variety "Amarilla de Maranganí" up to nearly 20% in variety "Negra Collana" produced in Peru (Vera et al., 2019; Table 7). Despite that, the fatty acid proportion and related nutritional quality are better than in amaranth with values reaching 33–69% (Tang et al., 2016; Paucar-Menacho et al., 2018).

Vitamin and minerals

Quinoa seeds generally contain a sufficient amount of minerals, such as Ca, Fe, Mg, Na, P, K, and Zn (Granado-Rodriguez et al., 2021a,b). As indicated by several authors, quinoa seeds have an even higher content of many minerals than common cereals (Martin et al., 2014; Nascimento et al., 2014; Mhada et al., 2020; Hussain et al., 2021). The content of minerals fluctuates due to genotype, soil type, year, and fertilization (Miranda et al., 2013; Prado et al., 2014; Pellegrini et al., 2018; Granado-Rodriguez et al., 2021a; Bock et al., 2022).

According to Granado-Rodriguez et al. (2021b), the content of P, Ca, and Fe remained unmodified between varieties, as opposed to K, Mg, and Na. Almost equivalent conclusions were defined by Matías et al. (2021), reporting significant fluctuations between cultivars in K and Mg contents, but also in P content, which conflicts with the previous study. Furthermore, Granado-Rodriguez et al. (2021a) stated that the content of Mg, Fe, and

Zn was not strongly modified by cultivar x year interactions. Reguera et al. (2018) noticed changes only in Zn between diverse locations, but not within cultivars, whereas De Bock et al. (2021b) recorded no variations in P and Ca content over the years but among the varieties. In addition, no difference between varieties was observed in P, Mg, and Fe concentrations; however, a higher accumulation of P was specific in dark-colored varieties. Higher content of P positively influenced the content of linoleic acid (C18:2) and negatively affected several MUFAs (Matías et al., 2021), which may explain, to some extent, why black seeded varieties contain higher PUFA content than red or white genotypes, as seen in Table 5. Strong correlations were also determined in P and protein content (Granado-Rodriguez et al., 2021b; Matias et al., 2022).

Significant contrasts in mineral concentration between cultivars were also analyzed between hot and cool years, which were probably caused due to little-understood heat-induced adaptation mechanisms and/or interactions among nutrients (Matías et al., 2021). Similar results were also confirmed by Tovar et al. (2020), who highlighted the relationship between heat exposure and specific stages of panicle development. Reguera et al. (2018) investigated aberrations in mineral content between varieties and the agro-ecological conditions they were grown in. According to their findings, the largest accumulation of Mg and Fe in seeds was characteristic of genotypes cultivated in Chile (Río Hurtado). Also, "Regalona" stored a larger amount of almost all analyzed minerals when cultivated in Chile, whereas "Salcedo-INIA" had a larger amount of Mg, Fe, Ca, and Zn when cultivated in Peru (Arequipa). In contrast to that, "Regalona", cultivated in Chile was characterized as the genotype with the lowest mineral content (Martin et al., 2014).

Genotypes "Pasto", "Dutchess", "Atlas", and "Summer Red" cultivated in Belgium had the highest amount of minerals, in contrast to the other studied genotypes in the experiment of De Bock et al. (2021b). Granado-Rodriguez et al. (2021b) also identified "Pasto", together with "Marisma", as genotypes with significantly higher mineral content. On the other hand, Matías et al. (2021) determined "Jessie" as the genotype with the highest mineral content. All genotypes in both studies were cultivated in Spain. In terms of adaptability to adverse conditions, Toderich et al. (2020) referred to the genotype "Q5" as suitable for saline environments since there was a remarkable increment of Fe, Zn, and Ca content under salinity. Mineral concentration varied under contrasting irrigation treatments, except for Mn concentration, which was not significantly different (Walters et al., 2016). The authors also estimated that heterogeneity in concentrations might occur due to the dilution effect.

Although there is not enough current data on overall vitamin content in quinoa, it was concluded in previous studies that quinoa has a satisfactory concentration of thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), folic acid, and vitamins A, C, and E (Koziol, 1992; Ruales and Nair, 1992). Vitamin E is a general term for tocopherols (α-, β-, γ-, and δ-) and tocotrienols (α-, β-, γ-, and δ-), also named vitamin E homologs. According to Fischer et al. (2013), vitamin E content in quinoa seeds was ranging between 1.04–1.28 mg.100g^{-1}, and overall content was not altered by escalated moisture deficit in genotypes "Regalona", "B080", and "AG2010". Tang et al. (2016) found significant variations in overall vitamin E content and the composition of vitamin E homologs. The most abundant vitamin E homolog in quinoa was γ-tocopherol followed by α-tocopherol, and δ-tocopherol, which is in accordance with the results of Pereira et al. (2019) and Granda et al. (2018). No tocotrienols were detected in any of mentioned studies. Pereira et al. (2019) also determined higher content of γ- and β-tocopherols in the black genotype, but higher α-tocopherol content in the red genotype.

Miranda et al. (2013) uncovered significant alterations in vitamin B content caused by distinct environmental conditions in two studied localities with the highest concentration of B vitamins in the arid locality Vicuña in Chile. Granda et al. (2018) also observed diverse content of vitamin B. While the content of B2 and B6 was relatively similar among varieties, diverse values were determined for B1. The highest concentration of B1 was found in non-pigmented varieties "Tunkahuan" and "Titicaca". Increased content of B2 appeared in colored varieties and the highest content of B6 was identified in pigmented variety "Pasankalla". The vitamin C content also shows some changes between distinctive locations with the highest content (49.30 mg 100.g^{-1} dw) in genotype "Villarrica" cultivated in location Temuco with a cold temperate climate (Miranda et al., 2013).

Summary

This overview provides a summary focused on current research of different quinoa genetic resources in diverse growing conditions. Quinoa is considered a highly nutritive crop that is also resistant to drought and salt suitable for marginal regions. According to our findings, the different environmental condition can have a strong impact on the nutritive compounds of quinoa seeds. Further, the adaptation of quinoa to adverse conditions has limitations in the case of elevated temperatures, high salinity levels, or a combination of weather extremes – heavy rainfall followed by temperatures over 30°C – together with cultivar-response may negatively affect growth and productivity which can result in changed content of nutritive compounds. However, an insight into the enormous variability of nutritive components possessed by quinoa germplasm cultivated in the different conditions of the world shows us how important it is to conserve and protect this richness, and to select outstanding accessions suitable to different conditions. It gives us the potential and hope to develop new varieties of quinoa adapted to different environments and production systems.

Author contributions

DJ and PC: conceptualization. PC and LD: resources and writing–original draft preparation. DJ, MJ, PC, and IV: writing–review and editing. DJ: supervision. DJ, PC, IV: funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This work was financially supported by the Ministry of Agriculture of the Czech Republic (No. RO0418) and by the Internal Grant Agency of the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague IGA (Project No. 20223105).

Acknowledgments

We gratefully acknowledge the Subsidy Programme-the National Programme for the Conservation and Use of Plant Genetic Resources and Agrobiodiversity (No. 6.2.5/51834/2017-MZE-17253) for information about quinoa genetic resources in the Czech Republic.

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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frontiers | Frontiers in Sustainable Food Systems

TYPE Hypothesis and Theory
PUBLISHED 23 February 2023
DOI 10.3389/fsufs.2023.1059066

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Aquatic Foods, a section of the journal Frontiers in Sustainable Food Systems

RECEIVED 30 September 2022
ACCEPTED 06 February 2023
PUBLISHED 23 February 2023

CITATION
Tetreault J, Fogle RL and Guerdat T (2023) Scalable coupled aquaponics design: Lettuce and tilapia production using a parallel unit process approach.
Front. Sustain. Food Syst. 7:1059066.
doi: 10.3389/fsufs.2023.1059066

Scalable coupled aquaponics design: Lettuce and tilapia production using a parallel unit process approach

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Coupled aquaponics is the integration of recirculating aquaculture systems (RAS) with hydroponic cropping systems (HCS) into a single system with shared water treatment units. Potential benefits of integration include water conservation, reduced reliance on finite mineral fertilizers, and intensive year-round location-independent production of lean proteins and fresh vegetables. However, coupled aquaponic practitioners have found minimal commercial success to date. This has been mostly due to the use of system designs which are not based on contemporary water treatment principles, especially those for commercial aquaculture. Instead, conventional coupled aquaponic system design has been based on a linear framework assuming fish wastes are readily utilized as plant fertilizers, with minimal emphasis on waste treatment or individual component hydraulic retention times. The result has been economic failures due to misbalancing the cost of inputs, the value of the outputs, and the time required to reach a marketable harvest size for both crops: fish and plants. This manuscript provides theoretical calculations based on existing standards in commercial RAS and HCS for sizing plant, fish, and biofiltration units focused on nitrogenous waste production from fish. Successful integration of HCS and RAS is defined as achieving industry standard production timelines for lettuce (seed to harvest time of 35 days) and Nile tilapia (fry to a 624 g average harvest weight in 35 weeks). Equations and examples to calculate lettuce yield, daily lettuce nitrogen requirement, fish feed rates to achieve specific nitrogen production rates, and fish tank and biofilter volumes are provided.

KEYWORDS

aquaponics system design, recirculating aquaculture system (RAS), commercial aquaponics production, controlled environment agriculture (CEA), nutrient bioeconomy

1. Introduction

Coupled aquaponics is the integration of a recirculating aquaculture system (RAS) and a hydroponic cropping system (HCS) where treated fish culture water is used as a nutrient solution for soilless plant production. Individually, RAS and HCS are prominent components of the controlled environment agriculture (CEA) industry where protected growing conditions are strictly manipulated to provide season-independent production to reduce water usage and increase yield in a minimized, location-independent growing area (Resh, 2013; Benke and Tomkins, 2017; Timmons et al., 2018). The CEA industry is an attractive option for decentralized urban food security, and it is expected to grow as the

demand for food and agricultural water are projected to increase by 56 and 60% by 2050, respectively, to meet the needs of the growing global population (Smith and Stwalley, 2018; Boretti and Rosa, 2019; van Dijk et al., 2021). Proponents of aquaponics claim that the integration of RAS and HCS into a coupled system can further optimize CEA production and result in improved environmental sustainability through minimized water and fertilizer use with increased profitability through the sale of multiple locally grown commodities (Love et al., 2014; Goddek et al., 2015; Atique et al., 2022). Additionally, urban food distribution models indicate a market for aquaponics as consumer preference is shifting to regional hubs that provide year-round fresh food production (Feldmann and Hamm, 2015; Broad et al., 2022). Despite the proposed environmental benefits and potential consumer market, commercial-scaled success has been limited.

In practitioner surveys, it was found that the majority of aquaponic systems were not profitable, and that fish production was a substantial source of financial loss (Love et al., 2014, 2015; Pattillo et al., 2022). While aquaponic systems without nutrient supplementation has demonstrated leafy green vegetable production with similar or faster growth rates than hydroponics, it has also been found that the cost of nitrogen (N) and phosphorus (P) by mass were, respectively, up to 14 and 88 times more expensive in fish feed than in inorganic fertilizer salts used in hydroponics (Colt and Schuur, 2021; Atique et al., 2022). These reports suggest that aquaponic systems where fish production is not profitable and is managed predominantly to mineralize feed that is used as the sole nutrient source for plants will likely struggle to cover production costs. A more appropriate production model would meet the fish growth and harvest rates at standards demonstrated to be profitable in the RAS industry. The potential environmental and financial benefits of integrated production could then be achieved by using excess nutrients in culture water for leafy green production commensurate with the HCS industry. This manuscript provides a coupled aquaponic system design template derived from contemporary RAS design principles. This approach focuses on a parallel unit process design for individual fish, plant, and water treatment unit hydraulic retention optimization to maximize fish growth and N production, differing from the conventionally used linear water flow design found throughout much of the published literature on coupled aquaponics.

2. Scaling limitations of traditional coupled aquaponic designs

Many self-designed aquaponic systems are adapted from a template developed at the University of Virgin Islands (UVI) where a single process flow directs nutrients produced by fish to plants (Rakocy et al., 2006; Love et al., 2014). Scalable commercial application is limited since there is little opportunity for controlling water flow rate, nutrient mass loading rates, and organic carbon accumulation as the specific unit processes are not operated under optimal conditions to meet the individual requirements for ideal fish and crop production (Baßmann et al., 2017; Knaus and Palm, 2017; Yang and Kim, 2020). Optimization of an integrated production system requires a design which utilizes hydraulic retention times (HRTs) for waste treatment and crop production unit processes (fish and plants) based on established principles in the RAS and HCS industries (Davison, 1997; Chen et al., 2006; Rusten et al., 2006; Resh, 2013; Summerfelt et al., 2016). Many of the single, in-series culture water process flow systems used in published aquaponics research did not meet the individual requirements for each unit process and therefore would be ineffective for developing scalable production systems (Baßmann et al., 2017; Knaus and Palm, 2017; Yang and Kim, 2020; Ani et al., 2021; Dusci et al., 2021) (Figure 1). Utilizing a system design approach proven to be commercially effective for aquatic food production would allow greater scalability and optimization in coupled aquaponic systems.

3. Potential benefits of adopting contemporary RAS design principles

Applying contemporary RAS engineering principles to develop system design guidelines for consistent productivity is required to enhance economic viability of commercial coupled aquaponics. Adopting a parallel unit process design modeled from RAS engineering principles could separate fish and plant production into isolated, recirculating "loops" within one culture system that shares water treatment unit processes. This retains the benefits of integration while allowing independent unit scaling and operation at contemporary RAS and HCS industry standards (Figure 2). This manuscript provides theoretical calculations derived from existing RAS and hydroponic system design methodologies to size the primary components to estimate fish and crop production rates in an aquaponics system using a parallel unit process approach.

4. Designing a coupled aquaponics system with a parallel unit process approach

4.1. System surface area and design assumptions

Greenhouse systems are popular because modifications can be made to allow season-independent growth while harnessing natural light and heat. This design is based on commonly available commercial greenhouse dimensions of 29.26 m long and 9.14 m wide, with a total surface area of 267.6 m^2. The floor area of the greenhouse is divided into four primary zones for efficient area utilization (Table 1). Most of the area is devoted to hydroponic production as this will provide the most consistent revenue source. Nile tilapia (*Oreochromis niloticus*) and lettuce (*Lactuca sativa*) are some of the most commonly grown fish and vegetable, respectively, according to recent aquaponic practitioner surveys and are the basis for all subsequent fish and plant management guidelines (Love et al., 2014; Pattillo et al., 2022). To ensure consistency in estimated yields, it is assumed that supplemental light, air temperature control, and water temperature control are used to maintain ideal growing conditions throughout the year.

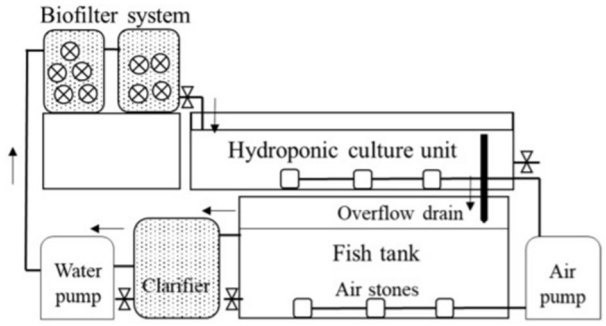

FIGURE 1
Basic single process flow design characterizing the UVI system design. Schematic of the single process flow system used in Yang and Kim (2020) where research was conducted to determine the effect of system retention time on water quality and fish and plant growth in coupled aquaponics. With a single loop design, only one water flow could be used in the system, preventing optimization of individual units.

4.2. Scalable hydroponic production

4.2.1. Deep water culture hydroponics

In deep water culture (DWC) hydroponics, plants float on polystyrene rafts in ponds that allow root systems to be fully submerged in nutrient-rich water (Resh, 2013). The ponds provide security against crop loss since sufficient standing water is retained in the event of equipment failure. Furthermore, supplemental lighting is simplified, and harvesting is streamlined in DWC because plants are easily accessible at a uniform elevation. Commercially available rafts are often 0.61 m wide and 1.22 m long, with a variety of options for spacing of grow holes.

Head lettuce, such as Butterhead lettuce, is well-suited to DWC production because it has a lightweight head that can be easily supported by a raft and a relatively small root mass that will not clog the pond. A 5-week seed to harvest timeline for Butterhead lettuce has been established where seedlings are kept in a separate germination area for 2 weeks before being transplanted into a DWC system for 3 weeks (Breckner and Both, 2013; Rakocy and Ebeling, 2018, p. 663–707). Initial transplants require less space than mature plants, and staggering growth into three 1-week phases provides consistent production and efficient space utilization. Ideal spacing for lettuce growth is used to determine the total number of heads across the three different phases and weekly harvest estimates for a system. Each week, lettuce on the phase 3 rafts is harvested, and plants within each phase progress into the next phase growing area. Based on the established growing area and the recommended spacing for lettuce, a 200.67 m^2 area could contain 11,067 plants and produce 3,689 heads per week or 191,828 heads per year (Table 2).

4.2.2. Nitrogen requirements

Nitrogen (N) is an essential macro-nutrient and is required for lettuce growth (Marschner, 2011). Smaller plants assimilate a smaller N mass each day while larger plants assimilate a greater mass each day. Maintaining a constant number of plants at each phase allows the calculation of an accurate average daily fish N production rate for ideal plant growing requirements using the following equation (Rakocy and Ebeling, 2018, p. 663–707):

$$P_N = \left(\left(plants\ phase^{-1} * phases\right) * assimilation_N\right) + SF \quad (1)$$

where P_N is the production of g N day^{-1} required for ideal lettuce growth, *phases* is the number of age-based growing sections, $assimilation_N$ is average g N day^{-1} required by a single lettuce plant, and *SF* is a safety factor to ensure an adequate nutrient mass is always available. The average N assimilation rate of a lettuce plant in a three-phased DWC growing method is 0.01837 g N $plant^{-1}$ day^{-1} (Rakocy and Ebeling, 2018, p. 663–707). Based on this assimilation rate and the addition of a 20% safety factor to ensure sufficient nutrient supply, a DWC pond with 11,067 lettuce plants evenly separated across three age-based phases would require 244 g N day^{-1}.

4.3. Scalable RAS production

4.3.1. Fish feed rate calculations

Fish waste contains high concentrations of total ammoniacal nitrogen (TAN) (Pulkkinen et al., 2019). A daily fish feed rate to produce a desired N loading rate from fish waste can be calculated using the following equation adapted from Timmons et al. (2018):

$$g\ feed = \frac{\frac{P_{TAN}}{PC}}{0.092} \quad (2)$$

where *g feed* is g feed day^{-1} required to produce a specific N mass, P_{TAN} is the specific production rate of N as g TAN day^{-1}, *PC* is the protein content of the feed (%), and 0.092 is the average percent of the feed mass excreted as ammonia. A feed rate of 6.63 kg day^{-1} is required to provide 244 g N day^{-1} from feed with a 40% protein content.

4.3.2. Fish production schedule

Staggered tilapia production is used to ensure a constant feed and N production rates and to increase fish harvest frequency. DeLong et al. (2009) and McGinty and Rakocy (2015) provide estimates for tilapia growth rates under intensive aquaculture production standards from fry to harvest weight. A daily feed rate per fish was calculated using those growth rates in conjunction with the following equation from Timmons et al. (2018):

$$g\ feed\ fish^{-1} = \frac{\left(weight_{final} - weight_{initial}\right)^{*} FCR}{\left(age_{final} - age_{initial}\right)} \quad (3)$$

where *g feed fish*$^{-1}$ is the average feed consumption day^{-1} $fish^{-1}$ over a chosen timeframe, $weight_{final}$ is the average weight $fish^{-1}$ in g at the end of this phase, $weight_{initial}$is the weight $fish^{-1}$ in g at the start of this phase, *FCR* is the average feed conversion ratio at the given age range, age_{final} is fish age in days at the end of the phase, and $age_{initial}$ is the fish age in days at the start of the phase. Phased production is determined by fish age, desired harvest frequency, or desired number of culture tanks. A five-phased production system can be utilized for a 7-week harvest

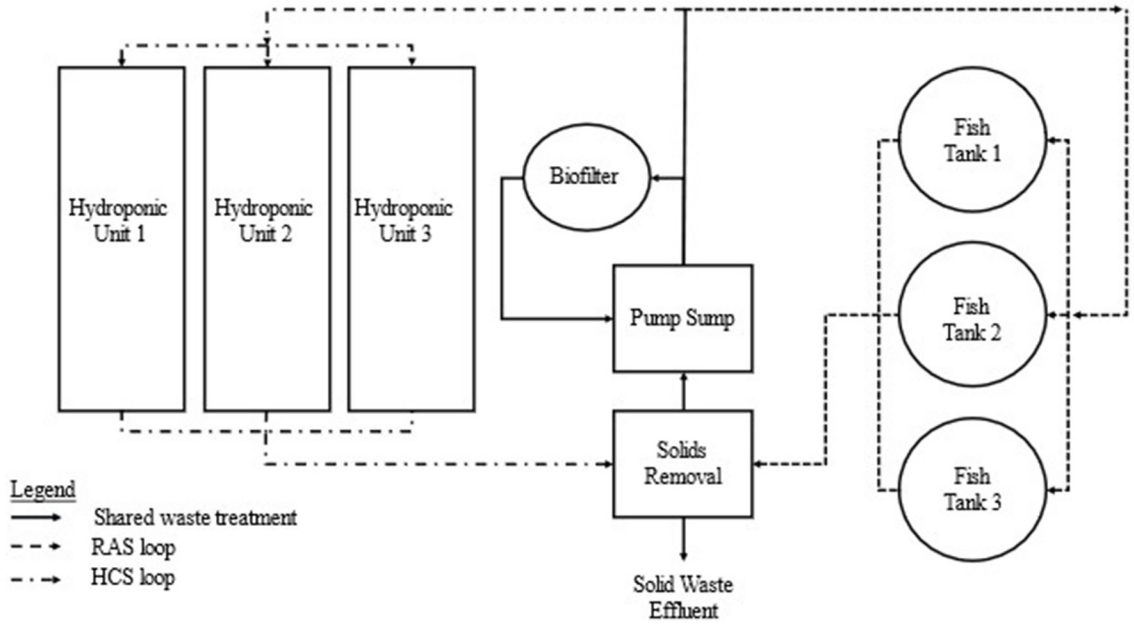

FIGURE 2
Basic components of a parallel unit process design. Flow schematic demonstrating the parallel "loops" used for individual fish tanks and hydroponic units with a shared waste treatment process for solids removal and biological nutrient conversion. Flow rate and HRT to each unit can be optimized to RAS and HCS industry standards and units can be added (or removed) as they are operated independently without affecting existing operating practices.

TABLE 1 Area allotment for greenhouse zones.

Greenhouse zone	% of area	Area (m^2)
Hydroponics	75%	200.67
Fish rearing and water treatment	15%	40.13
Harvesting space	5%	13.38
Storage	5%	13.38
Total	100%	267.56

The approximate percentage of the total area used for the primary zones of a commercial coupled aquaponics greenhouse are indicated.

interval with fry starting at 0.5 g and harvest when growth rates plateau and feed conversion ratio (FCR) increases upon reaching an average weight of 624 g (Losordo et al., 2000; McGinty and Rakocy, 2015) (Table 3).

The average feed rate $fish^{-1}$ day^{-1} across all phases is used to determine the total fish population required to consume a desired total feed rate to produce specific N mass each day, and can be calculated using the following equation:

$$average\ g\ feed\ fish^{-1} = \frac{(\sum_1^n phase_1 + phase_2 + \ldots + phase_n)}{n} \quad (4)$$

where *average g feed fish*$^{-1}$ is the average g feed $fish^{-1}$ day^{-1} across all growth phases, n is the total number of growth phases, and *phase* is the g feed $fish^{-1}$ day^{-1} for each phase. In the five-phased system described in Table 3, the average feed rate $fish^{-1}$ across all phases is 3.96 g day^{-1}. This average feed rate $fish^{-1}$ and the total system feed rate from Equation 2 can be used to calculate the total fish population that must remain evenly distributed across all growth phases to fully consume the total system feed rate to produce the required daily N loading rate. The total fish population can be calculated using the following equation:

$$fish_{total} = \frac{feed\ rate_{system}}{average\ g\ feed\ fish^{-1}\ phase^{-1}} \quad (5)$$

where $fish_{total}$ is the number of fish required across all growth phases to produce the desired mass of N day^{-1}, $feed\ rate_{system}$ is the g feed day^{-1} required to produce the desired mass of N day^{-1}, and average g feed $fish^{-1}$ $phase^{-1}$ is the average g feed $fish^{-1}$ day^{-1} across all growth phases. A system feeding 6.63 kg day^{-1} with an average feed rate of 3.96 g feed $fish^{-1}$ $phase^{-1}$, would require 1,674 fish evenly distributed across all growth phases. A five-phased grow-out would require 335 fish $phase^{-1}$. Each harvest would yield 209 kg of fish, for a yearly production of 1,553 kg.

4.3.3. Fish culture tank volume

The water volume of a tank can be calculated when the number of fish, final weight, and maximum stocking density are known using the following equation:

$$V = \frac{fish_{tank}}{\left(\frac{density_{final}}{weight_{harvest}}\right)} \quad (6)$$

Where V is the volume of water required in a fish tank in m^3, $fish_{tank}$ is the number of fish in each tank, $density_{final}$ is the maximum desired stocking density in kg m^{-3}, and $weight_{harvest}$ is the average weight in kg of fish at harvest. The

TABLE 2 Phases of hydroponic lettuce growth.

Phase	Age (days)	Spacing (m^2 $plant^{-1}$)	% of pond area	Area (m^2)	Rafts $phase^{-1}$	Plants $phase^{-1}$
1	14	0.003	5%	10.0	14	3,689
2	21	0.010	19%	38.1	52	3,689
3	28	0.041	76%	152.5	205	3,689

Hydroponic lettuce production was divided into three 1-week phases. Each phase increases in area as individual maturing plants required a greater spacing. The same number of plants at each phase allows consistent production rates and the development of an average daily N requirement for the hydroponic unit.

TABLE 3 Phased fish production based on desired daily *N* production rate.

Phase	Start age in days (weeks)	End age in days (weeks)	Start weight (g)	End weight (g)	FCR	Feed rate (g day^{-1} $fish^{-1}$)
1	1 (1)	49 (7)	0.5	24	1.1	0.54
2	50 (8)	98 (14)	24	130	1.2	2.65
3	99 (15)	147 (21)	130	277	1.4	4.29
4	148 (22)	196 (28)	277	439	1.6	5.40
5	197 (29)	245 (35)	439	624	1.8	6.94

This five-phased approach consistently maintains a desired system feed rate to produce the required daily N mass for ideal plant growth. Fish number is kept constant across each phase. Feed rate increases as fish weight and FCR increase to ensure growth rates commensurate with the RAS industry.

TABLE 4 Tank volume based on final fish weight and stocking density.

Phase	Fish population	Final fish weight (g)	Tank volume (m^3)
1	335	24	0.20
2	335	130	1.09
3	335	277	2.23
4	335	439	3.67
5	335	624	5.22

This five-phased approach maintains a consistent fish population at each phase but conserves the physical footprint of fish production by sizing each tank to the final estimate average weight of an individual fish at the end of each phase.

tank volume for each of this five-phased production method will house 335 fish and a maximum stocking density of 40 kg m^{-3} and is shown in Table 4. A 20% safety factor for fish numbers in Phase 1 may be beneficial to account for higher juvenile mortality rates.

4.3.4. Moving bed biofilm reactor volume

Moving bed biofilm reactors (MBBR) are commonly used in RAS to transform fish lethal TAN into the safer nitrate using multiple heterotrophic bacteria whose growth is facilitated on aerated media (Pulkkinen et al., 2019). Growth media volume in an MBBR is dependent on daily TAN production rates from fish waste, and can be calculated using the following equation (Timmons et al., 2018):

$$V_{media} = \left(\frac{P_{TAN}}{SSA_{media} + SF}\right) \quad (7)$$

where V_{media} is the volume of growth media in m^3, P_{TAN} is the daily TAN production in g day^{-1}, SSA_{media} is the specific surface area in m^2 m^{-3} of the media for bacteria growth, and *SF* is a safety factor to ensure complete nitrification occurs. A system that produces 244 g N day^{-1}, uses media with a 500 m^2 m^{-3} SSA, and has a 20% safety factor would require 0.59 m^3 of MBBR media. Research has demonstrated that MBBR is effective when 55% full of media (Timmons et al., 2018). An MBBR requiring 0.59 m^3 of media would necessitate a total volume of 1.07 m^3.

5. Discussion

Coupled aquaponic management improvements are often considered from only a fish or plant optimization perspective in systems designed with a single process flow (Yang and Kim, 2020; Ani et al., 2021). In an evaluation of tilapia stocking density on water quality, Ani et al. (2021) determined that lower densities resulted in more suitable dissolved oxygen (DO), TAN, nitrite, and nitrate conditions for ideal fish health than more densely stocked systems. These results have limited applicability to a CEA industry focused on intensive production and rapid yields. Based on diagrams in Ani et al. (2021), a single process flow system was used in the study and did not allow independent control over fish tank and biofilter HRTs, both of which have established parameters for maintaining appropriate DO, TAN, nitrite, and nitrate conditions in commercial RAS at stocking densities greater than any used in the study (Rusten et al., 2006; Summerfelt et al., 2016; Timmons et al., 2018; Ani et al., 2021).

For plant optimization, Yang and Kim (2020) compared three total system HRTs (6, 9, and 17 h) to determine ideal nutrient loading rates for crops in a single process flow system. The authors acknowledged that the HRTs were chosen based on DWC flow rates and would not be ideal for intensive fish production, again limiting commercial application where data suggest the fish production is key to financial success (Love et al., 2014; Yang and Kim, 2020; Colt and Schuur, 2021). The HRTs for optimal fish waste removal rates,

TABLE 5 Individual hydraulic management in a parallel unit process design.

Unit	HRT (min)	Example volume (m^3)	Example flow rate (gal min^{-1})	Citation
DWC pond	300	36.9	32.5	Resh, 2013
Fish tank (Phase 1)	35–53	0.20	1.51–1.00	Summerfelt et al., 2016
Fish tank (Phase 5)	35–53	5.22	39.4–26.0	Summerfelt et al., 2016
MBBR	3	1.07	94.2	Rusten et al., 2006

The HRT for each unit was recommended in RAS and HCS literature. The individual flow rates were based on those recommendations and the volume of each unit determined with the equations presented above.

DWC crop nutrient uptake, and TAN conversion are all different and a single process flow system cannot meet the requirements for each component within a coupled aquaponic system (Baßmann et al., 2017; Knaus and Palm, 2017; Yang and Kim, 2020; Ani et al., 2021; Dusci et al., 2021). These results demonstrate that scalability is limited and individual unit improvement without sacrificing optimization in a different unit is difficult to achieve in a linear approach.

In contrast, this work integrates principles proven to be effective in the RAS and HCS industries to provide practitioners with a design template from a N mass balance that permits individual unit control. The isolated loops in a parallel unit process design provide water flow rate control to each component, which can then be operated at ideal conditions regardless of scale (Table 5). Additional loops with different flow rates can be added, or removed, without affecting existing units. This allows multiple hydroponic methods to increase crop diversity and the opportunity to incorporate fingerling production with reduced flow rates, while maintaining the precise HRTs identified to meet differing fish and plant requirements.

6. Conclusions

Consumer interest in locally grown CEA produce combined with the growing limitations of traditional agricultural methods to meet vegetable and protein demands indicates the potential for a successful coupled aquaponics industry (Feldmann and Hamm, 2015; van Dijk et al., 2021; Broad et al., 2022). To date, minimal economic success has been achieved through aquaponics, especially if fish production fails to be profitable (Love et al., 2014; Colt and Schuur, 2021). Although RAS is one of the most prominent aquaculture methods, much of the recently published coupled aquaponics literature does not incorporate commercial RAS designs or operating standards into experimental systems when modeling production for practitioner application (Baßmann et al., 2017; Knaus and Palm, 2017; Food Agriculture Organization of the United Nations., 2020; Yang and Kim, 2020; Ani et al., 2021; Atique et al., 2022). This manuscript provides guidance on estimating plant and fish populations, yearly production, and unit volumes based on crop growing area, daily N mass requirements for lettuce in a 35-day seed to harvest schedule, and daily N mass production from tilapia in a 35-week fry to 624 g harvest schedule. A parallel unit process design adapted from RAS to incorporate an additional HCS loop is proposed to provide greater control over individual units. Unit volumes, HRTs, and fish feed and growth rates are calculated using established RAS production guidelines.

Additional research is required to further support the commercial aquaponics industry. Production data for yield estimates and comparisons to the individual RAS and HCS industries at commercial scale for additional fish species and vegetable varieties or cash crops would further de-risk practitioner adoption. Economic analysis to determine initial investment costs and potential return on investment is also required and may vary depending on environmental control costs by region as well as the fish and crop chosen. The optimization of aquaponic system design and operation is the first step to continue advancing a potential commercial industry.

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

JT: conceptualization, investigation, data curation, writing—original draft, writing—review and editing, and project administration. RF: conceptualization, validation, writing—review and editing, and visualization. TG: conceptualization, writing—review and editing, and project administration. All authors contributed to the article and approved the submitted version.

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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frontiers | Frontiers in Sustainable Food Systems

TYPE Original Research
PUBLISHED 30 June 2023
DOI 10.3389/fsufs.2023.1202015

OPEN ACCESS

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RECEIVED 07 April 2023
ACCEPTED 12 June 2023
PUBLISHED 30 June 2023

CITATION
Soro M, Pita JS, Somé K, Otron DH, Yéo E, Mutuku JM, Néya JB, Tiendrébéogo F and Koné D (2023) Genomic analysis and identification of potential duplicate accessions in Burkina Faso cassava germplasm based on single nucleotide polymorphism.
Front. Sustain. Food Syst. 7:1202015.
doi: 10.3389/fsufs.2023.1202015

Genomic analysis and identification of potential duplicate accessions in Burkina Faso cassava germplasm based on single nucleotide polymorphism

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Cassava adaptation to climate change and its resistance to diseases are essential prerequisites for achieving food security in sub-Saharan Africa. The accessions collected from farmers' fields are very important because they can provide new sources of genetic variability that are essential to achieve this goal. In this study, a panel of 184 accessions collected in Burkina Faso was genotyped using 36 single nucleotide polymorphism (SNP) markers. The accessions and markers that presented with more than 6% missing data were removed from the dataset and the remaining 34 markers and 166 accessions were retained for genetic diversity and population structure assessment. The average values of expected heterozygosity (0.46), observed heterozygosity (0.58), and polymorphic information content (0.36) indicated high genetic diversity within accessions. A complex genetic structure of 166 accessions was observed through the formation of 17 clusters using discriminant analysis of principal components (DAPC) and two clusters using Bayesian analysis. Out of the 166 accessions, 79 were unique multilocus genotypes (MLGs) and 87 were potentially duplicates. From the 79 MLGs, DAPC suggested eight clusters while the Bayesian analysis suggested seven clusters. Clusters shaped by DAPC appeared to be more consistent with a higher probability of assignment of the accessions within the clusters. Principal Coordinate Analysis (PCoA) showed a lack of clustering according to geographical origin. Information related to breeding patterns and geographic origin did not allow for a clear differentiation between the clusters according to the analysis of molecular variance (AMOVA). The results of this study will be useful for cassava germplasm conservation and breeding programs.

KEYWORDS

cassava germplasm, genotyping, genetic diversity, SNPs markers, population structure, duplicate accessions

1. Introduction

Cassava (*Manihot esculenta* Crantz, Family: Euphorbiaceae) is a staple food in most of the tropical regions of Africa, Asia, and Latin America (Zinga et al., 2016). It originated in the northern Amazonian basin (Olsen and Schaal, 1999; Léotard et al., 2009) and was introduced by the Portuguese to the African continent during the sixteenth century (Fauquet and Fargette, 1990). The global production of cassava was estimated to be 302.7 million tons in 2020. West Africa's production was 100.6 million tons, in the same year, representing more than 33.2% of the world's production of cassava (FAOSTAT, 2022). Cassava is an allogamous species propagated predominantly by cuttings (Elias et al., 2001; Oliveira et al., 2014). The genetic diversity studies in the genus Manihot seemed to support a single event of domestication from the wild form *M. esculenta* ssp. *flabellifolia* (Roa et al., 1997; Olsen and Schaal, 1999, 2001). The cassava hybrids resulting from interspecific crosses involving cassava and wild Manihot species can be highly fertile (Second et al., 1997; Nassar, 1999). In addition, interbreeding between different cassava genotypes within and between fields is common, and the seeds produced may fall and germinate, leading to an increase in the genetic diversity of cassava (Elias et al., 2001). Some studies reported the presence of a high diversity of accessions in farmers' fields due to *in situ* conservation and the exchange of planting materials between farmers (Park et al., 2005). This high genetic diversity could be used to develop new varieties with drought-tolerant, disease-resistant, high-quality, and high-yield attributes (Oliveira et al., 2014). However, through the exchange of planting materials between farmers, the same accessions are often given different names, or conversely, different accessions are given the same name, resulting in the existence of duplicate accessions collected in different localities (Salick and Cellinese, 1997; Rao et al., 2002). The costs of cassava germplasm maintenance are relatively high because the plants are kept in the field and/or *in vitro* (Albuquerque et al., 2019). Therefore, it is important to conduct studies aimed at identifying duplicate accessions in order to optimize the physical storage space both in the laboratory and in the field and to reduce the cost of maintaining the collection (Van Treuren and Van Hintum, 2003). Identification of genetic diversity in cassava germplasm has been already done using biochemical markers (Lefèvre and Charrier, 1993), quantitative and qualitative descriptors (Kawuki et al., 2011; Kamanda et al., 2020), and molecular markers such as microsatellites (Tiago et al., 2017; Adjebeng-Danquah et al., 2020) and the single nucleotide polymorphism (SNP; Oliveira et al., 2014; de Albuquerque et al., 2018; Prempeh et al., 2020). Most of the morphological descriptors, especially the quantitative ones, are not very reliable due to the strong influence of genotype-environment interaction (Al-Fares and Abu-Qaoud, 2012). Molecular markers are stable, easily detectable, and not influenced by the environment (Asare et al., 2011; Mezette et al., 2013). Among the molecular markers, SNPs seemed to have attracted research attention because of their genome abundance, chromosome-specific localization, low mutation rate, and ease of automation (Mammadov et al., 2012). SNP markers are used in genomic selection studies (Oliveira et al., 2012; Wolfe et al., 2016), the identification of sources of disease resistance by marker-assisted selection (Carmo et al., 2015), the identification of duplicate accessions (Albuquerque et al., 2019) and the characterization of germplasm (Oliveira et al., 2014; Mtunguja et al., 2015; Tiago et al., 2017; de Albuquerque et al., 2018; Prempeh et al., 2020).

Cassava was introduced to Burkina Faso from some neighboring countries (Ghana and Côte d'Ivoire) and has long been considered a neglected crop (Guira et al., 2017). In recent years, cassava production has increased through many government initiatives including the introduction of improved varieties in 2013 by the International Institute of Tropical Agriculture (IITA). However, despite these efforts, cassava production in Burkina Faso is relatively low compared to the demand. Indeed, the demand for cassava was estimated to be 124,917 tons in 2017 with the annual production estimated to be 22,104 tons (MAAH, 2019). This might be due to the use of susceptible varieties to pests and diseases, but also the high sensitivity to harsh environmental conditions (Akinwale et al., 2011). It may then be necessary to develop new varieties that are resistant to diseases and better adapted to these environments. Any progress made in breeding programs depends on a better understanding of the genetic variability present in the existing population (Adjebeng-Danquah et al., 2016). Unfortunately, since the introduction (formally or informally) of cassava accessions to Burkina Faso until today, no study on their genetic diversity has yet been conducted using molecular markers. The aim of this study was to assess the genetic diversity of cassava grown in Burkina Faso and to identify duplicate accessions in order to provide breeding programs with unique genotypes and reduce the conservation costs of cassava germplasm in the field and *in vitro*.

2. Materials and methods

2.1. Plant material

In 2017, a total of 164 accessions from seven major cassava-growing regions of Burkina Faso (Figure 1), 13 genotypes from seed germination at the Institut de l'Environnement et de Recherches Agricoles (INERA, Burkina Faso) located in Centre region, and 7 cassava varieties from the International Institute of Tropical Agriculture (IITA) were used for this study. In each field, cassava accessions were collected on the basis of their morphological differences (apical leaves color, petiole color, leaf color, number of leaf lobes, and leaf vein color). All the accessions that have been collected are maintained at the INERA station in Kamboinsé. The global positioning system (GPS) coordinates have been recorded for each location where cassava cuttings were collected. We decided to consider the cassava varieties from IITA as accessions. One cutting (20 cm) per accession was grown in a pot containing an autoclaved mixed media (two measures of soil, one measure of sand, and one measure of organic manure) for 1 month to obtain fully expanded leaves. Leaves from each cassava accession were sampled using the BioArk Leaf sample collection kit[1] and sent to LGC Biosearch Technologies, UK, for DNA extraction and genotyping. The collection and shipment of the samples were carried out according to the LGC company protocol.[2]

1 https://www.biosearchtech.com/bioark-sampling-kits

2 https://biosearchassets.blob.core.windows.net/assetsv6/guide_bioark-leaf-collection-kit.pdf

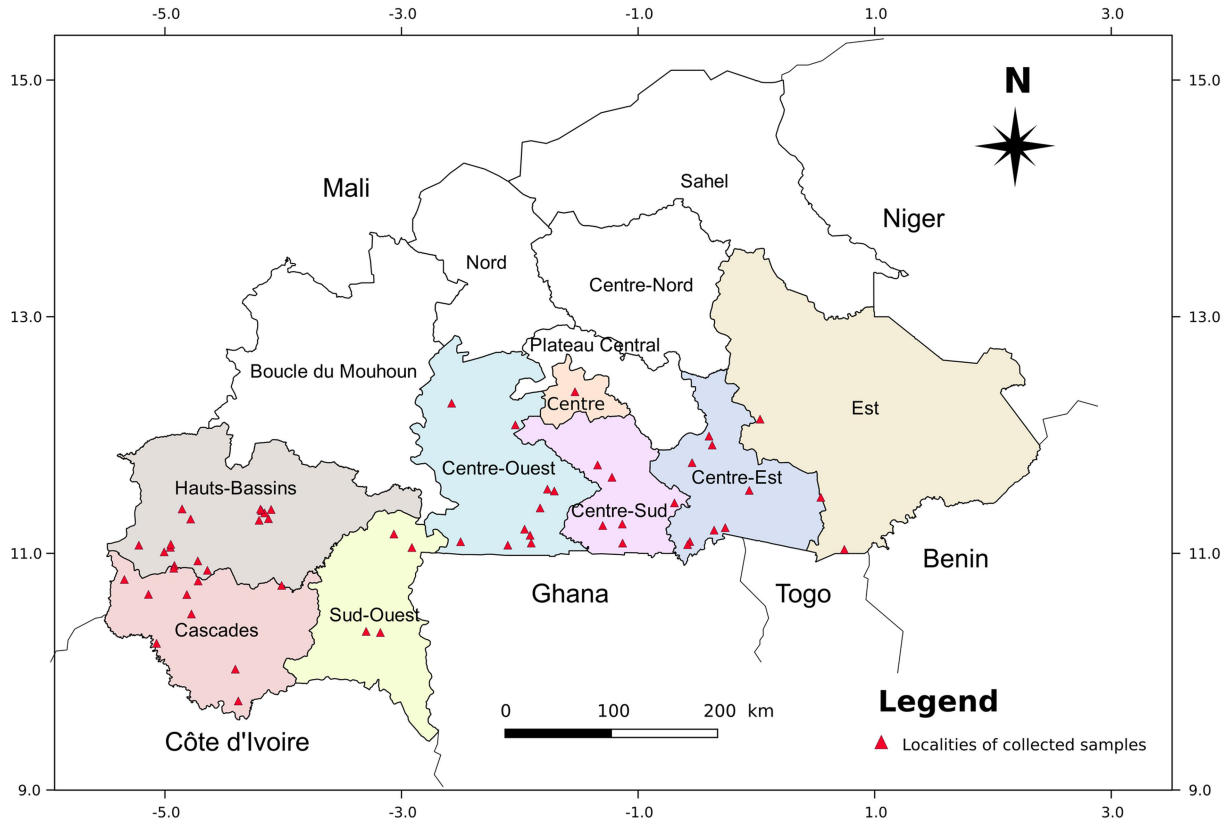

FIGURE 1
Map of Burkina Faso showing the regions and localities where cassava cuttings were collected.

2.2. SNPs markers selection

A total of 36 single nucleotide polymorphism (SNP) markers (Supplementary Table S1) were used for genotyping the 184 cassava accessions. These markers were selected from a list of markers identified by Ferguson et al. (2012) from the expressed sequence tag (EST) databases. Markers were selected based on their position on the cassava genome (to cover all 18 chromosomes) and their polymorphic information content (PIC) value. All the markers selected from the SNP markers identification and validation study conducted by Ferguson et al. (2012) had PIC values greater than 0.365. Kompetitive allele-specific PCR—polymerase chain reaction—(KASP) primers were designed for each SNP by LGC Biosearch Technologies, UK.

2.3. DNA extraction and genotyping

Total genomic DNA was extracted from cassava leaves using LGC's sbeadex™ DNA extraction, and SNP genotyping was performed using KASP™ genotyping assays.[3] DNA extraction and genotyping were performed at LGC Biosearch.[4]

2.4. Analysis of genetic diversity

The missing data percentage of each SNP marker and cassava accession was calculated using the function *missingno* in the package *poppr* (Kamvar, 2019) as implemented in R v. 4.0.2. Markers and accessions which had more than 6% missing data were removed from the dataset as recommended by Ferguson et al. (2019). The genotype accumulation curve was performed using the function *genotype_curve* in the package *poppr* to ensure that the remaining markers were sufficient to assess the genetic diversity of cassava accessions. The retained markers were subjected to various genetic diversity analyses such as polymorphic information content (PIC), major allele frequency (MaF), observed heterozygosity (H_o), and expected heterozygosity (H_e) under the Hardy–Weinberg equilibrium (HWE). PIC and MaF were obtained using PowerMarker v. 3.2.5 (Liu and Muse, 2005) while H_o and H_e were computed using the function *basic.stats* in the package *hierfstat* (Goudet et al., 2020) as implemented in R v. 4.0.2. Wright's F-statistics were calculated using the package *hierfstat* (Goudet et al., 2020). The HWE for each locus was computed using the package *pegas* (Paradis, 2010).

2.5. Analysis of genetic structure

A principal coordinate analysis (PCoA) was done using the package *cmdscale* on a dissimilarity matrix constructed with the function *vegdist* in the package *vegan* (Oksanen et al., 2020).

3 http://www.biosearchtech.com/kasp
4 http://www.biosearchtech.com/products/dna-extraction-kits

The Bray-Curtis method was used. The graph was generated using the function *ggplot* in the package *ggplot2* (Villanueva and Chen, 2019). All the packages used were implemented in R v. 4.0.2.

A Ward's minimum variance hierarchical clustering dendrogram was built using the function *hclust* in the package *stats*. The optimal number of clusters was assessed using the function *best.cutree* in the package *JLutils* (Larmarange, 2021) assuming the number of clusters to be between 1 and 20. The duplicate accessions were identified from the dendrogram on the basis of genetic distances. A threshold of 0.05 (based on the genetic distance between two representatives of the same accession) was defined as the minimum distance for considering that two genotypes were different. Any cassava accessions below this threshold were clustered into the same unique multilocus genotype (MLG). The identification of duplicates was also carried out based on the detection of MLGs using the function *mlg.id* in the package *poppr*. The same threshold was used and any cassava accessions below that were clustered into the same MLG.

The population structure was inferred using the Admixture model-based clustering algorithm as implemented in STRUCTURE v. 2.3.4 (Pritchard et al., 2000). The *ad hoc* number of clusters (k) varied from 1 to 20, with 50,000 burn-in steps, followed by 500,000 Markov chain Monte Carlo simulations. For each k, 15 independent iterations were implemented. The most likely number of k was determined by the *ad hoc* Δk statistics (Evanno et al., 2005) embedded in Structure Harvester (Earl and vonHoldt, 2012). Accessions with membership proportions (*Q*-value) ≥80% were assigned to groups, while those with membership probabilities of less than 80% were designated as admixtures.

A discriminant analysis of principal components (DAPC) was carried out using the package *adegenet* (Jombart et al., 2010) implemented in R v. 4.0.2. The optimal number of clusters was assessed using the function *find.clusters* implemented in the package *adegenet*. The function *xval.Dapc* was used to assess the best number of principal components and discriminant functions use for the DAPC. DAPC was performed using the function *dapc* in the package *adeneget* and the results of DAPC were visualized using the function *scatter.dapc* in the package *adegenet*.

Analysis of molecular variance (AMOVA) was performed by decomposition of the principal components into different hierarchical levels: (a) geographical origin (Cascades, Centre, Centre-Est, Centre-Ouest, Centre-Sud, Est, Hauts-Bassins, and Sud-Ouest), (b) breeding patterns (improved variety or landrace), (c) theoretical clusters obtained according to DAPC; and (d) theoretical clusters obtained according to Bayesian analysis. These analyses were performed using the function *poppr.amova* in the package *poppr*.

3. Results

3.1. Genetic diversity parameters

The selected SNP markers were all successfully amplified except for the marker Me_MEF_c_1418. The marker Me_MEF_c_0869, which had 12% missing data, and 18 accessions with missing data between 8 and 60%, were removed from the initial dataset, leaving a final dataset consisting of 34 SNP markers and 166 accessions. The genotype accumulation curve obtained from this dataset showed that 33 markers randomly selected from the list of 34 remaining markers make it possible to identify 100% of the unique multilocus genotypes (79 accessions) present in the population of 166 accessions (Figure 2). The common genetic parameters and genetic differentiation parameters estimated for each marker are reported in Table 1. The MaF, H_e, H_o, F_{IT}, F_{IS} F_{ST}, and PIC values estimated for the 166 cassava accessions averaged 0.06, 0.46, 0.58, −0.24, −0.27, 0.03, and 0.36, respectively. For 76.47% of the loci, H_o was greater than H_e. F_{IT} and F_{IS} were below zero for 76.47 and 82.35% of loci, respectively.

3.2. Population structure and genetic relationships

3.2.1. Principal coordinates analysis

The principal coordinate analysis (PCoA) of the 166 cassava accessions generated a graphical representation of the relationship between the accessions based on a dissimilarity matrix calculated using the Bray-Curtis method (Figure 3). The graphical representation was made using the first two principal coordinates (Cord.1 and Cord.2). These two coordinates accounted for 48.13% of the total variation. The PCoA showed an absence of clustering cassava accessions according to their geographical origins.

3.2.2. Hierarchical clustering analysis and identification of potential duplicates

The Ward's minimum variance hierarchical clustering dendrogram (Figure 4A) and optimal clusters number assessment (Figure 4B) showed that the 166 cassava accessions could be gathered into two clusters. The dendrogram revealed the presence of 87 (52.41%) potential duplicates in the dataset of 166 cassava accessions. The potential duplicates belonged to 17 unique multilocus genotypes (Figure 4A). These results were confirmed by the results of duplicate identification. The duplicate MLGs are distributed over eight regions of Burkina Faso (Figure 4C). The highest percentage of potential duplicates (70.59%) and the highest number of duplicate MLGs (10) were found in the Cascades region. The lowest percentage of potential duplicates (10.53%) was recorded in the Centre region and the lowest number of duplicate MLGs (2) was observed in the Sud-Ouest and Centre regions (Table 2). MLGs V and XIV were found only in the Cascades region, MLGs IX and XI were found only in the Hauts-Bassins region, and MLG XVI was only found in the Centre-Sud region. The other duplicate MLGs were found at least in 2 regions.

3.2.3. Bayesian analysis

Population structure analysis of the 166 cassava accessions based on Evanno's method showed that the optimal number of groups that would best explain the structure of the accessions is two with a Δk of 474.94 (Figure 5). Using an 80% membership probability threshold, 118 accessions (71.08%) were successfully assigned to both groups. In contrast, 48 accessions (28.92%) with assignment probabilities less than 80% were considered admixtures (Supplementary Table S2). Fifty-two (31.33%) accessions were assigned to Group 1 with an average assignment probability of 96%. These accessions belonged to the Cascades, Centre-Est, Centre-Ouest, Centre-Sud, Est and Hauts-Bassins regions. Sixty-six accessions (39.76%) were assigned to Group 2 with an average assignment probability of 96%. The accessions of Group 2 belonged to the Cascades, Centre, Centre-Est, Centre-Ouest, Centre-Sud, Est, Hauts-Bassins, and Sud-Ouest

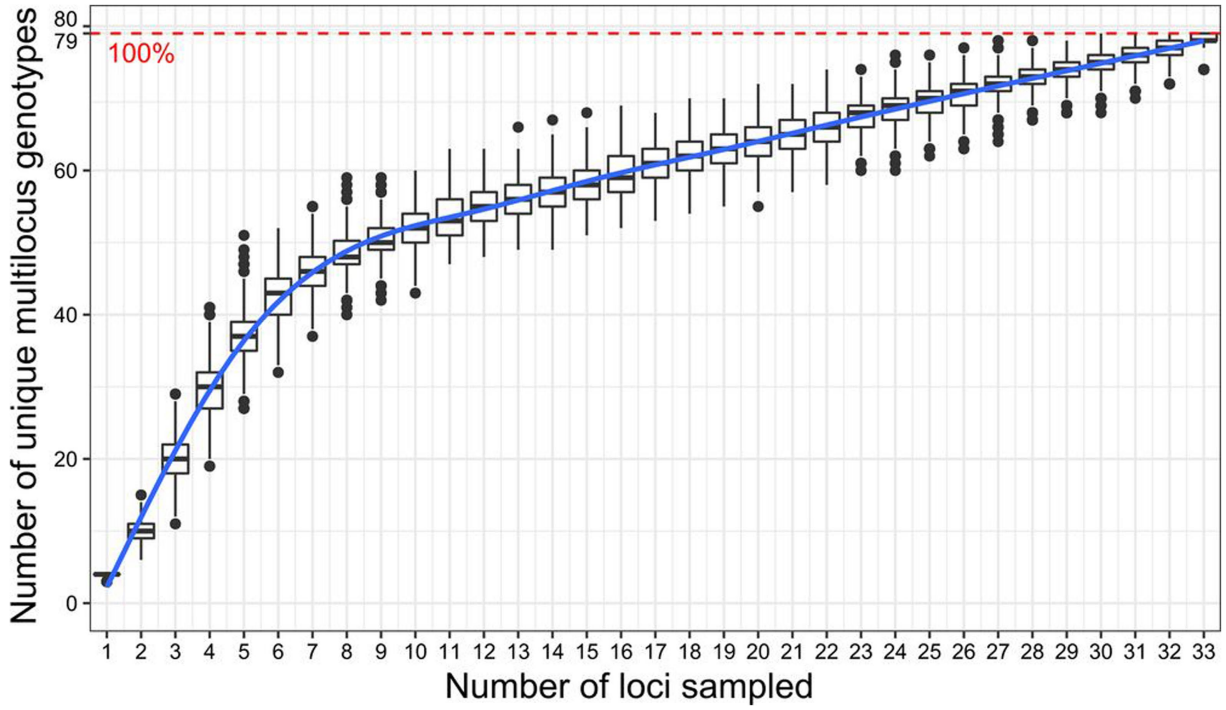

FIGURE 2
Genotype accumulation curve for 166 cassava accessions genotyped over 34 loci. The horizontal axis represents the number of loci randomly sampled without re-placement up to n–1 loci, the vertical axis shows the number of unique multilocus genotypes observed. The red dashed line represents 100% of the total observed unique multilocus genotypes (79 MLGs).

regions. The admixtures belonged to the Cascades, Centre, Centre-Est, Centre-Ouest, Centre-Sud, Est, Hauts-Bassins, and Sud-Ouest regions.

Population structure analysis of the 79 unique MLGs based on Evanno's method showed that the seven groups would best explain the structure with a Δk of 61.51 (Figure 6). Using an 80% membership probability threshold, 60 MLGs (75.95%) were successfully assigned to seven groups. Nineteen MLGs (24.05%) with assignment probabilities less than 80% were considered admixtures (Supplementary Table S3). Group 1 consisted of seven MLGs belonging to the Centre-Est, Centre-Ouest, Centre-Sud, and Hauts-Bassins regions. Group 2 consisted of eight MLGs belonging to the Cascades, Centre-Est, Est, and Hauts-Bassins regions. Group 3 (10 MLGs) belonged to the Centre, Est, Hauts-Bassins, and Sud-Ouest regions. Groups 4 (6 MLGs) and 7 (3 MLGs) belonged to the Centre region. Group 5 (11 MLGs) belonged to the Cascades, Centre-Est, Centre-Ouest, Centre-Sud, and Hauts-Bassins regions. Group 6 (14 MLGs) belonged to the Centre-Est, Centre-Ouest, Centre-Sud, Est, and Hauts-Bassins regions. MLGs were assigned to the different groups with average assignment probabilities of 94% (Group 1), 93% (Group 2), 96% (Group 3), 93% (Group 4), 97% (Group 5), 91% (Group 6), and 93% (Group 7).

3.2.4. Discriminant analysis of principal components

The discriminant analysis of principal components was first performed using the regions as predefined groups. The first 20 principal components (PCs) (explaining 96.8% of the total variance retained by PCA) and seven discriminant functions were used for the DAPC. The first two discriminant functions explaining 62 and 14% of the total genetic variation, respectively, were used for the graphical representation of the DAPC results (Figure 7). Accessions were assigned to the different regions with average assignment probabilities of 12.5% (Est), 25.0% (Centre-Ouest), 31.2% (Centre-Sud), 50% (Sud-Ouest), 55.9% (Cascades), 66.7% (Centre-Est), 69.8% (Hauts-Bassins), and 73.7% (Centre). The HWE test performed on accessions collected in each region revealed that none of the sub-populations were at HWE (Figure 8).

For the 166 accessions, the lowest Bayesian Information Criterion (BIC) value (62.09) was obtained for an optimal number of 17 clusters (Figure 9A). This number of clusters was used for the DAPC. The first 10 principal components (PCs) (which explained 82.6% of the total variance retained by PCA) and 10 discriminant functions were used for the DAPC. The first two discriminant functions, which explained, respectively, 53.1 and 19.6% of the total genetic variation, were used for the graphical representation of the DAPC results (Figure 9B). Accessions were assigned to each of the 17 clusters with an individual assignment probability of 100% (Figure 9C).

The lowest BIC value (120.97) indicating an optimal distribution of the 79 MLGs into 8 clusters was obtained (Figure 10A). The first five principal components (PCs), explaining 57.3% of the total variance retained by PCA, and five discriminant functions were retained for the DAPC. The first two discriminant functions, which explained, respectively, 53.9 and 22.5% of the total genetic variation, were used for the graphical representation of the DAPC results (Figure 10B). MLGs were assigned to the 8 clusters with an individual assignment probability ranging from 94 to 100% except for the BFM152 accession which was assigned to cluster 2 with a probability of 74%. MLGs in clusters 1, 3, 6, and 8 were assigned with an individual assignment probability of 100% (Figure 10C).

TABLE 1 Common genetic parameters and F-statistics for each locus.

Markers	MaF	H_e	H_o	F_{IT}	F_{IS}	F_{ST}	PIC
Me_MEF_c_0556	0.71	0.37	0.44	−0.12	−0.19	0.06	0.33
Me_MEF_c_0566	0.92	0.15	0.17	−0.09	−0.10	0.01	0.14
Me_MEF_c_0587	0.66	0.44	0.60	−0.35	−0.34	0.00	0.35
Me_MEF_c_0936	0.57	0.49	0.84	−0.71	−0.69	0.00	0.37
Me_MEF_c_0979	0.67	0.44	0.58	−0.29	−0.30	0.00	0.34
Me_MEF_c_0981	0.62	0.45	0.48	−0.01	−0.06	0.05	0.36
Me_MEF_c_1018	0.67	0.45	0.32	0.31	0.30	0.01	0.34
Me_MEF_c_0363	0.62	0.46	0.60	−0.30	−0.32	0.01	0.36
Me_MEF_c_1074	0.60	0.44	0.36	0.27	0.17	0.12	0.37
Me_MEF_c_1081	0.54	0.46	0.64	−0.29	−0.38	0.07	0.37
Me_MEF_c_1094	0.58	0.49	0.53	−0.09	−0.09	0.00	0.37
Me_MEF_c_1179	0.53	0.48	0.67	−0.33	−0.39	0.04	0.37
Me_MEF_c_1186	0.56	0.49	0.86	−0.74	−0.74	0.00	0.37
Me_MEF_c_0153	0.52	0.49	0.78	−0.56	−0.58	0.02	0.37
Me_MEF_c_1187	0.57	0.49	0.86	−0.76	−0.76	0.00	0.37
Me_MEF_c_3217	0.58	0.50	0.58	−0.18	−0.17	0.00	0.37
Me_MEF_c_0262	0.69	0.39	0.47	−0.16	−0.20	0.03	0.34
Me_MEF_c_2368	0.60	0.45	0.45	0.05	−0.01	0.06	0.36
Me_MEF_c_1361	0.74	0.39	0.39	0.00	−0.01	0.01	0.31
Me_MEF_c_2268	0.52	0.48	0.56	−0.12	−0.18	0.05	0.37
Me_MEF_c_3025	0.52	0.50	0.78	−0.56	−0.56	0.00	0.37
Me_MEF_c_1568	0.65	0.47	0.39	0.17	0.17	0.00	0.35
Me_MEF_c_1585	0.52	0.49	0.76	−0.52	−0.55	0.02	0.37
Me_MEF_c_1671	0.52	0.50	0.74	−0.48	−0.47	0.00	0.37
Me_MEF_c_0227	0.56	0.48	0.44	0.13	0.09	0.05	0.37
Me_MEF_c_2177	0.52	0.50	0.73	−0.45	−0.45	0.00	0.37
Me_MEF_c_2297	0.67	0.44	0.61	−0.38	−0.39	0.01	0.34
Me_MEF_c_2515	0.63	0.45	0.63	−0.37	−0.39	0.02	0.36
Me_MEF_c_0284	0.53	0.49	0.81	−0.61	−0.66	0.03	0.37
Me_MEF_c_2574	0.50	0.45	0.56	−0.13	−0.26	0.10	0.37
Me_MEF_c_2644	0.61	0.47	0.73	−0.54	−0.54	0.00	0.36
Me_MEF_c_2911	0.57	0.49	0.39	0.22	0.21	0.01	0.37
Me_MEF_c_3142	0.60	0.47	0.70	−0.44	−0.49	0.03	0.36
Me_MEF_c_0126	0.56	0.0.47	0.19	0.63	0.60	0.07	0.37
Mean	**0.60**	**0.46**	**0.58**	**−0.24**	**−0.27**	**0.03**	**0.36**

MaF, major allele frequency; He, expected heterozygosity; Ho, observed heterozygosity; F_{IT}, inbreeding coefficient of an individual into the whole population; F_{IS}, within-population inbreeding coefficient; F_{ST}, coefficient of differentiation and PIC, polymorphic information content.

3.2.5. Analysis of molecular variance

Analysis of molecular variance of the 166 cassava accessions based on geographical origin (regions), breeding patterns, and Bayesian analysis clusters showed that the most significant differences in the molecular variance of the SNPs were within individuals with 92.88, 84.27, and 66.52% of the total molecular variance, respectively. When using the clusters identified by DAPC, most of the variability was found among clusters (83.95%; Table 3). Analysis of the molecular variance of the 79 MLGs based on geographical origin and breeding patterns showed that the most significant differences in the molecular variance were within individuals with 93.50 and 87.19% of the total molecular variance, respectively. The AMOVA based on the DAPC clusters revealed that the molecular variance was slightly higher between clusters at 53.07% compared to the variance within individuals (46.93%). Using Bayesian analysis clusters, the results showed that the molecular variance between clusters and within individuals were almost the same with 50.41 and 49.59% of the total variance, respectively (Table 4). The mean indexes of genetic

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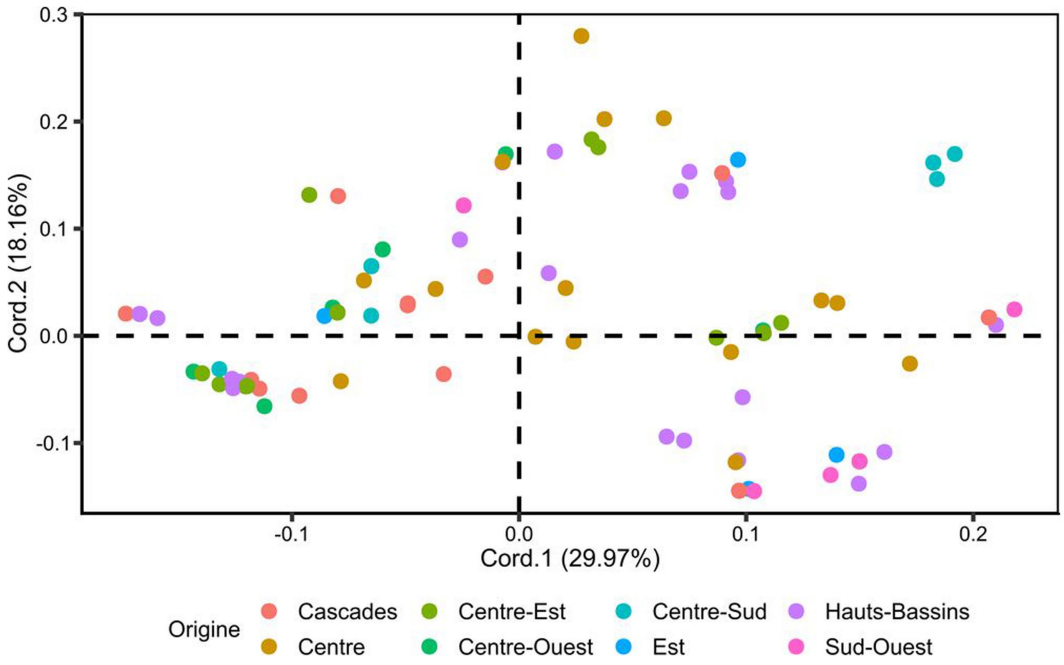

FIGURE 3
Principal coordinate analysis (PCoA) based on a dissimilarity matrix calculated using the Bray-Curtis method. Accessions are colored according to geographical origin.

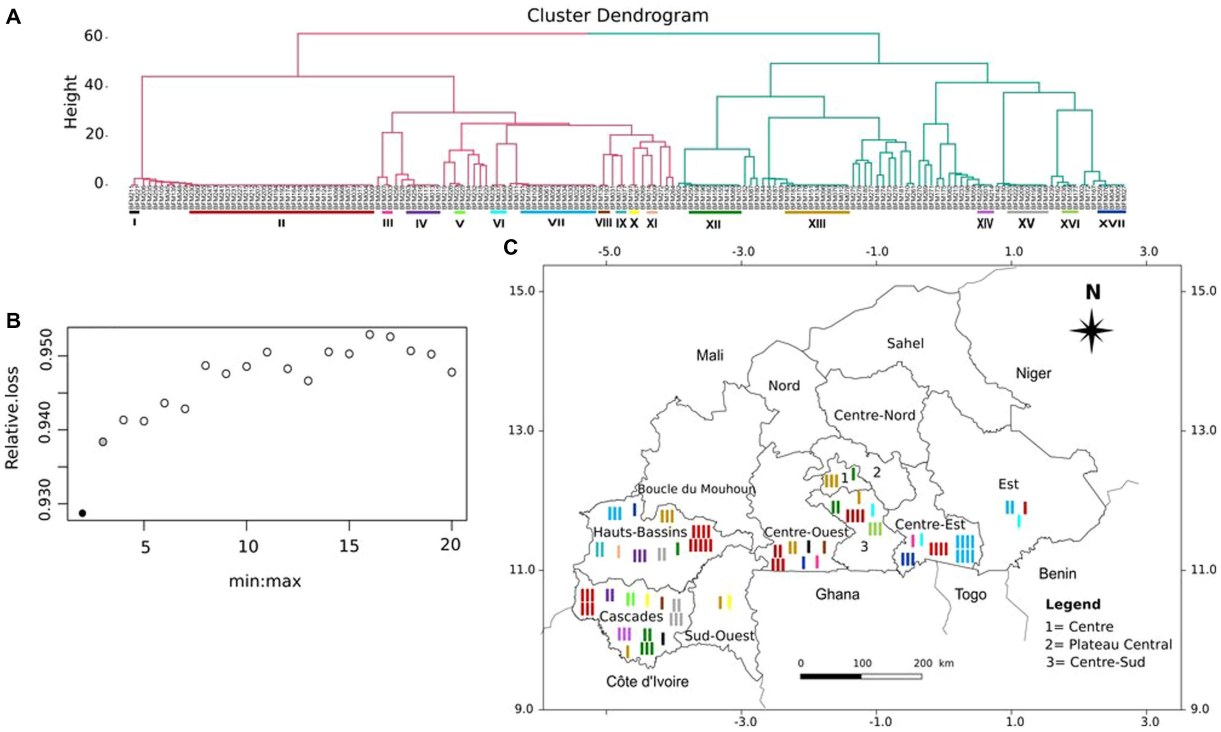

FIGURE 4
Hierarchical clustering of the 166 cassava accessions and geographical distribution of potential duplicates. **(A)** Ward's minimum variance ascending hierarchical clustering of the 166 cassava accessions. The colored bars represent the 17 duplicate MLGs identified. **(B)** The black dot indicates the optimal number of clusters. **(C)** Distribution of duplicate MLGs in different cassava growing regions of Burkina Faso. Each color represents a duplicate MLG. For each duplicate MLG, the number of bars corresponds to the number of times the GLM has been found in the same region.

TABLE 2 Summary table of collected cassava accessions characteristics per region.

Regions	Accessions	Unique MLGs	Potential duplicates	Duplicates percentage (%)	Duplicate MLGs
Cascades	34	10	24	70.59	I, II, IV, V, VIII, X, XII, XIII, XIV, XV
Centre	19	17	2	10.53	XII, XIII
Centre-Est	24	9	15	62.50	II, III, VI, VII, XVII
Centre-Ouest	16	7	9	56.25	I, II, III, VIII, XIII, XVII
Centre-Sud	16	6	10	62.50	II, VI, XII, XIII, XVI
Est	8	5	3	37.50	II, VI, VII
Hauts-Bassins	43	20	23	53.49	II, IV, VII, IX, XI, XII, XIII, XV, XVII
Sud-Ouest	6	5	1	16.67	X, XIII
Total	**166**	**79**	**87**	**52.41**	**17 duplicate MLGs**

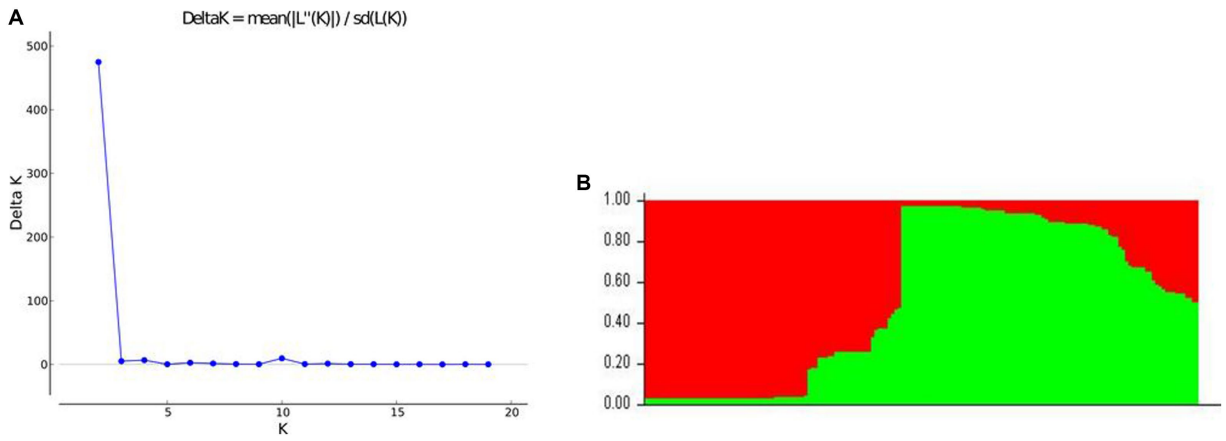

FIGURE 5
Graphical representation of the population structure of 166 cassava accessions. **(A)** Plot of mean likelihood of delta K against the number of K groups. The highest peak observed at *K*=2 signifies the grouping of accessions into two groups. **(B)** The colors represent two groups of 166 accessions. The separation of accessions into Group 1 (red) and Group 2 (green) was based on membership probability ≥80%.

differentiation of the 166 accessions and 79 MLGs according to geographical origin, breeding patterns, DAPC clusters, and Bayesian analysis clusters are recorded in Table 5.

4. Discussion

The minimum number of markers required to adequately determine genetic diversity in a population varies with the genetic diversity of the population, the scale of the study, and the type of marker used (Grünwald et al., 2017). Species that are highly inbred require a larger number of markers than those that are naturally heterogeneous, like cassava, for the detection of allelic variations. In addition, the number of alleles varies with the type of marker. SSR markers can have a large number of alleles at a locus while SNP markers have a fixed number of alleles (Adjebeng-Danquah et al., 2020). Thus, fewer microsatellite markers may be needed when compared with SNP markers to achieve the same degree of resolution (Schlötterer, 2004; Arnaud-Haond et al., 2007; Grünwald et al., 2017). Whatever the type of marker used or the diversity within the species, it is important to determine the appropriate number of markers for which the diversity observed in the population will not increase significantly if an additional marker is added (Arnaud-Haond et al., 2007). The genotype accumulation curve that was made to determine this appropriate number of markers showed that the selected markers were sufficient for the discrimination of the cassava accessions grown in Burkina Faso. Moreover, it revealed the existence of 79 unique multilocus genotypes among the 166 accessions.

Further analysis revealed that the retained 34 SNP markers were highly polymorphic. The high number of polymorphic SNP markers could be explained by the open-pollination mode of reproduction and the level of genetic variation in cassava (Oliveira et al., 2014). This finding could be a reflection of the diversified origin of cassava accessions grown in Burkina Faso through the informal introduction of accessions from other countries (Guira et al., 2017). Despite the high polymorphism of the SNP markers used in this study, the average PIC value was lower than those of other markers such as SSR (Asare et al., 2011; Pedri et al., 2019; Adjebeng-Danquah et al., 2020). This difference could be explained by the bi-allelic nature of

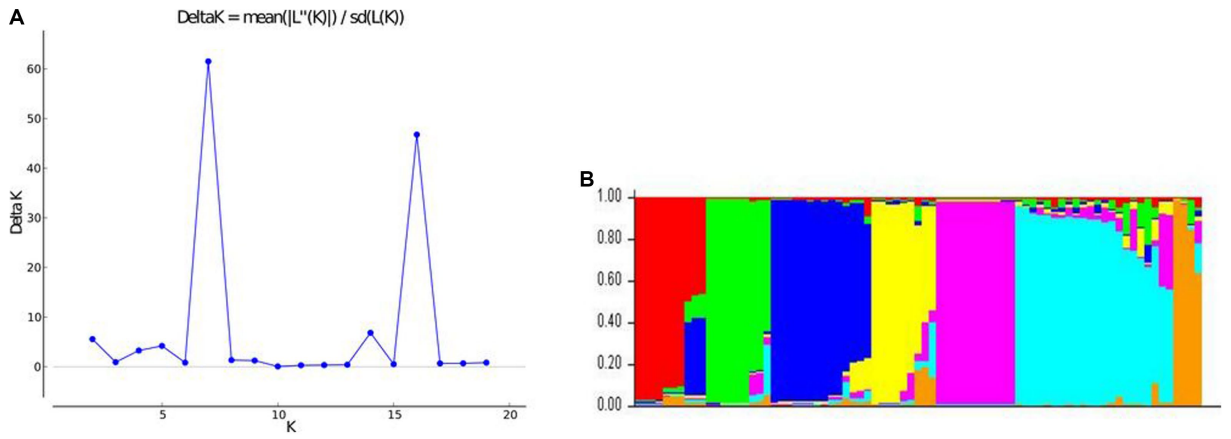

FIGURE 6
Graphical representation of the population structure of the 79 MLGs. **(A)** Plot of mean likelihood of delta k against the number of k groups. The highest peak observed at *k*=7 signifies the grouping of accessions into seven groups. **(B)** The colors represent seven groups of 79 was based on membership probability ≥80%.

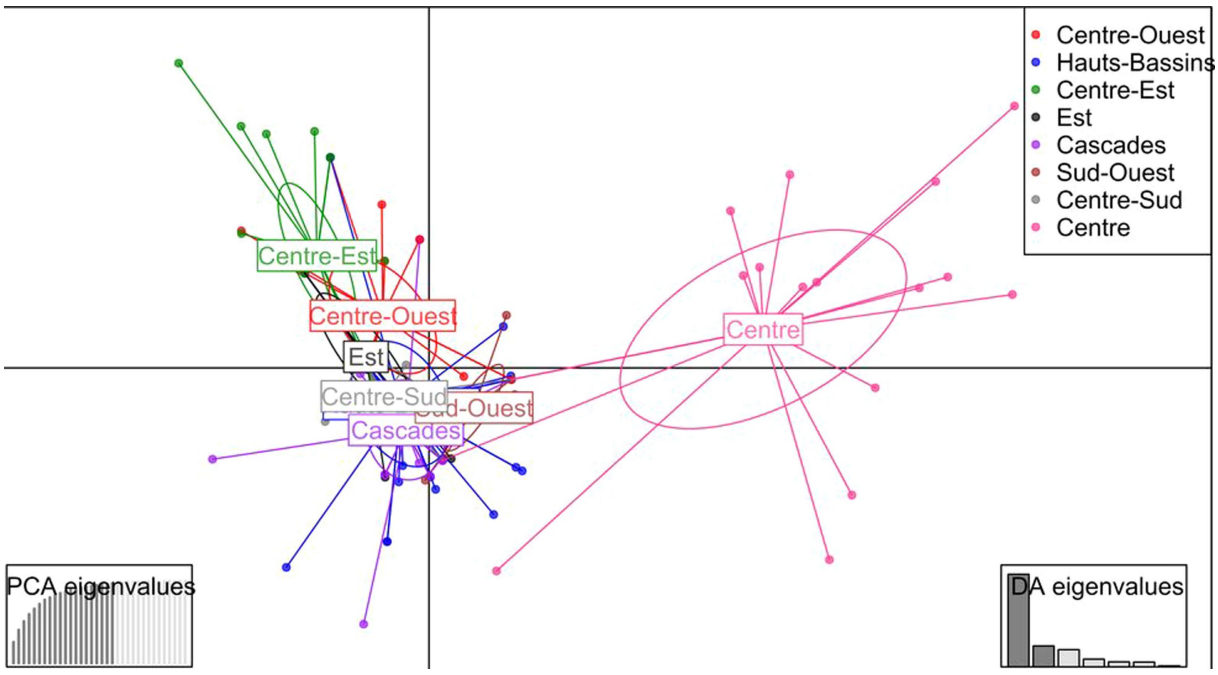

FIGURE 7
Discriminant analysis of principal components (DAPC) using the regions as predefined groups. The axes represent the first two discriminant functions. Each color represents a region, while each dot represents an accession.

SNP markers whose PIC values vary between 0.0 and 0.5, unlike SSR which are multi-allelic and can have PIC values up to 1 (Prempeh et al., 2020). Therefore, our results indicate that most of the SNPs used were sufficiently informative and can be used to study the genetic diversity of cassava accessions. The only exception we found was the Me_MEF_c_0566 marker which was moderately informative. Furthermore, the mean PIC value observed in this study was higher than those observed previously in some genetic diversity studies of cassava accessions (Oliveira et al., 2014; Kamanda et al., 2020; Karim et al., 2020; Prempeh et al., 2020).

The average heterozygosity observed in this study was higher than the expected average heterozygosity suggesting a heterozygote excess within cassava accessions under the HWE. This heterozygote excess was confirmed by negative values of the F_{IS} and F_{IT} fixation indexes. Curiously, there are studies that have reported heterozygote excess within cassava accessions in relation to that expected under the HWE (Kamanda et al., 2020), and other studies reporting heterozygote deficit in relation to that expected under the HWE (de Albuquerque et al., 2018; Prempeh et al., 2020). Nevertheless, the differences observed between the mean values of PIC, H_o, and H_e in

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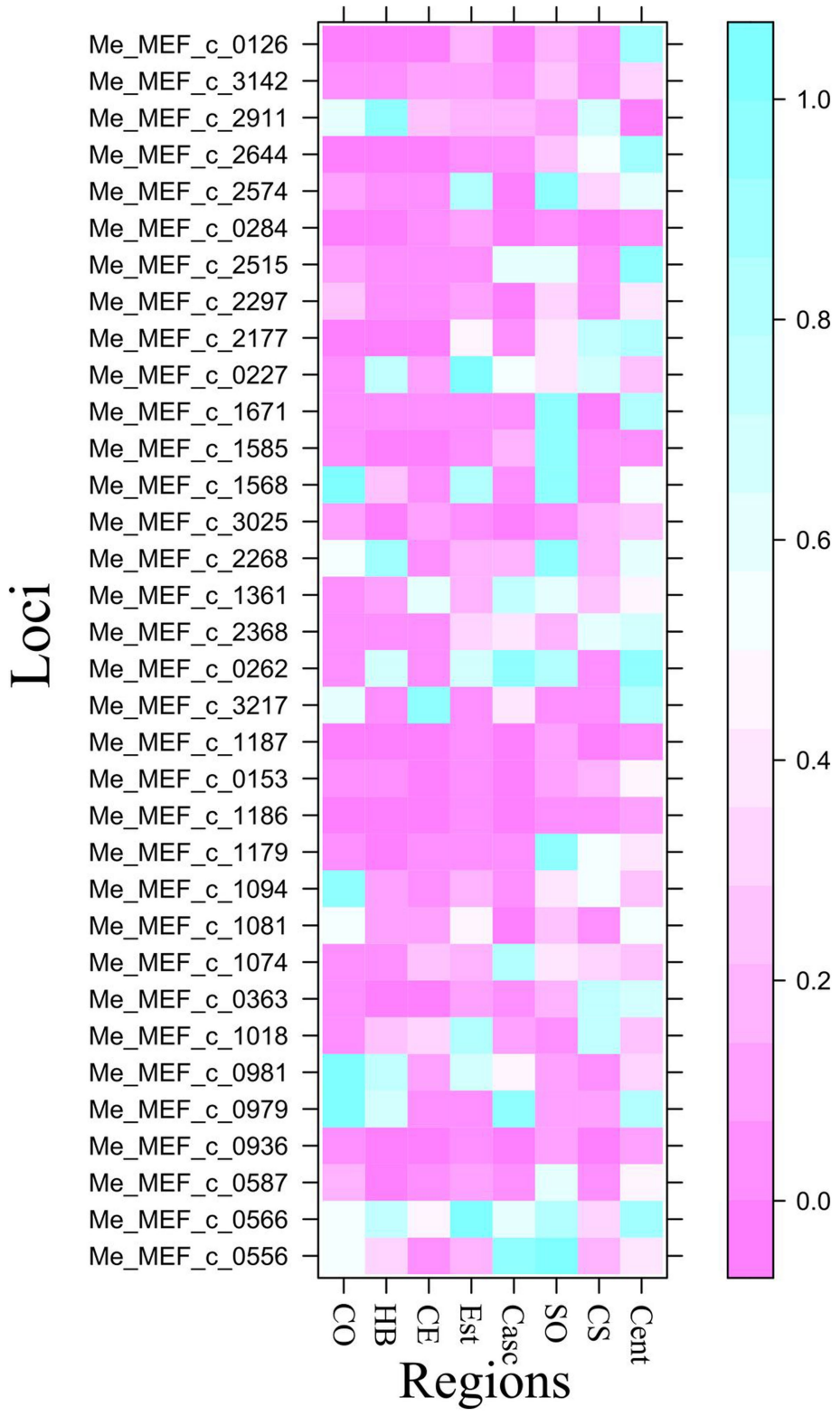

FIGURE 8
Graph showing significant deviations from the HWE. Each row represents a locus, and each column represents a subpopulation (CO, Centre-Ouest; HB, Hauts-Bassins; CE, Centre-Est; Casc, Cascades; SO, Sud-Ouest; CS, Centre-Sud; Cent, Centre). The presence of pink color in a column at a given locus indicates that the subpopulation is not at the HWE for that locus with a probability $p \leq 0.05$.

this study and those of other studies could be explained by the specificity of each cassava germplasm studied and the SNP markers used.

Molecular profiling of cassava accessions revealed a high rate of potential duplicates among the 166 accessions and a high variability in the proportions of potential duplicates across regions. The

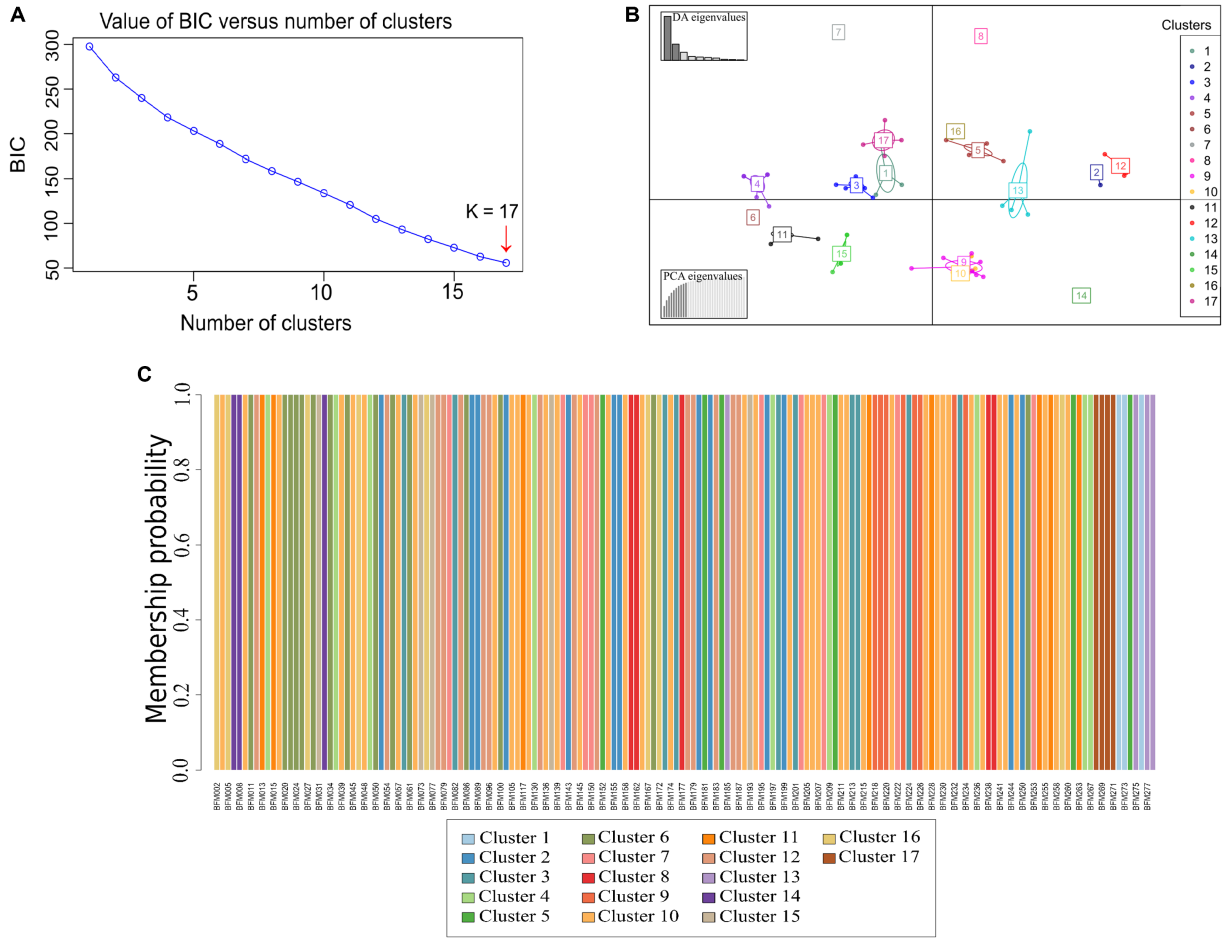

FIGURE 9
Discriminant analysis of principal components (DAPC) of 166 cassava accessions obtained from the analysis of 34 single nucleotide polymorphism (SNP) markers. **(A)** Optimal number of clusters. **(B)** Graphical representation of the DAPC results. Clusters are represented by colors according to the legend. The graph below to the left represents the contribution of the eigenvalues of the principal components selected, while the graph above to the left indicates the variance explained by the eigenvalues of the discriminant functions. **(C)** Membership probability of cassava accessions. Each accession is represented by a vertical line and the colors correspond to the probability of assignment in each of the 17 groups.

analysis also revealed that these potential duplicates belong to several genetic profiles and that the number of profiles varies between regions. This could be due to a high rate of exchange of cassava cuttings within regions. In addition, most of the potential duplicate profiles were found in several regions, indicating a high rate of exchange of cassava cuttings between regions. Similar results were obtained in Brazil in a study conducted on the identification of duplicates in the Embrapa cassava germplasm bank (Albuquerque et al., 2019). Accessions with profiles found in several regions probably have quite interesting characteristics for farmers (Rabbi et al., 2015). From the PCoA analysis, it was not possible to differentiate cassava accessions according to geographical origin. This lack of differentiation was confirmed by the low value of the genetic differentiation index ($F_{ST} = 0.03$) and by the low assignment probabilities of accessions to different regions. Furthermore, the AMOVA results revealed that more than 92%, for the 166 accessions, and more than 93%, for the 79 MLGs, of the total molecular variance were within accessions. This could be explained by the fact that some accessions are grown in several regions. The determination of the best number of clusters using the function *best.cutree* showed that the cassava accessions studied can be grouped into two large groups. The truncation was done at the top of the dendrogram and therefore does not accurately give the diversity of accessions. A lower truncation would more precisely give the real diversity of cassava accessions cultivated in Burkina Faso. The Bayesian analysis performed on the 166 accessions also grouped the accessions into two clusters, with more than 28% of the accessions not being assigned to a cluster at the 80% membership probability threshold. The result of the Bayesian analysis could be an underestimation of the diversity of cassava accessions. Indeed, as this analysis is based on the HWE model, it then assumes that the population is panmictic, that it is infinite, that there is no selection, mutation, or migration, and that successive generations are discrete (Oliveira et al., 2014; Rabbi et al., 2015). For populations with clonal and/or partially clonal reproduction such as cassava, these assumptions are violated because alleles are not always transmitted independently from one generation to another (Kamvar et al., 2014; Rabbi et al., 2015). The significant deviations from the HWE we detected during our analysis confirm this assertion as none of the subpopulations were at HWE. The DAPC

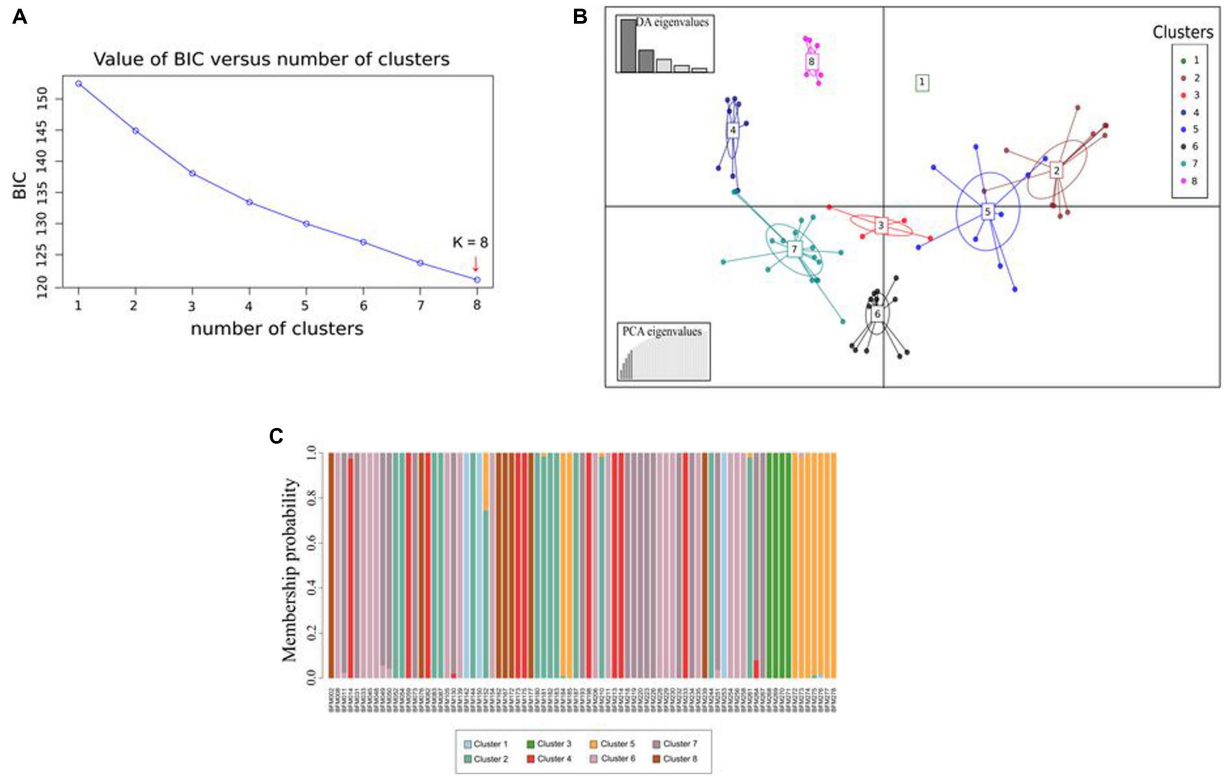

FIGURE 10
Discriminant analysis of principal components (DAPC) of 79 MLGs obtained from the analysis of 34 single-nucleotide polymorphism (SNP) markers. **(A)** Optimal number of clusters. **(B)** Graphical representation of the DAPC results. Clusters are represented by colors according to the legend. The graph below to the left represents the contribution of the eigenvalues of the principal components selected, while the graph above to the left indicates the variance explained by the eigenvalues of the discriminant functions. **(C)** Membership probability of MLGs. Each MLG is represented by a vertical line and the colors correspond to the probability of assignment in each of the eight groups.

TABLE 3 AMOVA of the 166 accessions considering (a) geographical origins, (b) breeding patterns, (c) theoretical clusters obtained according to DAPC, and (d) theoretical clusters obtained according to Bayesian analysis.

Source of variation	Geographical origin			Source of variation	Breeding patterns		
	df	Mean Sq	% of variation		df	Mean Sq	% of variation
Between clusters	7	14.52	7.12	Between clusters	1	43.06	15.73
Within individuals	158	5.77	92.88	Within individuals	164	5.91	84.27
Total	165	6.14	100.00	Total	165	6.14	100.00

Source of variation	DAPC			Source of variation	Bayesian analysis		
	Df	Mean Sq	% of variation		df	Mean Sq	% of variation
Between clusters	16	53.30	83.95	Between clusters	1	208.70	33.48
Within individuals	149	1.07	16.05	Within individuals	164	4.90	66.52
Total	165	6.14	100.00	Total	165	6.14	100,00

performed on the 166 cassava accessions divided the accessions into 17 clusters with a higher individual assignment probability (100%) of accessions into clusters. The difference between the results of the Bayesian analysis (2 clusters) and the DAPC (17 clusters) could be explained by the multivariate approach used by the DAPC but also by the presence of many duplicates in the cassava germplasm. There are reports suggesting that the type of population structure influences the precision of the method (Jombart et al., 2010; Oliveira et al., 2014). In addition, the analysis of the population structure of the cassava germplasm involving samples from different genetic origins in different and unknown proportions leads to linkage disequilibrium between non-linked loci (Ersoz et al., 2007; Oliveira et al., 2014). In this case, DAPC would be more appropriate as it uses an approach that can identify genetic structures in the absence of any assumptions about the genetic model of the population (Jombart et al., 2010). Indeed, the results of the AMOVA of the 166

TABLE 4 AMOVA of the 79 MLGs considering: (a) geographical origins, (b) breeding patterns, (c) theoretical clusters obtained according to DAPC, and (d) theoretical clusters obtained according to Bayesian analysis.

Source of variation	Geographical origins			Source of variation	Breeding patterns		
	df	Mean Sq	% of variation		df	Mean Sq	% of variation
Between clusters	7	9.54	6.50	Between clusters	1	28.55	12.81
Within individuals	71	5.75	93.50	Within individuals	77	5.80	87.19
Total	78	6.09	100.00	Total	78	6.09	100.00

Source of variation	DAPC groups			Source of variation	Bayesian analysis		
	Df	Mean Sq	% of variation		df	Mean Sq	% of variation
Between clusters	7	36.46	53.07	Between clusters	6	39.72	50.41
Within individuals	71	3.10	46.93	Within individuals	72	3.29	49.59
Total	78	6.0.09	100.00	Total	78	6.09	100.00

TABLE 5 The fixation index F_{ST} of cassava accessions to (a) geographical origin, (b) breeding patterns, (c) DAPC, and (d) Bayesian analysis.

166 accessions		79 MLGs	
Type of clustering	F_{ST}	Type of clustering	F_{ST}
Geographical origins	0.03	Geographical origins	0.02
Breeding patterns	0.03	Breeding patterns	0.03
DAPC clusters	0.34	DAPC clusters	0.25
Bayesian clusters	0.08	Bayesian clusters	0.23

cassava accessions in relation to the clusters formed by the Bayesian analysis (STRUCTURE clusters) and by the DAPC (DAPC clusters) showed that the DAPC allows for better discrimination of cassava accessions. It was found that more than 80% of the total molecular variance was between the DAPC clusters, compared to only 16.05% within the accessions. In contrast to the DAPC, the molecular variance between the Bayesian analysis clusters represented 33.48% of the total molecular variance compared to 66.52% within the accessions. This moderate differentiation of the accessions by Bayesian analysis was confirmed by the low value of the genetic differentiation index ($F_{ST} = 0.08$). The criteria for classifying F_{ST} values showed that F_{ST} between 0 and 0.05 indicates low differentiation; F_{ST} between 0.05 and 0.15 reflects moderate differentiation; F_{ST} between 0.15 and 0.25 suggests high differentiation and above 0.25, the F_{ST} illustrates very high differentiation (Wright, 1978). These classification criteria showed that there was indeed moderate differentiation of the cassava accessions according to the clusters formed by the Bayesian analysis. Furthermore, the DAPC allowed a significant differentiation of the cassava accessions with an F_{ST} value higher than 0.25. The number of clusters proposed by the Bayesian analysis of the 79 MLGs (seven clusters) was very close to that proposed by the DAPC (eight clusters) which suggests that the elimination of duplicates improves the accuracy of the Bayesian analysis as mentioned in a previous study (Kamvar et al., 2014). Indeed, the results of the AMOVA in relation to the clusters formed by the Bayesian analysis (50.41% between clusters) and the F_{ST} value (0.23) showed a clear increase in the discriminatory power of the Bayesian analysis. The analysis also showed a weak differentiation of the accessions in relation to breeding patterns (improved varieties and landraces) with an F_{ST} of 0.03. This weak differentiation is probably due to the fact that most of the improved varieties were grown in cassava fields, but the difficulty in identifying them correctly led us to include them in the set of farmer accessions.

Data availability statement

The names of the repository/repositories and accession number(s) can be found through this link: https://www.ebi.ac.uk/biostudies/studies/S-BSST1040?key=6eb67c56-96f6-44da-b772-14dc9d750616.

Author contributions

MS, FT, and KS: conceptualization. MS and JP: methodology. MS, DO, and EY: formal analysis. MS: data curation and writing—original draft preparation. FT, KS, JP, JM, JN, and DK: writing—review and editing. DK, JP, FT, and KS: supervision. FT and JP: project administration and funding acquisition. All authors have read and agreed to the submitted version of the manuscript.

Funding

This work was supported by the Bill and Melinda Gates Foundation and The United Kingdom Foreign, Commonwealth and Development Office (FCDO; grant no. OPP1082413) to the Central and West African Virus Epidemiology (WAVE) Program for root and tuber crops—through a subgrant from Université Félix Houphouet-Boigny (UFHB) to the Institut de l'Environnement et de Recherches Agricoles (INERA). Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission.

Acknowledgments

The authors thank farmers for providing plant material.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1202015/full#supplementary-material

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frontiers | Frontiers in Sustainable Food Systems

TYPE Original Research
PUBLISHED 20 July 2023
DOI 10.3389/fsufs.2023.1204293

OPEN ACCESS

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RECEIVED 13 April 2023
ACCEPTED 06 July 2023
PUBLISHED 20 July 2023

CITATION
Luo X, Dong R, Siddique KHM, He J and Jin Y (2023) Soybean breeding in southwestern China improved P and N utilization efficiencies by increasing phosphorus and nitrogen partitioning to pods.
Front. Sustain. Food Syst. 7:1204293.
doi: 10.3389/fsufs.2023.1204293

Soybean breeding in southwestern China improved P and N utilization efficiencies by increasing phosphorus and nitrogen partitioning to pods

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Introduction: Soybean breeding in southwestern China has vastly improved soybean yields with the increasing demand for nutrients such as phosphorus (P) and nitrogen (N). This study aimed to assess the impact of soybean breeding on P and N utilization efficiencies.

Methods: Field experiments with split-plot experimental designs were conducted at two locations [Dafang (DF) and Shiqian (SQ)] in the 2019 growing season to determine the agronomic efficiency of P fertilizer (AEp), P and N utilization efficiencies, and P and N accumulation and partitioning in different soybean organs under 0 (P0) and 35 (P35) kg ha^{-1} P supply.

Results: The results showed that soybean breeding targeting high seed yield also improved AEp ($p < 0.05$) and P ($p < 0.05$) and N utilization efficiencies ($p < 0.05$), with the improvement in AEp associated with the high yield response to P supply. P and N accumulation significantly increased in pods ($p < 0.05$) and leaves ($p < 0.05$) but not in stems or roots with year of release, while P and N concentrations did not change in any organ with year of release. In addition, only pod dry weight significantly increased ($p < 0.01$) with year of release, and P and N partitioning increased to pods ($p < 0.05$) but decreased to stems ($p < 0.05$) with year of release. Correlation and PCA analyses revealed P and N utilization efficiencies positively correlated with P and N partitioning to pods but negatively correlated with P and N partitioning to stems. While P supply increased P and N accumulation, it reduced P utilization efficiency.

Discussion: We conclude that (1) soybean breeding improved AEp and P and N utilization efficiencies; (2) the increased P and N partitioning to pods but decreased partitioning to stems contributed to the high P and N utilization efficiencies in new soybean cultivars, reducing the demand for N and P; (3) P supply increased nutrient accumulation but reduced P utilization efficiency. These results highlight the significance of appropriate resource allocation among organs and efficient P management for enhancing nutrient utilization and reducing fertilizer requirements.

KEYWORDS

nutrient utilization efficiency, genetic improvement, yield response, nutrient partitioning, nutrient accumulation

1. Introduction

Soybean (*Glycine max* (L.) Merrill) is an important crop cultivated for its rich protein content (Wu et al., 2015). However, in China, soybean production faces a shortfall in domestic soybean production, relying heavily on imports (>80%). Southwestern China, a key soybean production area, has undertaken numerous efforts, including soybean breeding, to improve soybean yields (Yang et al., 2022; Zhang et al., 2022). Breeding programs aimed at increasing seed yield have significantly improved yields (Todeschini et al., 2019; de Felipe et al., 2020), primarily through increased biomass in China (Jin et al., 2010; Yang et al., 2022), United States (Cafaro La Menza et al., 2017) and South America (Todeschini et al., 2019). Increases in harvest index have contributed to soybean yield gains worldwide (He et al., 2016; Todeschini et al., 2019; Tamagno et al., 2020; Feng et al., 2022). Changes in yield components, such as seed number and seed size, have also been associated with increased soybean seed yields in China (Wang et al., 2016; Qin et al., 2017; Zhang et al., 2022), United States (Kumudini et al., 2001), and South America (de Felipe et al., 2016). Genetic gains in soybean seed yield range from 0.4 to 2.2% y^{-1} worldwide and about 2.0% y^{-1} in southwestern China (Yang et al., 2022). Improved lodging resistance has also helped boost yields during soybean breeding (Jin et al., 2010; Kumudini et al., 2001; Rogers et al., 2015; Milioli et al., 2022). However, the impact of soybean breeding on P and N utilization efficiencies in southwestern China remains unclear.

Nutrient uptake, especially nitrogen (N) and phosphorus (P) is vital for crop growth and productivity (Jat and Bijay-Singh, 2014; Salvagiotti et al., 2021; Meng et al., 2022). Both P and N are involved in leaf photosynthesis, essential for crop growth, and high accumulation of these nutrients is important for achieving high seed yields (Tamagno et al., 2017) and efficient nutrient utilization (Meng et al., 2022). High nutrient accumulation is needed to accumulate high biomass (Cafaro La Menza et al., 2017), associated with high seed number and/or seed size and, thus, seed yield (He et al., 2019; Meng et al., 2022). Thus high-yielding soybean cultivars have high N (Salvagiotti et al., 2008; Gaspar et al., 2017) and P accumulation (He et al., 2017b, 2019), requiring increased P and N uptake through root inputs and/or modifications to root structure characteristics, such as increased adventitious root density and shallow root angle (He et al., 2017b; Lynch, 2019). Recent studies have shown that N accumulation increased with seed yield improvement during soybean breeding in Argentina (de Felipe et al., 2020) and United States (Donahue et al., 2020). However, the relative contributions of increased biomass and nutrient concentration to nutrient accumulation during soybean breeding are poorly understood. Enhancing nutrient utilization efficiencies (seed yield/total nutrient accumulation) under low fertilizer inputs can enhance yields and potentially reduce N and P demands, promoting sustainable agriculture and increasing food security (An et al., 2018; Wu et al., 2019). Nutrient utilization efficiencies have been associated with harvest index, a key trait determining grain yield (Meng et al., 2022). Genetic variations in nutrient utilization efficiencies have been reported for various crops, including wheat (Ortiz-Monasterio et al., 1997; Sadras and Lawson, 2013), maize (Ciampitti and Vyn, 2012), barley (Muurinen et al., 2006; Bingham et al., 2012), and cotton (Rochester and Constable, 2015). However, the effects of breeding on nutrient use efficiencies vary among different species. For example, P and N utilization efficiencies significantly increased in cotton cultivars released in Australia from 1973 to 2006 (Rochester and Constable, 2015), while N utilization efficiency did not change with year of release in barley cultivars (Muurinen et al., 2006). These inconsistent results suggest that changes in nutrient utilization efficiency during cultivar improvement may be species-specific.

Understanding changes in nutrient partitioning among plant organs could help reduce N and P demands by directing limited nutrients to essential organs, such as reproductive organs. For example, increased seed biomass accumulation changes the N partitioning between other plant organs (Sinclair, 1998). Nutrient partitioning is associated with biomass partitioning (Donald and Hamblin, 1976; Tamagno et al., 2017). While soybean breeding has increased plant biomass (Yang et al., 2022), it remains unclear if dry weights and nutrient accumulation have improved in all organs, how P and N partitioning among organs has changed, and how P and N utilization efficiencies have been affected.

This study investigated changes in the agronomic efficiency of P fertilizer (AEp), P and N accumulation and utilization efficiencies, and their partitioning to different organs in a historic set of 12 soybean cultivars bred for high seed yield. The study was condcuted under two P rates [0 (P0) and 35 (P35) kg ha^{-1} P] at two field sites [Dafang (DF) and Shiqian (SQ)] during the 2019 growing season. The hypotheses tested were: (1) soybean breeding has increased P and N utilization efficiencies with seed yield; (2) enhanced P and N utilization efficiencies are associated with high P and N partitioning to pods.

2. Materials and methods

This study evaluated a historic set of 12 soybean cultivars (released from 1995 to 2016, Supplementary Table S1) grown by local farmers (past and present) at two field sites [Shiqian (SQ) and Dafang (DF)] in Guizhou Province, China, in 2019. The cultivars were collected from three provinces in southwest China (Sichuan, Yunnan, and Guizhou), where soybean breeding focused on increasing seed yield. All cultivars can grow in Guizhou province, with maturity times ranging from 115 to 121 days after sowing (DAS) for SQ and 124 to 130 DAS for DF. The soil pH, total P, and plant available soil P were 6.8, 0.77 g kg^{-1} and 33 mg kg^{-1} for SQ, and 6.7, 0.92 g kg^{-1} and 33 mg kg^{-1} for DF, respectively. The mean temperature and precipitation were 849 mm and 20.2°C for SQ and 573 mm and 23.6°C for DF, respectively (Supplementary Figure S1). The split-plot design had two P levels [zero P (P0) and 35 kg ha^{-1} (P35) applied as calcium superphosphate] as the main plots, with cultivars as the sub-plots. Each cultivar in each main plot had three replicates, for a total of 72 plots at each site. Each plot was 12.8 m^2 (3.2 m wide × 4 m long), with rows spaced 0.4 m apart. The straight line between the two main plots was 2 m. Two days before sowing, N and K fertilizers were applied to all plots as urea (75 kg N ha^{-1}) and K_2SO_4 (40 kg K ha^{-1}) according to our previous study (Zhang et al., 2022), with the same N and K rates used at both experimental sites. The fertilizers were broadcast and mixed into the soil using a rotary cultivator. The seeds were sown (April 2019) at about 5 cm depth with a 40 cm row spacing. After germination, each plot was thinned to 18 seedlings per m^2. No irrigation was applied. Weeds were removed by hand, with pesticides used as needed. The upper 20 cm of soil was collected to analyze the basic nutrient status before

applying the fertilizer. Weather data were collected from weather stations near the field sites (straight-line distance ranged from 0.5–19.2 km).

2.1. Plant sampling at the R6 growth stage in 2019

Plant samples were harvested at the R6 stage when the pods contained full-sized green beans on one of the four uppermost nodes with a completely unrolled leaf (Fehr et al., 1971). For each plot, about 0.5 m^2 of soybean plants were cut just above the soil surface, placed in paper bags, transported to the laboratory, divided into pods, leaves, and stems, and oven-dried at 60°C for 72 h. After drying, the samples were weighed, stored, and later used to determine P and N concentrations. After shoot removal, a standard spade was used to excavate roots to 20 cm depth (depth of most roots), which were washed carefully to remove root-attached soil, oven-dried at 80°C for 48 h, and weighed. The root samples were stored for later determination of P and N concentrations.

2.2. P and N concentrations, accumulation, and partitioning

P and N concentrations were measured according to He et al. (2019). All samples were ground to a fine powder using an Ultra Centrifugal Mill (ZM200, Retsch, GmbH, Düsseldorf, Germany). Samples (~0.2 g) were digested with H_2SO_4-H_2O_2 to determine total N concentration using the Kjeldahl method (SKD-800, Shanghai Peiou Analytical Instruments Co. Ltd., Shanghai, China) and total P concentration using the molybdenum–stibium anti-spectrophotometry method (UV-1800 Spectrophotometer, Shanghai Meipuda Instrument Co. Ltd., Shanghai, China). P (N) accumulation in pods (stems, leaves, roots) was obtained by multiplying pod (stem, leaf, root) P (N) concentration by pod (stem, leaf, root) DW. Total P (N) accumulation was obtained by summing P (N) accumulation in different plant parts. P (N) partitioning to pod (stem, leaf, root) = P (N) accumulation in pod (stem, leaf, root)/total P (N) accumulation (Feng et al., 2021). The P and N utilization efficiencies calculated as (Meng et al., 2022):

P utilization efficiency = seed yield/total P accumulation.

N utilization efficiency = seed yield/total N accumulation.

2.3. Agronomic efficiency of P fertilizer

Two center rows (0.8 m × 4 m = 3.2 m^2) in each plot were harvested at physiological maturity (He et al., 2017a) before placing the pods into bags, transporting them to the laboratory, and oven-drying at 60°C for 72 h. Dried pods were threshed by hand to remove the seeds, which were weighed to calculate seed yield (seed weight/harvest area). The agronomic efficiency of P fertilizer (AEp) and yield response to P were calculated as:

AEp = (seed yield at P35 − seed yield at P0)/P fertilizer application rate.

Yield response to P = (seed yield at P35 − seed yield at P0)/seed yield at P0.

2.4. Statistical analyses

A three-way analysis of variance (ANOVA) analyzed the effects of genotype, P level, location, and their interactions on P and N utilization efficiencies, pod, leaf, stem, and root dry weights, P and N concentrations, and P and N accumulation, and P and N partitioning to pod, leaves, stems, and roots using the GenStat 19.0 statistical package (VSN International Ltd., Rothamsted, England). Changes in the measured parameters with year of release were fitted with a linear model for each site. The linear or sigmoid model was used to evaluate the relationships between N utilization efficiency and N accumulation and partitioning to pods, leaves, stems, and roots and between P utilization efficiency and P accumulation and partitioning to pods, leaves, stems, and roots. All data determined in the field experiment were combined to perform principle component analysis (PCA) with Origin (Pro 2023, Origin Lab, Northampton, MA, United States).

3. Results

3.1. P and N utilization efficiencies and the agronomic efficiency of P fertilizer

Soybean genotype and experimental site significantly affected P and N utilization efficiencies, while P level only affected P utilization efficiency (Supplementary Table S2). Genetic variation in P and N utilization among the 12 soybean cultivars occurred ($p < 0.001$). The P utilization efficiencies at Shiqian (SQ) ranged from 92–150 g g^{-1} (average 121 g g^{-1}) under 35 kg P ha^{-1} (P35) supply and 115–152 g g^{-1} (average 137 g g^{-1}) under 0 kg P ha^{-1} (P0) supply, and at Dafang (DF) ranged from 73–117 g g^{-1} (average 100 g g^{-1}) under P35 and 90–138 g g^{-1} (average 116 g g^{-1}) under P0 (Figure 1). P supply significantly decreased P utilization efficiency at SQ (19%; $p < 0.001$) and DF (16%; $p < 0.001$; Supplementary Table S2; Figures 1A,B). The yield response to P application ranged from 5.5–32.7 at SQ and 7.8–39.5 at DF (Figure 1).

3.2. P and N concentrations, accumulation, and partitioning to different plant parts

Soybean genotype, P level, experimental site, and their interactions significantly affected P and N concentrations in pods, leaves, stems, and roots, genotype and P level significantly affected leaf and pod dry weights, and genotype and experimental site significantly affected root and stem dry weights. For genotypes, root dry weights ranged from 0.95 to 2.78 g plant^{-1} ($p < 0.001$), and stem dry weights ranged from 5.77 to 9.45 g plant^{-1} ($p < 0.001$). Average root dry weights were 1.8 g plant^{-1} at SQ and 1.6 g plant^{-1} at DF ($p < 0.001$), and average stem dry weights were 6.7 g plant^{-1} at SQ and 7.2 g plant^{-1} at DF ($p < 0.05$; Supplementary Table S2). P supply significantly increased pod (18%, $p < 0.001$) and leaf (16%, $p < 0.001$) dry weights but did not affect stem or root dry weights (Supplementary Table S2; Figure 2). Only soybean genotype and P level affected pod and leaf P accumulation (Supplementary Table S2). P supply increased P accumulation in pods (25%, $p < 0.001$), leaves (31%, $p < 0.001$), and stems (69%, $p < 0.001$; Supplementary Table S2; Figures 3A,C,E).

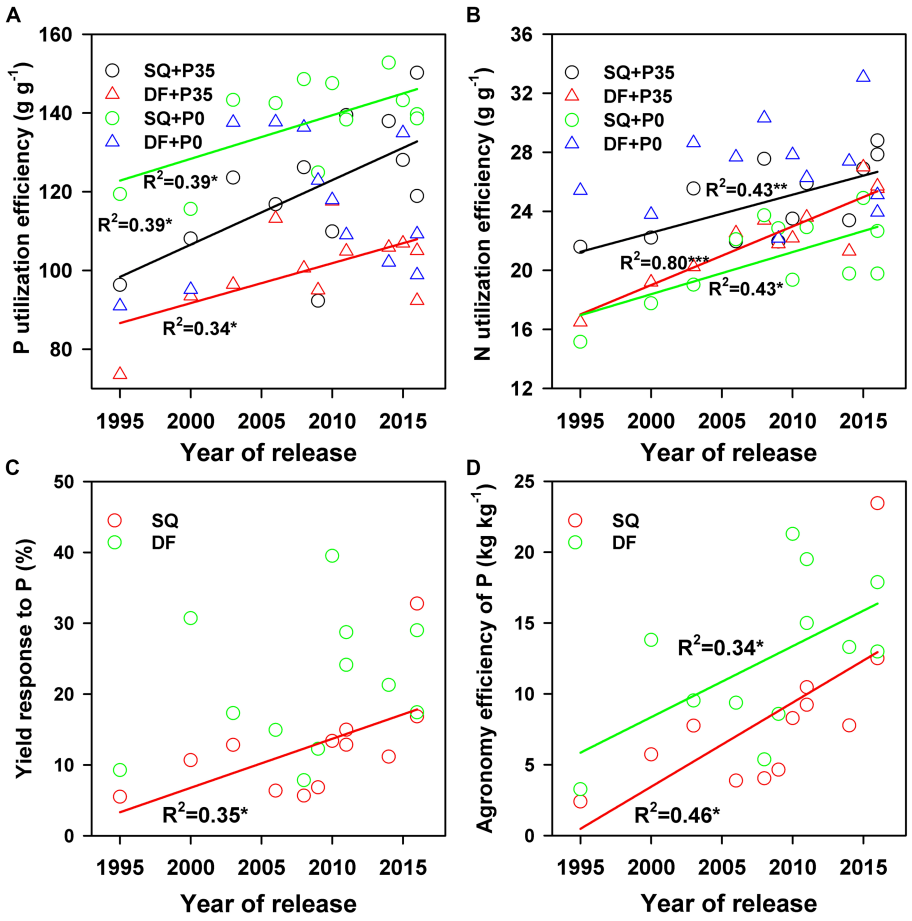

FIGURE 1
Changes in soybean **(A)** P and **(B)** N utilization efficiencies with year of release under 0 (P0) and 35 (P35) kg ha^{-1} P supply and changes in **(C)** yield response to P and **(D)** agronomy efficiency of P with year of release at Shiqian (SQ) and Dafang (DF). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Genetic variations in nutrient partitioning to different plant parts occurred ($p < 0.001$), which were significantly affected by soybean genotype, P level, experimental site, and their interactions (Supplementary Table S2). Pods had the highest P and N partitioning (average 47% for P, 51% for N), while roots had the lowest (3.5% for P, 1.3% for N; Figure 4). P supply decreased P and N partitioning to pods (9.4% for P, $p < 0.001$; 6.9% for N, $p < 0.001$) but increased P partitioning to stems (18.5% for P, $p < 0.001$; 5.9% for N, $p < 0.05$) (Supplementary Table S1; Figures 4A,E). SQ had significantly higher N partitioning to pods than DF ($p < 0.001$), and the reverse was true for N partitioning to stems ($p < 0.001$; Figures 4B,F).

3.3. Correlation analysis

P utilization efficiency positively correlated with pod P accumulation ($r = 0.44$, $p = 0.009$) but negatively correlated with leaf ($r = -0.52$, $p < 0.001$) and stem ($r = -0.62$, $p < 0.001$) P accumulation (Figure 5). N utilization efficiency positively correlated with pod N accumulation ($r = 0.55$, $p < 0.001$) but negatively correlated with stem P accumulation ($r = -0.58$, $p < 0.001$; Figure 6). P and N utilization efficiencies positively correlated with pod P ($r = 0.59$, $p < 0.001$) and N ($r = 0.51$, $p < 0.001$) partitioning but negatively correlated with stem P ($r = -0.50$, $p < 0.001$) and N ($r = -0.50$, $p < 0.001$) partitioning, respectively (Figures 5, 6). The principal component analysis showed a clear separation into two groups related to P rate (Figure 7). PC1 and PC2 represent 52.7% of the variation, with P and N utilization efficiencies, pod and root P and N partitioning, pod N concentration, and root dry weight tending to increase under P0. In contrast, P and N accumulation, leaf and pod dry weights, and leaf and pod P and N accumulation tend to increase under P35.

3.4. Changes in AEp and P and N accumulation, partitioning, and utilization efficiencies with year of release

P and N utilization efficiencies significantly ($p < 0.05$) increased from 1995 to 2016 under both P levels at both sites (Supplementary Table S2; Figures 1A,B). The yield response to P fertilizer significantly increased with year of release ($p = 0.04$) at SQ (Figure 1C). The agronomic efficiency of P fertilizer (AEp) also significantly increased ($p = 0.04$ for DF and $p = 0.016$ for SQ) during soybean breeding from 1995–2016 (Figure 1D), ranging from

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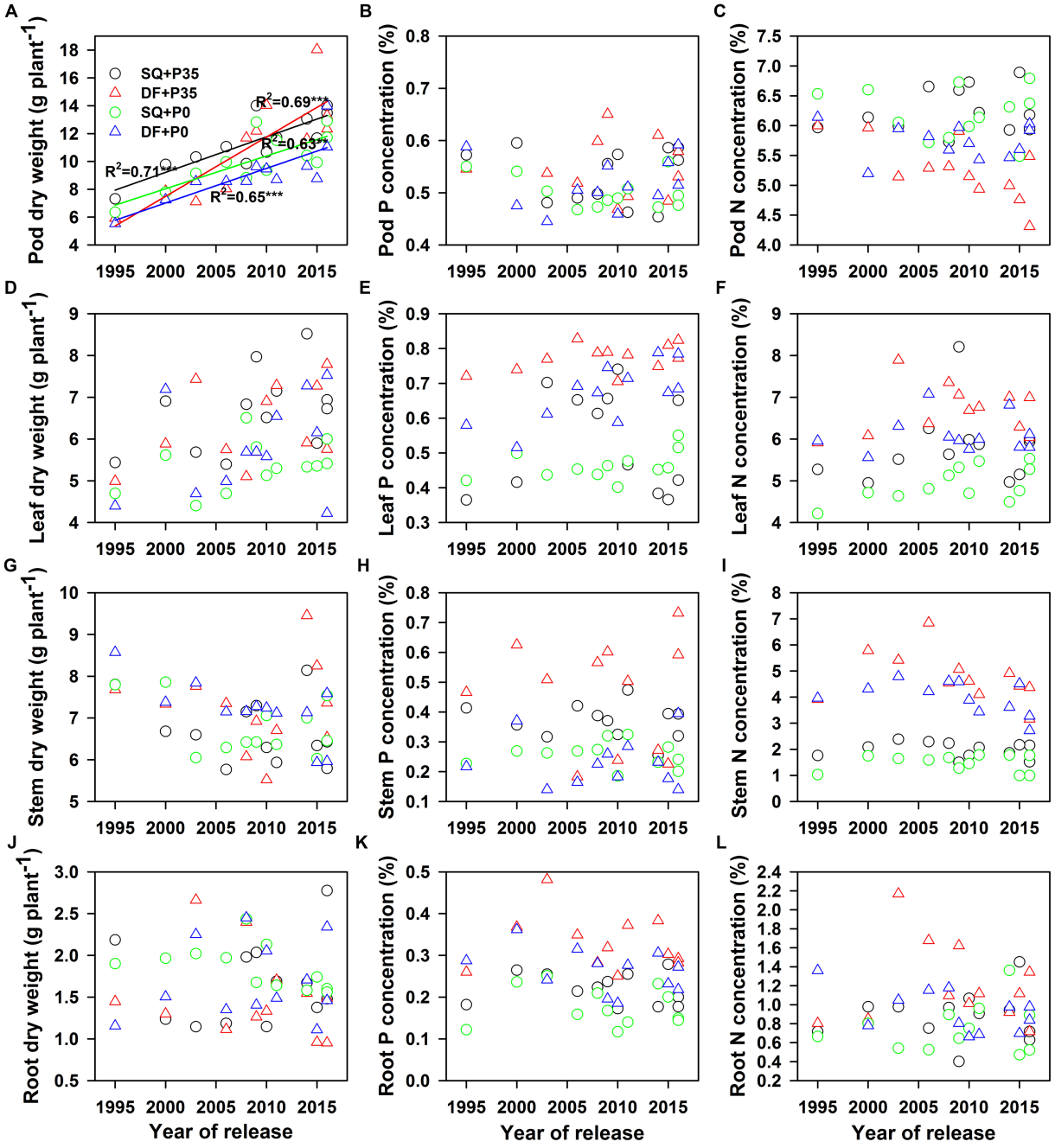

FIGURE 2
Changes in soybean **(A)** pod, **(B)** leaf, **(C)** stem, and **(D)** root dry weights and **(E)**, **(I)** pod, **(F)**, **(J)** leaf, **(G)**, **(K)** stem, and **(H)**, **(L)** root P and N concentrations with year of release under 0 (P0) and 35 (P35) kg ha^{-1} P supply at Shiqian (SQ) and Dafang (DF). $^{**}p < 0.01$ and $^{***}p < 0.001$.

2.4–23.4 at SQ and 3.3–21.3 at DF. Pod dry weight significantly increased ($p < 0.05$) with year of release at SQ and DF under P35 and P0, while leaf, stem, and root dry weights did not change (Figure 2). P and N concentrations in pods, leaves, stems, and roots did not change with year of release (Figure 2). Pod P ($p < 0.01$) and N ($p < 0.05$) accumulation significantly increased with year of release (Figure 3). Leaf P accumulation significantly increased ($p < 0.05$) with year of release; leaf N accumulation had a weak positive correlation ($p = 0.07$) with year of release under P35 (Figures 3C,D). Stem and root P and N accumulation did not change with year of release (Figures 3E–H). P and N partitioning significantly increased to pods ($p < 0.05$) but decreased to stems ($p < 0.05$) with year of release (Figure 4). P and N partitioning to leaves and roots did not change with year of release (Figure 4).

4. Discussion

The newer soybean cultivars exhibited higher seed yields and greater yield responses to P application than older cultivars. The

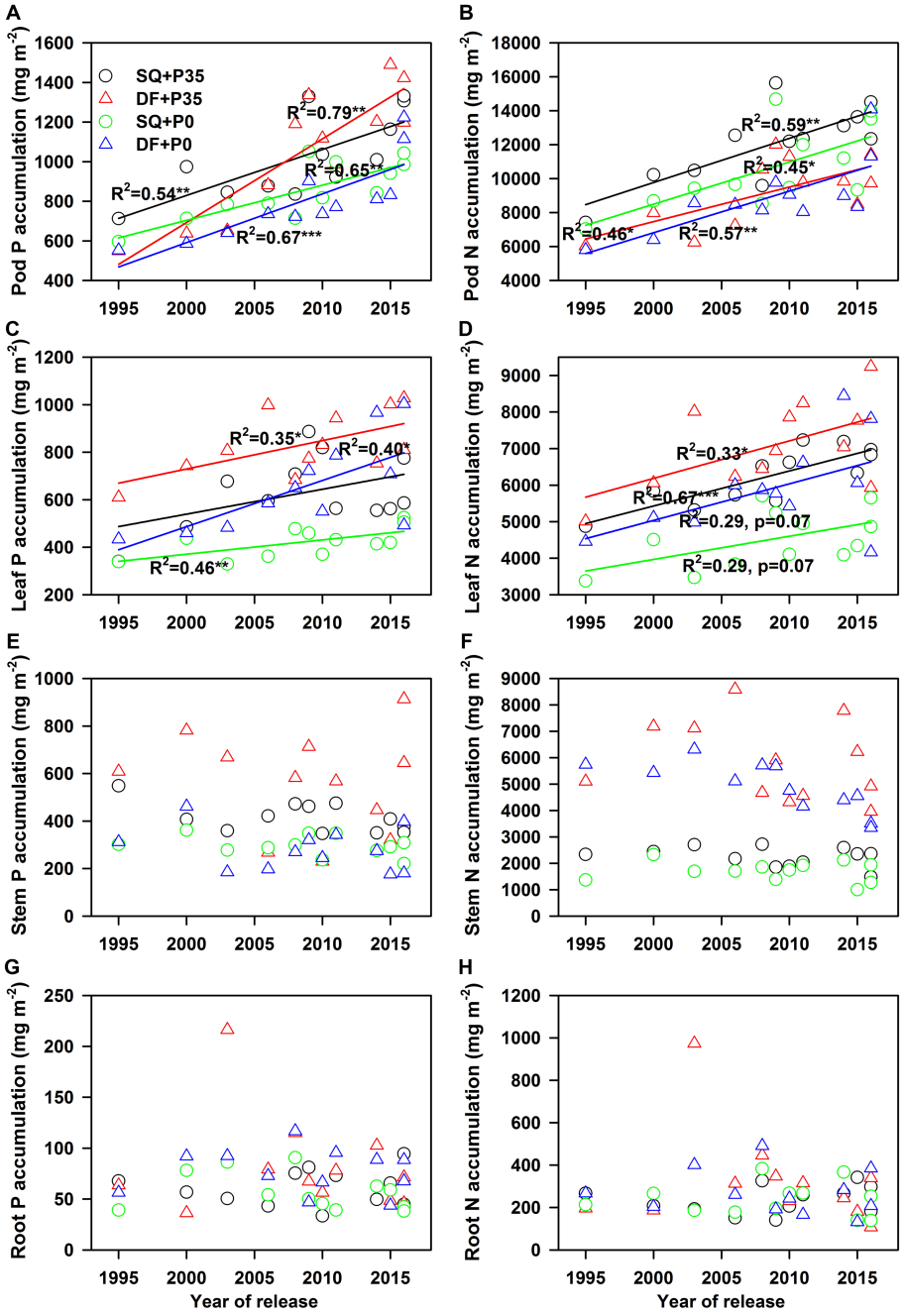

FIGURE 3
Changes in soybean **(A)**, **(B)** pod, **(C)**, **(D)** leaf, **(E)**, **(F)** stem, and **(G)**, **(H)** root P and N accumulation with year of release under 0 (P0) and 35 (P35) kg ha^{-1} P supply at Shiqian (SQ) and Dafang (DF). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

higher seed yields of newer cultivars are the result of soybean breeding efforts worldwide (Jin et al., 2010; Todeschini et al., 2019; de Felipe et al., 2020; Yang et al., 2020, 2022). The greater yield response to P application in the newer cultivars can be attributed to the significant yield improvements compared to older cultivars under P supply, leading to improved agronomic efficiency of P fertilizer (AEp). In maize, increased yield with N fertilizer supply was associated with increased grain numbers (Liu et al., 2022), indicating the important role of yield components in the yield response to fertilizer supply, such as seed number and seed size (Kumudini et al., 2001; de Felipe et al., 2016; Wang et al., 2016; Qin et al., 2017; Zhang et al., 2022). Understanding the underlying mechanisms responsible for soybean's high response to P supply, particularly related to yield components, would be valuable for future research.

Soybean breeding simultaneously increased yield and P and N accumulation, consistent with similar studies in Argentina (de Felipe et al., 2020) and United States (Donahue et al., 2020). High N accumulation positively correlated with leaf and pod biomass (Figure 7), indicating that increased soil N uptake sustains leaf and seed development (He et al., 2019; Jin et al., 2022). Similarly, high P

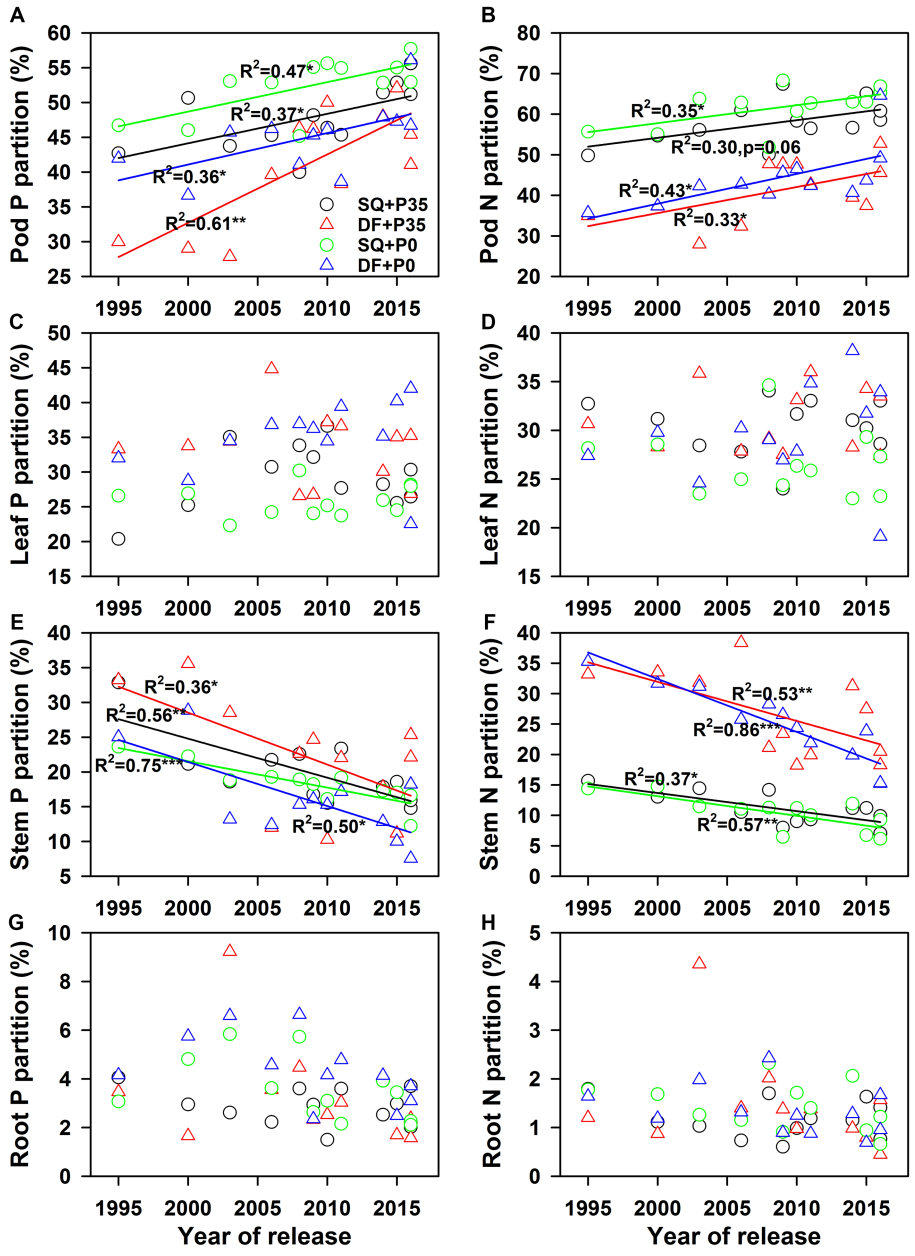

FIGURE 4
Changes in soybean **(A)**, **(B)** pod, **(C)**, **(D)** leaf, **(E)**, **(F)** stem, and **(G)**, **(H)** root P and N partitioning with year of release under 0 (P0) and 35 (P35) kg ha^{-1} P supply at Shiqian (SQ) and Dafang (DF). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

accumulation may be associated with root traits associated with P acquisition (He et al., 2017b, 2021; Lynch, 2019). While soybean breeding did not change root dry weights, which did not correlate with P or N accumulation (Figure 7), other root traits, such as shallow root growth angle, could improve P uptake (Lynch, 2019) and contribute to P and N accumulation.

In this study, biomass accumulation increased during soybean breeding, consistent with other studies (Jin et al., 2010; Todeschini et al., 2019; de Felipe et al., 2020; Yang et al., 2022), which could play a role in increasing nutrient accumulation. Soybean breeding did not change P and N concentrations despite the increase in biomass, suggesting that the increase in biomass accumulation primarily drives P and N accumulation, which is influenced by factors such as the duration after flowering (Yang et al., 2022) and/or high photosynthesis rate (Todeschini et al., 2019). However, it is important to consider the role of soil nutrient status in nutrient accumulation. For example, DF with high soil-available P had higher P and N concentrations than SQ with low soil-available P, which were associated with the high leaf and stem P and N accumulation driven by the high stem and leaf biomass. Thus, soil nutrient status can increase nutrient accumulation by increasing biomass accumulation.

P and N accumulation increased with P supply. In addition, P and N accumulation positively correlated with leaf and pod dry weights (Figure 7) without diluting P and N concentrations,

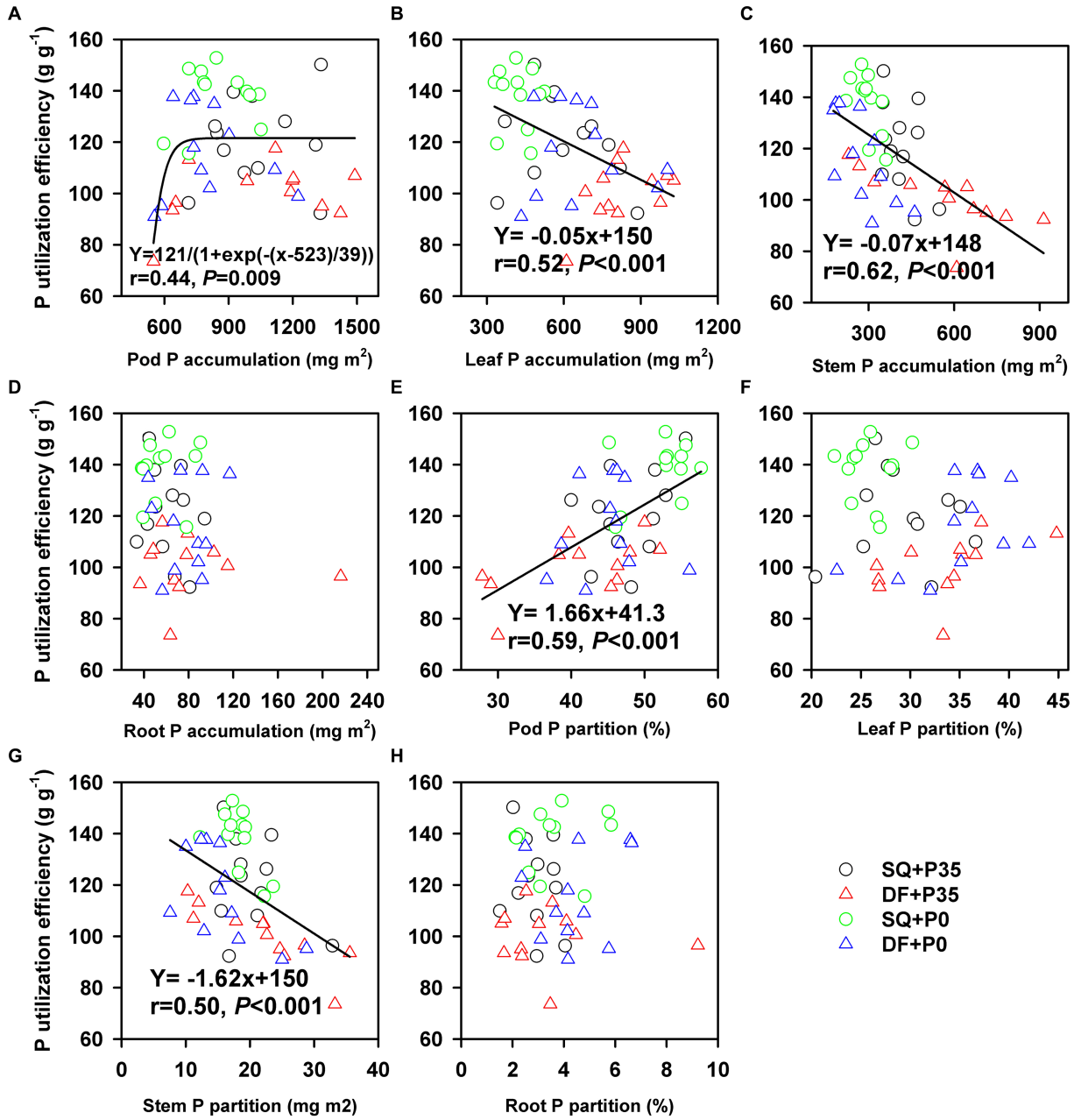

FIGURE 5
Relationship between P utilization efficiency and **(A)** pod P accumulation, **(B)** leaf P accumulation, **(C)** stem P accumulation, **(D)** root P accumulation, **(E)** pod P partitioning, **(F)** leaf P partitioning, **(G)** stem P partitioning, and **(H)** root P partitioning under 0 (P0) and 35 (P35) kg ha^{-1} P supply at Shiqian (SQ) and Dafang (DF).

indicating that biomass accumulation with P supply primarily drove pod and leaf P accumulation. Pods had significantly higher genetic gains in P and N accumulation (average 26.9 mg m^{-2} y^{-1} for P, 239 mg m^{-2} y^{-1} for N) than leaves (11.9 mg m^{-2} y^{-1} for P, 91 mg m^{-2} y^{-1} for N), demonstrating that pods contributed more to P and N accumulation than leaves. The high genetic gains of pod P and N accumulation were also associated with the high demand for P and N during seed development; furthermore, the partitioning of P and N from leaves to seeds also contributed to pod P and N accumulation (Gaspar et al., 2017). Thus, enhanced pod P and N accumulation was associated with high seed numbers, a key driver for seed yield improvement during soybean breeding (Jin et al., 2010; He et al., 2019; Yang et al., 2022).

4.1. Nutrient partitioning and utilization efficiency

Soybean breeding improved P and N utilization efficiencies, supporting our first hypothesis. Seed yield had a higher genetic gain (average 2.0% y^{-1}) than P (1.2% y^{-1}) or N accumulation (1.0% y^{-1}), contributing to improved P and N utilization efficiencies. Similar

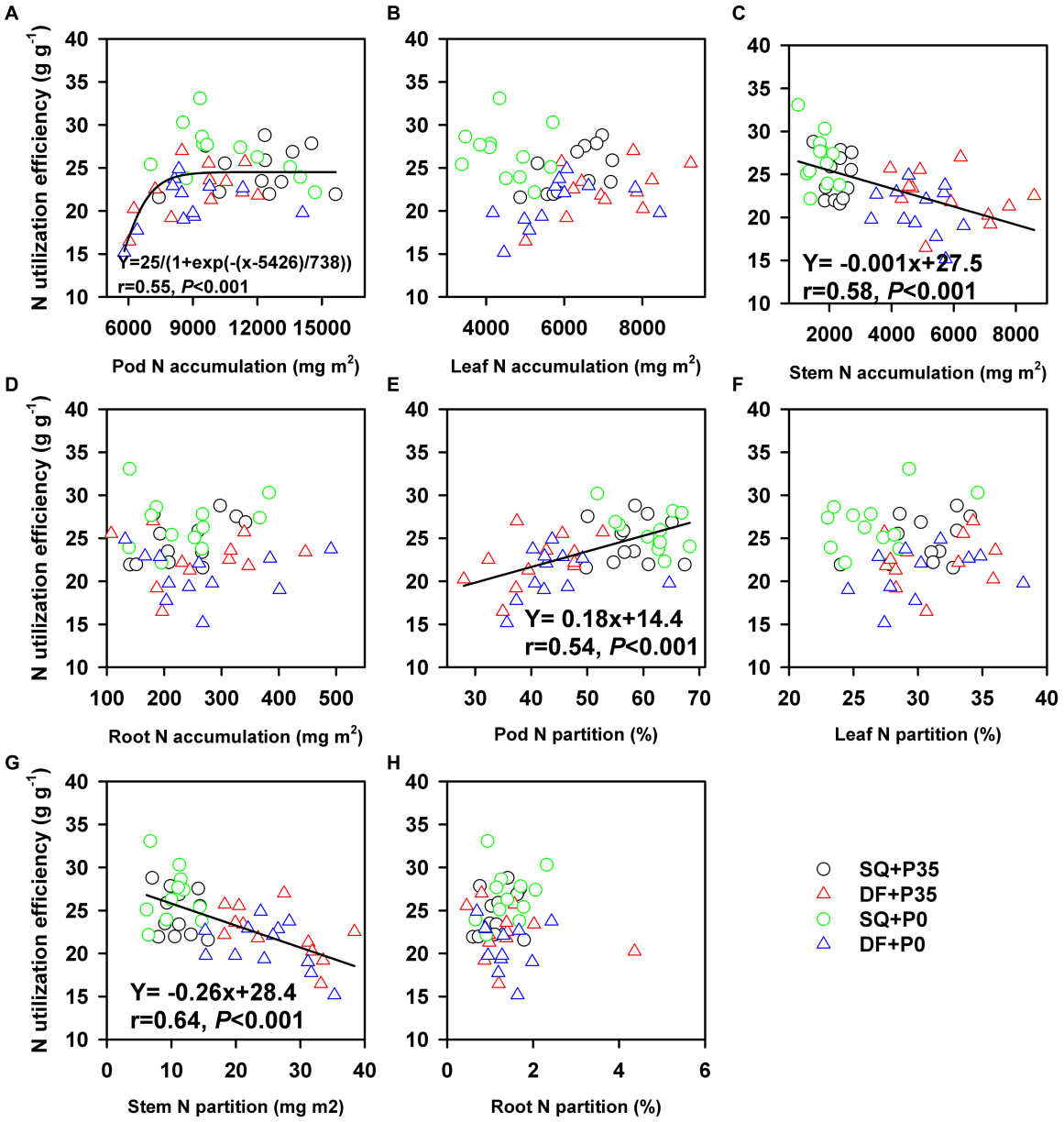

FIGURE 6
Relationship between N utilization efficiency and **(A)** pod N accumulation, **(B)** leaf N accumulation, **(C)** stem N accumulation, **(D)** root N accumulation, **(E)** pod N partitioning, **(F)** leaf N partitioning, **(G)** stem N partitioning, and **(H)** root N partitioning under 0 (P0) and 35 (P35) kg ha^{-1} P supply at Shiqian (SQ) and Dafang (DF).

improvements in P and N utilization efficiencies have been observed in other crops, such as wheat (Ortiz-Monasterio et al., 1997; Sadras and Lawson, 2013), cotton (Rochester and Constable, 2015), and rice (Meng et al., 2022). However, it is worth noting that P and N utilization efficiencies can vary among cultivars and crops (Dhugga and Waines, 1989; Ortiz-Monasterio et al., 1997; Ciampitti and Vyn, 2012), as observed in the soybean cultivars used in this study. Soybean had higher P and N utilization efficiencies (74–152 g g^{-1} for P, 15–22 g g^{-1} for N) than cotton (65–80 g g^{-1} for P, 12–15 g g^{-1} for N; Rochester and Constable, 2015), but lower P and N utilization efficiencies than rice (159–180 g g^{-1} for P, 45–54 g g^{-1} for N; Meng et al., 2022), indicating room for improvement in soybean. The P supply reduced the P utilization efficiency (Figure 7) at both sites, possibly because the rate of seed yield improvement (16.8%) with P supply was lower than the rate of P accumulation (33.2%). Despite the impact of soybean breeding on P utilization efficiency, the PUE was significantly affected by the interaction of genotype and P rate ($p < 0.01$). Moreover, SQ with low plant-available soil P had a significantly higher P utilization efficiency than DF with high soil-available P, highlighting the importance of soil P status and P management practices in regulating P utilization efficiency. One possible explanation for the difference between the two sites is the lower average P accumulation at SQ (1.8 g m^{-2}) than DF (2.2 g m^{-2}) but similar seed yield.

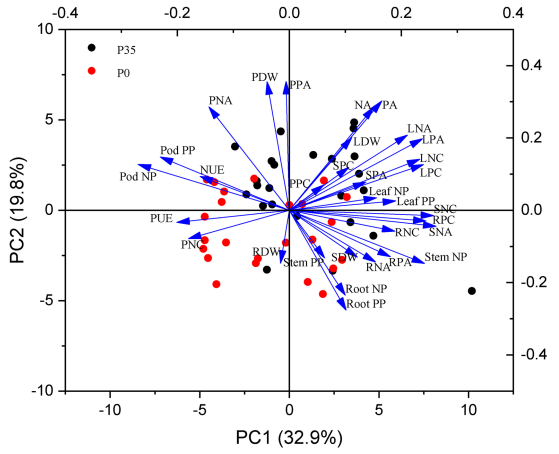

FIGURE 7
The principle component analysis (PCA) of the measured parameters in a historic set of 12 soybean cultivars under 0 (P0) and 35 (P35) kgPha^{-1} supply in two experiment sites. PUE, P utilization efficiency; NUE, N utilization efficiency; PDW, pod dry weight; LDW, leaf dry weight; SDW, stem dry weight; RDW, root dry weight; PPC, pod P concentration; LPC, leaf P concentration; SPC, stem P concentration; RPC, root P concentration; PNC, pod N concentration; LNC, leaf N concentration; SNC, stem N concentration; RNC, root N concentration; PPA, pod P accumulation; LPA, leaf P accumulation; SPA, stem P accumulation; RPA, root P accumulation; PA, P accumulation; PNA, pod N accumulation; LNA, leaf N accumulation; SNA, stem N accumulation; RNA, root N accumulation; NA, P accumulation; Pod PP, pod P partition; Leaf PP, leaf P partition; Stem PP, stem P partition; Root PP, root P partition; Pod NP, pod N partition; Leaf NP, leaf N partition; Stem NP, stem N partition; Root NP, root N partition.

In this study, P and N partitioning significantly increased to pods but decreased to stems during soybean breeding, with this trend consistent across the two P rates tested. Decreased nutrient partitioning to low-demand organs such as stems and increased partitioning to seeds can enhance yield (Weiner, 2019). This trade-off between P and N partitioning in pods and stems during soybean breeding was important for improving nutrient utilization efficiencies (Ciampitti and Vyn, 2012, 2013). The increase in P and N partitioning to seeds is likely associated with the increase in seed number, the key driver of yield improvement during crop breeding (Jin et al., 2010; Qin et al., 2017; Meng et al., 2022; Zhang et al., 2022). More seeds require increased P and N to support seed development and quality, including protein formation. Thus, increasing seed yield through higher seed numbers could increase P and N partitioning to seeds and subsequently improve P and N utilization efficiencies. In addition, nutrient partitioning may be related to dry matter partitioning, as indicated by the increased harvest index (pod harvest index) and decreased dry matter partitioning to stems during soybean breeding (Yang et al., 2022). On the other hand, the trade-off between P and N partitioning to pods and stems could reduce P and N demand, as supported by the lower genetic gains for P (average 1.2% y^{-1}) and N accumulation (1.0% y^{-1}) than seed yield (2.0% y^{-1}). Thus P and N partitioning can help improve P and N utilization efficiencies, supporting our second hypothesis.

5. Conclusion

This study evaluates whether soybean breeding increased P and N utilization efficiencies in southwestern China. We confirmed that selecting for high seed yield during soybean breeding improved P and N utilization efficiencies, attributed to increased P and N partitioning to pods while reducing their partitioning to stems. The increased P and N accumulation in pods, driven by pod biomass, played a significant role in the overall P and N accumulation during soybean breeding. We conclude that soybean breeding increased P and N utilization efficiencies by regulating P and N accumulation and partitioning.

Data availability statement

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

Author contributions

JH and YJ conceived the experiments. XL and RD performed the experiments. XL, YJ, and RD analyzed the data. XL, KS, and JH wrote this manuscript. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the National Natural Science Foundation of China (32060427), Guizhou Science and Technology Support Program Project (Qiankehezhicheng (2021) yiban217).

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/fsufs.2023.1204293/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 18 September 2023
DOI 10.3389/fpls.2023.1151765

OPEN ACCESS

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RECEIVED 26 January 2023
ACCEPTED 14 August 2023
PUBLISHED 18 September 2023

CITATION
Choi H, Back S, Kim GW, Lee K, Venkatesh J, Lee HB, Kwon J-K and Kang B-C (2023) Development of a speed breeding protocol with flowering gene investigation in pepper (*Capsicum annuum*). *Front. Plant Sci.* 14:1151765. doi: 10.3389/fpls.2023.1151765

Development of a speed breeding protocol with flowering gene investigation in pepper (*Capsicum annuum*)

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Pepper (*Capsicum* spp.) is a vegetable and spice crop in the Solanaceae family with many nutritional benefits for human health. During several decades, horticultural traits, including disease resistance, yield, and fruit quality, have been improved through conventional breeding methods. Nevertheless, cultivar development is a time-consuming process because of the long generation time of pepper. Recently, speed breeding has been introduced as a solution for shorting the breeding cycle in long-day or day-neutral field crops, but there have been only a few studies on speed breeding in vegetable crops. In this study, a speed breeding protocol for pepper was developed by controlling the photoperiod and light quality. Under the condition of a low red (R) to far-red (FR) ratio of 0.3 with an extended photoperiod (Epp) of 20 h (95 $\pm$ 0 DAT), the time to first harvest was shortened by 75 days after transplant (DAT) compared to that of the control treatment (170 $\pm$ 2 DAT), suggesting that Epp with FR light is an essential factor for flowering in pepper. In addition, we established the speed breeding system in a greenhouse with a 20 h photoperiod and a 3.8 R:FR ratio and promoted the breeding cycle of C. *annuum* for 110 days from seed to seed. To explain the accelerated flowering response to the Epp and supplemented FR light, genome-wide association study (GWAS) and gene expression analysis were performed. As a result of the GWAS, we identified a new flowering gene locus for pepper and suggested four candidate genes for flowering (*APETALA2* (*AP2*), *WUSCHEL-RELATED HOMEOBOX4* (*WOX4*), *FLOWERING LOCUS T* (*FT*), and *GIGANTEA* (*GI*)). Through expression analysis with the candidate genes, it appeared that Epp and FR induced flowering by up-regulating the flowering-promoting gene *GI* and down-regulating *FT*. The results demonstrate the effect of a combination of Epp and FR light by genetic analysis of flowering gene expression. This is the first study that verifies gene expression patterns associated with the flowering responses of pepper in a speed breeding system. Overall, this study demonstrates that speed breeding can shorten the breeding cycle and accelerate genetic research in pepper through reduced generation time.

KEYWORDS

pepper, speed breeding, photoperiod, far-red light, genome-wide association study, gene expression analysis

1 Introduction

Pepper is a great source of vitamin C and β-carotene, and the only plant genus that synthesizes capsaicinoids (Srivastava and Mangal, 2019). Pepper capsaicinoids are used in food ingredients due to their unique taste, and also have physiological and pharmacological health benefits including anticancer, anti-inflammatory, and anti-obesity properties (Aza-González et al., 2011). Owing to these nutritional benefits, producing high-quality cultivars is one of the major goals of pepper breeding.

The long generation time of pepper is a bottleneck in breeding programs; it takes 7-8 years to develop a cultivar. To mitigate this issue, there have been various technologies to shorten the generation time. The most well-known approach is shuttle breeding, which uses two distinct fields with different climates (Ortiz et al., 2007). Shuttle breeding enables only two generations per year by growing breeding populations at two locations where plants can produce seeds (Ortiz et al., 2007). For a successful shuttle breeding approach, two fields with different environmental requirements are needed for testing and generation advancement, which can be laborious and expensive. Doubled haploid (DH) technology can generate homozygous lines rapidly by bypassing the process of inbreeding (Gerald et al., 2013). However, each step of DH is strongly genotype-dependent, and this method is laborious, expensive, and requires high skills to obtain a successful product (Gerald et al., 2013). Genome-editing technologies can also generate desired plants by directly editing target genes (Araki and Ishii, 2015). However, this approach is difficult to applying to certain species and requires skilled technicians (Araki and Ishii, 2015). To overcome these issues, Watson et al. (2018) proposed a simple but effective technology, 'speed breeding'.

The response of crops to continuous light conditions has been the subject of scientific investigation for many years. In the 1980s, NASA conducted an experiment in which crops, including wheat, soybean, lettuce, and potato, were grown under constant light conditions. It was observed that the total biomass of these crops strongly depended on the amount of light provided to the plants (Wheeler et al., 1996). O'Connell (2007) focused specifically on exploring how wheat plants respond to continuous light and investigated the potential implications of continuous light on wheat growth and development. O'Connor et al. (2013) described a speed breeding technique that utilized controlled environment conditions, continuous light, and a single seed descent breeding strategy in a greenhouse to reduce the generation time of full-season maturity peanut cultivars from 145 to 89 days, potentially accelerating the development of new varieties. Inspired by previous works, Watson et al. (2018) coined the term 'speed breeding' and developed protocols for shortening the generation time of long-day field crops by extending the photoperiod. They achieved up to 6 generations per year for wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), chickpea (*Cicer arietinum*), and pea (*Pisum sativum*) compared to the 2 to 3 generations of traditionally grown crops. Although speed breeding is a powerful alternative for reducing generation time in long-day plants, this method has rarely been applied to short-day plants. Recently, Jähne et al. (2020) demonstrated a protocol for short-day crops soybean (*Glycine max*), rice (*Oryza sativa*), and amaranth (*Amaranthus* spp.), adjusting the photoperiod to 10 h and adjusting the light intensity and quality. Far-red (FR) light allowed flowering time reduction in some amaranth and rice genotypes, but there was no impact on soybeans. Therefore, crop-specific LED lighting schemes will need to be developed (Jähne et al., 2020). Previous studies on speed breeding primarily focused on field crops, but research for vegetable crops is scarce. Liu et al. (2022) developed a speed breeding scheme for hot pepper through light environment modification. They investigated the growth and development of hot pepper varieties ('Xiangyan 55' and 'Xiangla 712') under various light intensities (240, 300, 360, and 420 PPFD), photoperiods (14 h, 16 h, 18 h, and 20 h), and R:FR ratios (2.1, 2.7, 3.8, and 6.3) (Liu et al., 2022). They reported that light intensities of 300 and 420 PPFD, a photoperiod of 20h, and an R:FR ratio of 3.8 were effective in shortening the flowering time for hot peppers (Liu et al., 2022). However, they did not confirm the effect of combining all the conditions of light intensity, photoperiod, and supplementary FR. Since the plant's response to the speed breeding conditions in growth chambers or plant factories can be different from in actual greenhouse conditions, further study for applying comprehensive speed breeding in the greenhouse needs to be done. Moreover, the genetic and molecular basis of speed breeding has not been fully elucidated.

Flowering time is controlled by the interaction of endogenous and exogenous signals such as photoperiod, temperature, and plant hormones (Srikanth and Schmid, 2011). Depending on the environmental signals, the pattern of flowering gene expression is diverse (Cho et al., 2017; Ding et al., 2020). Previous studies on Arabidopsis, a model plant, have revealed many flowering-associated genes and flowering gene expression patterns according to environmental controls (Mouradov et al., 2002). In contrast, there are few studies on the expression patterns of flowering genes in pepper under environmental change (extending day length, adding FR, etc.). Moreover, although flowering genes are conserved between species, they are likely to have very different functions in each species. For example, *SHORT VEGETATIVE PHASE* (*SVP*) acts as a major flowering repressor together with *FLOWERING LOCUS C* (*FLC*) in Arabidopsis (Li et al., 2008), while its homolog, *CaJOINTLESS*, acts as a major flowering activator in pepper (Cohen et al., 2012). Therefore, a genetic study is required for each particular species. Furthermore, it is necessary to analyze the changes in flowering gene expression in a controlled environment specific to each crop speed breeding system. This genetic analysis would identify the cause of accelerated flowering time under speed breeding.

In this paper, we established a speed breeding system suitable for pepper by shortening the generation time of pepper *via* extending the day length to 20 h, controlling light quality by supplementing FR, and adjusting the R:FR ratio. We then demonstrated the effectiveness of this protocol on a genetic and molecular basis. Moreover, we applied the speed breeding system in the greenhouse and established a speed breeding protocol for *C. annuum*, enabling us to shorten the breeding pipeline in less than three years. To identify the genetic loci associated with flowering time, we performed a genome-wide association study (GWAS) and investigated the expression levels of candidate genes for comparative analysis of the flowering gene expression patterns between the speed breeding system and the normal environmental growing conditions. It is expected that this study will have a significant impact on breeding and genetic research by

shortening the breeding cycle, and the GWAS-identified candidate genes for loci associated with shortened flowering time provide valuable information about the genetic control of flowering time in pepper.

2 Materials and methods

2.1 Plant materials

For setting the speed breeding system for pepper, *Capsicum annuum* 'MicroPep Red' ('MR') from germplasm of the Horticultural Crops Breeding and Genetics Laboratory (Seoul National University, Republic of Korea) was used as the plant material of this study. 'MR' has a rapid generation time and short plant height compared to other cultivars.

For GWAS, 220 C. *annuum* accessions were used (Lee et al., 2020). The 220 accessions comprising the GWAS population were grown in a greenhouse at the RDA-GenBank (Jeonju, Republic of Korea) in 2015, 2016, and 2017. Over three years, six plants per accession were randomly planted, and three plants per accession were evaluated for flowering time (Lee et al., 2020).

2.2 Extending the photoperiod experiment

For each treatment, a multi-room chamber (Hanbaek, Bucheon, Republic of Korea) was programmed to have a day and night cycle of 12/12 h, 16/8 h, 20/4 h, and 24/0 h. In all the treatments, the temperature was set at 26°C during the day and 20°C during the dark (Table 1). The light source was a fluorescent lamp, and the light intensity was adjusted to 66.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at bench height (Table 1). The seeds were sown in a 72-hole seedling tray, and the seedlings were grown for 20 days in a walk-in chamber under a 16/8-hour day/night cycle and temperature conditions of 25/20°C. Subsequently, individual seedlings were transplanted into pots with a diameter of 9.2 cm and a height of 8.8 cm. The pots were filled with approximately 90% of a commercial substrate called 'Barokuh' (SeoulBio, Eumseong, Republic of Korea). The composition and ratio of the 'Barokuh' substrate were as follows: zeolite 4%, perlite 7%, pumice 6%, coco peat 68%, peat moss 14.73%, fertilizer 0.201% (containing N, P, K, Ca, Mg, Fe, Cu, Zn, B, and other elements), wetting agent 0.064%, and pH adjuster 0.005%. The physical properties of the substrate included a moisture content range of 40-60%, an air-filled porosity range of 30-50%, and a bulk density of 0.15-0.25 Mg/m^3. The chemical properties of the substrate were

TABLE 1 Growth conditions for extending the photoperiod, far-red (FR) light intensity, pepper-specific speed breeding system, and application of a speed breeding system in a greenhouse (GH).

Treatment	Photoperiod (h) (day/night)	Temperature (°C) (day/night)	Light source	Light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)			R:FR ratio
				White (W: 400-700 nm)	Red (R: 600-700 nm)	Far-red (FR: 700-780 nm)	
12/12 h	12/12	26/20	Fluorescent lamp	66.2	15.3	2.7	5.7
16/8 h	16/8	26/20	Fluorescent lamp	66.2	15.3	2.7	5.7
20/4 h	20/4	26/20	Fluorescent lamp	66.2	15.3	2.7	5.7
24/0 h	24/0	26/20	Fluorescent lamp	66.2	15.3	2.7	5.7
W45	20/4	27/24	Warm-white LED	45.0	22.2	3.0	7.3
W45+LFR	20/4	27/24	Warm-white LED +FR LED	45.2	22.3	18.8	1.2
W45+MFR	20/4	27/24	Warm-white LED +FR LED	45.3	23.0	38.7	0.6
W45+HFR	20/4	27/24	Warm-white LED +FR LED	45.2	23.3	78.2	0.3
Ctl	12/12	26/20	Fluorescent lamp	66.2	15.3	2.7	5.7
Ctl+FR	12/12	26/20	Fluorescent lamp +FR LED	66.2	17.0	56.5	0.3
Epp	20/4	26/20	Fluorescent lamp	66.2	15.4	2.7	5.8
Epp+FR	20/4	26/20	Fluorescent lamp +FR LED	66.2	17.0	57.1	0.3
Normal GH	Natural light	Suwon farm condition	Natural light	152.8	55.1	40.3	1.4
Epp GH	20/4	Suwon farm condition	Natural light +LED (400-700 nm)	319.7	151.3	31.8	4.8
Epp+FR GH	20/4	Suwon farm condition	Natural light +LED (400-772 nm)	450.7	283.2	74.7	3.8

characterized by a pH range of 5.5-7.0 and an electrical conductivity (EC) of 0.65 ds/m. Throughout the cultivation period, no additional fertilizers were applied. Each pot was watered once daily in the morning using approximately 150-200 ml of tap water. Eight plants of 'MR' at two leaves (at least 1cm) were transplanted for testing the effect of extended photoperiod on flowering time and phenotyped for number of days to transition (floral meristem appearance) and flowering from days after transplanting (DAT).

2.3 FR light intensity experiment

The FR light intensity experiment was conducted in the walk-in chamber at Seoul National University Farm (Suwon, Republic of Korea). This walk-in chamber was programmed to run a 20 h photoperiod and 4 h dark period with a temperature of 27°C during the day and 24°C during the night (Table 1). The relative humidity was set to 70%. Warm-white (W) and FR LEDs were used to test the effect of FR light on accelerating the transition from the vegetative to the reproductive stage. Light intensity (400-700nm) was set at 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (W LEDs; W45) in all four treatments: W45 treatment (non-FR), W45+LFR (low FR), W45+MFR (medium FR), and W45+HFR (high FR) (Table 1). The light intensity of FR light in LFR, MFR, and HFR was 18.8, 38.7, and 78.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ each (Table 1 and Supplementary Figure 1). The 'MR' seeds were directly sown in the 72 hole seedling tray and grown in each treatment of the walk-in chamber without undergoing a separate seedling growth process. The basic cultivation conditions, such as pot size, amount of media, composition of the media, and water management, remained consistent with those described in the "Extending the photoperiod experiment" section. Ten plants of 'MR' were grown in each treatment and plant height (distance from the shoot apical meristem to the soil) and time to floral meristem were recorded.

2.4 Pepper-specific speed breeding system

Plants were grown in a multi-room chamber (Hanbaek, Bucheon, Republic of Korea). For all the treatments: Control (Ctl), Control+Far-red light (Ctl+FR), Extended photoperiod (Epp), and Extended photoperiod+Far-red light (Epp+FR), each chamber room was programmed at 26°C during the day and 20°C at night (Table 1). In Ctl and Ctl+FR treatments, each chamber room was set to have a 12 h photoperiod and 12 h dark period (Table 1). In the case of the Epp and Epp+FR treatments, the day and night lengths were set to 20 h and 4 h (Table 1). In Ctl+FR and Epp+FR treatments, FR light was supplemented with R:FR=0.3 (Table 1 and Supplementary Figure 2). The light intensity of the fluorescent lamp for all the treatments was adjusted to 66.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Table 1). The seeds were sown in the 72 hole seedling tray, and the seedlings were cultivated for 20 days in a walk-in chamber with a day/night cycle of 16/8 hours and temperature settings of 25/20°C. The basic cultivation conditions, such as pot size, amount of media, composition of the media, and water management, remained consistent with those described in the "Extending the photoperiod experiment" section. In each experimental treatment, a total of 10 'MR' plants at the 1-2 leaf stage (with a minimum length of 1 cm) were transplanted. Various parameters were recorded, including the duration of the transition phase, the timing of the first flower appearance, the onset of first fruit production, the ripening of the fruits, the number of fruits produced, and the distance from the shoot apical meristem to the soil surface. The observed data pertaining to these parameters have been organized and presented in Tables 2, 3. Specifically, the parameter labeled as 'Transition' denotes the critical moment when the floral meristem becomes visible, signaling the emergence of the first flower bud. 'First flower' represents the precise point in time when the initial flower blossoms on the plant. 'First fruit' characterizes the developmental stage when the plant generates its first fruit. 'First ripening' captures the significant stage at which the first fruit reaches full maturity, acquiring a vibrant red coloration referred to as the mature red stage.

2.5 Application of a speed breeding system in the greenhouse

For applying the pepper-specific speed breeding system in the greenhouse, a system was devised in the greenhouse at Seoul National University Farm (Suwon, Republic of Korea). The 'MR' seeds were directly sown in the 72-hole seedling tray and grown in each treatment of the greenhouses during the fall season in Suwon, Republic of Korea. The basic cultivation conditions, such as pot size, amount of media, composition of the media, and water management, remained consistent with those described in the "Extending the photoperiod experiment." However, approximately 85 days after transplantation in the greenhouse, an additional granular fertilizer (Inovatec, Jeongeup, Republic of Korea) was applied at a rate of approximately 5 pellets per pot. The fundamental cultivation conditions, including pot size, amount of media, composition of the media, and water management, remained consistent with those described in the "Extending the photoperiod experiment". For the normal greenhouse (Normal GH) condition, the natural light and temperature in Suwon Farm were used. For the treatments of extended photoperiod in GH (Epp GH) and extended photoperiod with supplemented FR in GH (Epp+FR GH), the 20 h photoperiod and 4 h dark period were used (Table 1). Three plants for each treatment were analyzed. The light spectrum of LED (Bissol, Seoul, Korea) was 400-700 nm for Epp GH and 400-772 nm for Epp+FR GH (Table 1). The ratio of red to FR was adjusted to 4.8 in Epp GH and 3.8 in Epp+FR GH (Table 1).

2.6 Genomic DNA extraction

Total genomic DNA (gDNA) was extracted from three young leaves of plants using a modified cetyltrimethylammonium bromide (CTAB) method (Porebski et al., 1997). The extracted DNAs were quantified with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The gDNA concentration was measured and diluted to 20 $ng \cdot \mu L^{-1}$.

TABLE 2 Development of *Capsicum annuum* 'MicroPep Red' ('MR') under the treatments of extending the photoperiod experiment, far-red (FR) light intensity experiment, pepper-specific speed breeding system, and application of speed breeding in greenhouse (GH).

Experiment	Treatment	Days after transplant to **			
		Transition	First flower	First fruit	First ripening
Extending the photoperiod experiment	12/12 h	28.9±2.5 [a]	43.6±3.7 [a]	–	–
	16/8 h	28.9±2.5 [a]	43.1±4.3 [a]	–	–
	20/4 h	28±0 [a]	37.5±2.1 [b]	–	–
	24/0 h	23.6±3.6 [b]	36.5±2.1 [b]	–	–
FR light intensity experiment (day/night: 20/4 h)	W45	25.9±3.4 [a]	–	–	–
	W45+LFR	23.9±4.4 [ab]	–	–	–
	W45+MFR	21.7±4.3 [bc]	–	–	–
	W45+HFR	18.6±0.5 [c]	–	–	–
Pepper-specific speed breeding system (day/night - Ctl: 12/12 h - Epp: 20/4 h)	Ctl	36±2 [a]	56±2 [a]	113±5 [a]	170±2 [a]
	Ctl+FR	29±5 [b]	50±6 [b]	106±8 [a]	163±2 [b]
	Epp	24±5 [c]	45±5 [c]	88±6 [b]	130±3 [c]
	Epp+FR	19±4 [d]	36±2 [d]	53±8 [c]	95±0 [d]
Application of speed breeding in GH (day/night - Normal: natural light - Epp: 20/4 h)	Normal GH	28± [a]	51±0 [a]	71±0 [a]	128±0 [a]
	Epp GH	21±0 [b]	28±0 [b]	41±0 [b]	84±0 [b]
	Epp+FR GH	7±0 [c]	21± [c]	28±0 [c]	71±0 [c]

*Means ± SD with the same letter in the stages of each experiment are not significantly different at the P = 0.05 level according to Duncan's multiple range test.
**Days after germination in the FR light intensity experiment.

2.7 GWAS and candidate gene identification

The GWAS accessions were genotyped using the genotyping-by-sequencing (GBS) method based on the *PstI*/*MseI* and *EcoRI*/*MseI* restriction enzymes as previously described (Lee et al., 2020). The digested DNA was ligated to adapters and the libraries were amplified using 'TA' primers (Lee et al., 2020). The libraries were pooled in five tubes, and the pooled libraries were sequenced using an Illumina HiSeq2000 sequencing system (Illumina, San Diego, CA, United States) at Macrogen (Seoul, South Korea). Filtered raw reads of GWAS population accessions were aligned to the *C.*

TABLE 3 Plant height and number of fruits of *Capsicum annuum* 'MicroPep Red' ('MR') from far-red (FR) light intensity experiment, pepper-specific speed breeding system, and application of speed breeding in greenhouse (GH).

Experiment	Treatment	Plant height (cm)	No. of fruit
FR light intensity experiment (day/night: 20/4 h)	W45	9.3±0.6 [a]	–
	W45+LFR	9.2±0.9 [a]	–
	W45+MFR	10.6±0.9 [b]	–
	W45+HFR	16.4±1.0 [c]	–
Pepper-specific speed breeding system (day/night - Ctl: 12/12 h - Epp: 20/4 h)	Ctl	8.6±0.5 [a]	1.7±0.8 [a]
	Ctl+FR	21.3±0.8 [b]	2.8±1.2 [a]
	Epp	9.5±0.5 [c]	2.7±1.0 [a]
	Epp+FR	21.7±1.1 [b]	3.0±1.7 [a]
Application of speed breeding in GH (day/night - Normal: natural light - Epp: 20/4 h)	Normal GH	2.9±0.2 [a]	3±0 [a]
	Epp GH	2.9±0.2 [b]	3±0 [a]
	Epp+FR GH	5.5±0.4 [b]	27.8±3.7 [b]

*Means ± SD with the same letter in the stages of each experiment are not significantly different at the P = 0.05 level according to Duncan's multiple range test.

annuum 'Dempsey' reference genome (Lee et al., 2022) using the Burrows-Wheeler Aligner (Hong et al., 2020). Alignment procedures yielded various types of variants, from which single nucleotide polymorphisms (SNPs) were exclusively extracted. These SNPs underwent a filtering process conditioned on parameters of a quality score (QUAL) < 30, a mapping quality (MQ) < 30.00, a Strand Odds Ratio (SOR) > 4.000, and a depth of coverage (DP) < 3 to ensure data reliability. A further filtering step followed, operating under a Minor Allele Frequency (MAF) cutoff of 0.05% and a call rate threshold of 50%. This filtering procedure resulted in the acquisition of 221,487 SNPs for utilization in the GWAS. Prior to conducting the GWAS, an exploratory principal component analysis (PCA) of the genotypic data earmarked for GWAS revealed that two principal components accounted for over 95% of the population variance. This insight dictated the analytical strategy for the subsequent GWAS, integrating the two principal components to consider population structure appropriately. The GWAS based on the compressed mixed linear model (CMLM) was conducted using the R Package Genomic Association with default settings (Wang and Zhang, 2021). The significance threshold was set after the Bonferroni multiple-test correction, setting the significant threshold level (Bonferroni, 1936). Candidate gene prediction for flowering time was performed based on the 'Dempsey' reference genome (Lee et al., 2022).

2.8 Sequence analysis of GWAS candidate genes

PCR was performed using 5 µL of 5x GXL buffer, 100 ng of template DNA, 10 pmol of each primer, 2 µL of 10mM dNTPs, and 0.5 µL of Taq polymerase (PrimeStar GXL; Takara, Shiga, Japan). Primers were designed to amplify all the coding sequences of candidate genes. The amplified products were resolved in 1% agarose gel (Lonza, Lockland, ME, USA) and gel eluted and purified using a PCR Clean-up Kit (Cosmogenetech, Seoul, Korea). The amplicons were sequenced at Macrogen (Macrogen, Seoul, Korea) and analyzed using SeqMan (Ver 5.00, DNASTAR Inc., Madison, WI, USA).

2.9 Expression analysis of GWAS candidate genes using quantitative real-time PCR

For gene expression analysis, the young leaves (1 cm) of 'MR' grown in the pepper-specific speed breeding system were sampled when the fourth and the sixth leaf were initiated. The leaves were frozen immediately in liquid nitrogen after sampling. Total RNA was extracted from the leaves using the MG Total RNA extraction kit (MGmed, Seoul, Republic of Korea). Total RNA (1µg) was used for cDNA synthesis by reverse transcription (RT) PCR using AccuPower RT PreMix (Bioneer, Daejeon, Republic of Korea). For qRT-PCR, three biological replicates were used for each sample. Based on the exon sequencing results of the 'MR', primers for qRT-PCR have been designed. The identified variations in the exons have the potential to explain the rapid flowering of the 'MR'. The qRT-PCR was performed using the primers AP2_qRT (Borovsky et al., 2015), WOX4_qRT, FT_qRT, and GI_qRT. The qRT-PCR was performed in a 20 µL reaction volume containing 2 µL of 5x diluted cDNA, 2 µL of 10mM dNTPs, 2 µL of 10x reaction buffer, 0.5 µL of SYTO 9 (Thermo Fisher Scientific Korea, Seoul, Republic of Korea), 0.5 µL of 10 pmol primers, and 0.4 µL of R Taq (Takara Bio). A Rotor-Gene 6000 real-time PCR thermocycler (Corbett Research, Sydney, Australia) was used with the following PCR amplification conditions: 95°C for 5 min; 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The relative expression levels of candidate genes were normalized against *CaUBIQUITIN* (DQ975458.1) using the primer UBQ_qRT (Borovsky et al., 2015). The reliability and reproducibility were ensured with three independent replicates per plant. Statistical analysis was conducted by Tukey's honest significant difference (HSD) test for pairwise comparisons and significant differences of means (Tukey, 1977) using R program.

3 Results

3.1 A photoperiod extended to 20 h was the best condition for accelerating the flowering time

To find out the best photoperiod for the flowering of the pepper, as shown in Figure 1, pepper plants were grown under various conditions with 12 h, 16 h, 20 h, and 24 h of photoperiod. As the photoperiod extended, the 'MR' variety demonstrated a reduction in the number of days required for transition, indicating an accelerated emergence of floral meristem (Table 2). Flowering time was shortened by 6-7 days under the 20/4 h and 24/0 h conditions compared to the 12/12 h and 16/8 h conditions (Table 2). However, the plants under 24 h showed physiological disorders with wrinkled and yellowing leaves (Supplementary Figure 3). Thus, it was demonstrated that extending the day length to 20 h effectively promotes the flowering time and is the best photoperiod for speeding up the flowering of 'MR' (Figure 1 and Table 2).

3.2 A lower R:FR ratio promoted the flowering of *Capsicum annuum*

In the above experiment, it was found that extending the day length to 20 h could promote the flowering time of pepper. To further shorten the flowering time of 'MR', diverse R:FR ratios were tested. The plants under higher FR light treatment showed accelerated floral induction (Figure 2 and Table 2). FR light stimulation facilitated floral differentiation, as evidenced by a reduction in the number of days required for the transition of the 'MR' variety (Table 2). However, it is important to note that FR light also induced undesired stem elongation. As the intensity of FR light increased, there was a significant promotion of stem elongation (Table 3). Overall, this study indicates that supplemented higher FR was effective for shortening the time to floral induction of 'MR', although unwanted stem elongation was observed.

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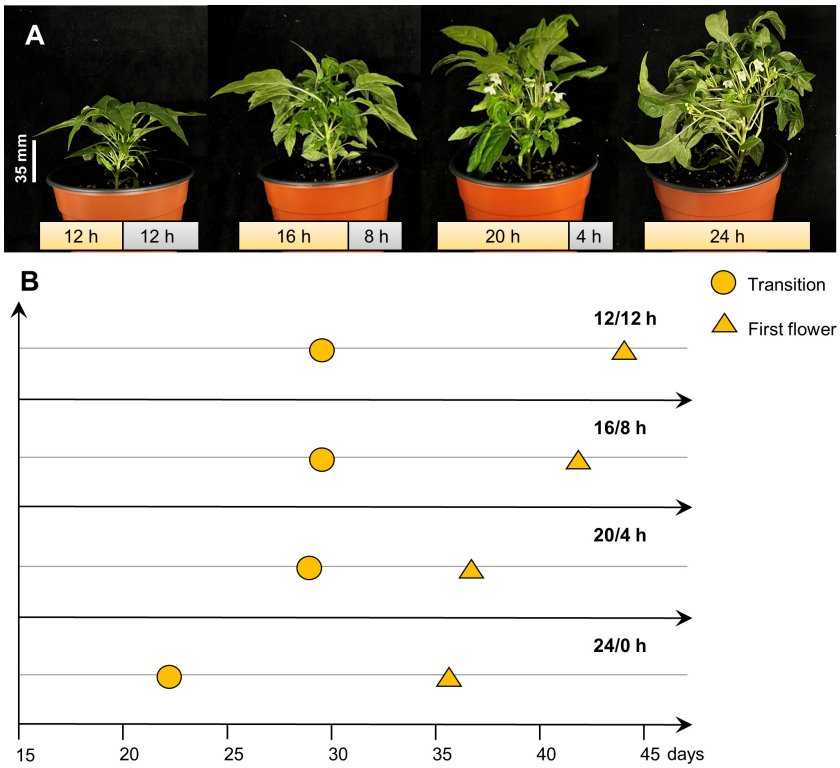

FIGURE 1
The growth and development of *Capsicum annuum* 'MicroPep Red' ('MR') in extending the photoperiod experiment. **(A)** 'MR' at 39 days after transplant (DAT) showed transition stage (appearance of flower buds) under the 12/12 h and 16/8 h of light/dark cycle. Concurrently, 'MR' showed flowering under the 20/4 h and 24/0 h of light/dark cycle. **(B)** Representative graph depicting the development stages of 'MR' under the various photoperiod conditions.

3.3 The combination of extended photoperiod and FR light as a pepper-specific speed breeding system shortened the generation time effectively

Through extending the photoperiod experiment and the FR light intensity test, it was found that increasing the light period to 20 h and higher FR light intensity could promote the flowering of 'MR'. Therefore, to shorten pepper generation time dramatically, a pepper-specific speed breeding system was devised by combining extended photoperiod and supplementing FR light. A 20 h photoperiod and an R:FR ratio of 0.3 was adopted from the previous experiment, which was effective in promoting transition and flowering. Compared to other treatments (Ctl, Ctl+FR, Epp), transition, flowering, fruit, and ripening time were significantly reduced under Epp+FR treatment (Figure 3 and Table 2). Specifically, compared to Ctl (56 ± 2 DAT), the combination of extended photoperiod and FR light (Epp+FR) shortened the flowering time significantly (36 ± 2 DAT) (Figure 3 and Table 2). In addition to transition and flowering, fruit setting and ripening times were also significantly decreased in Epp+FR, suggesting that the

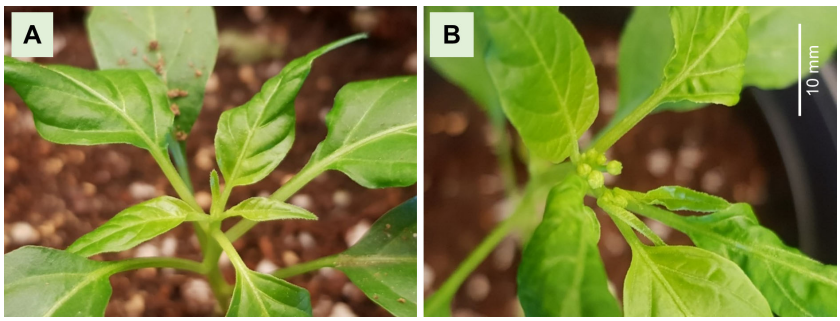

FIGURE 2
The appearance of flower buds in far-red (FR) light intensity experiment. **(A)** *Capsicum annuum* 'MicroPep Red' ('MR') at 23 days after germination showed vegetative shoot apical meristem under W45. **(B)** 'MR' at 23 days after germination showed inflorescence meristem under W45+HFR (R:FR = 0.3).

generation time of pepper can be remarkably reduced due to a combination of extended photoperiod and supplemented FR, which can be a speed breeding system suitable for pepper (Figure 3). In addition, there were significant differences in stem length among the treatments (Ctl, Ctl+FR, Epp, and Epp+FR). Plants grown under FR light (Ctl+FR and Epp+FR) had higher stem lengths as compared to those grown under other conditions without FR light (non-FR) (Figure 3 and Table 3).

3.4 Application of a pepper-specific speed breeding system in a greenhouse accelerated the breeding cycle of *Capsicum annuum*

Employing the results of the experiments in the chambers, speed breeding conditions with extended light and supplemented FR light were applied in the greenhouse. Since it was confirmed that the plant height was elongated when the FR light was higher than the red light in 'MR', an R:FR ratio of 3.8 was set in the greenhouse to prevent over-growth (Liu et al., 2022). Days to transition, flowering, first fruit, and first ripening were significantly shortened under Epp+FR GH compared to Normal GH and Epp GH (Figure 4). Days to flowering was 51, 28, and 21 DAT under Normal, Epp, and Epp+FR GH, respectively (Table 2). Days to first fruit and ripening were 28 and 71 DAT under Epp+FR GH (Table 2). Concurrently, the corresponding plants under Normal GH had only reached floral induction, while the plants under Epp+FR GH had already reached the first fruit stage (Figure 4 and Supplementary Figure 4). Unlike the stem elongation under the higher R:FR of the FR light intensity experiment (Figure 3 and Table 3), the ratio of R:FR at Epp+FR GH did not cause stem elongation (Figure 4 and Table 3). In addition to speeding up the flowering, Epp+FR condition upregulated the number of fruits, which is important to harvest more seeds for breeding peppers (Table 3).

3.5 Genome-wide association study provided candidate flowering-time genes for *Capsicum annuum*

Through previous experiments, it was confirmed that pepper generation time is different depending on the day length and the

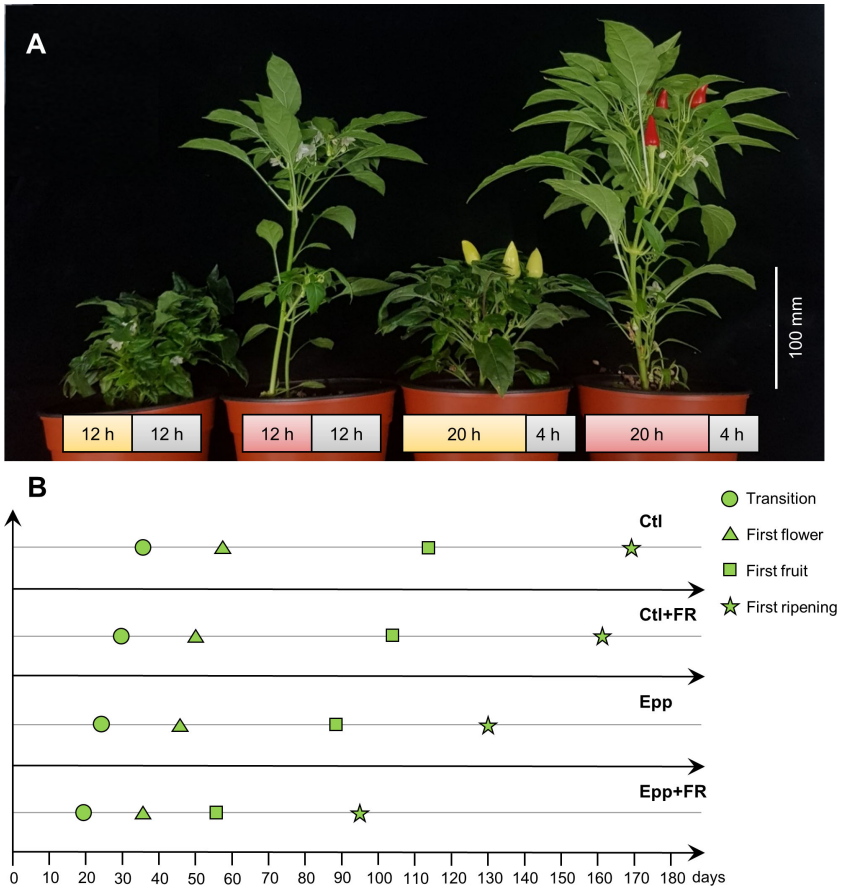

FIGURE 3
Growth and development of *Capsicum annuum* 'MicroPep Red' ('MR') in pepper-specific speed breeding system. **(A)** 'MR' at 100 days after transplant (DAT) under the Ctl (12/12 h and 26/20°C of light/dark cycle), Ctl+FR (12/12 h, 26/20°C of light/dark cycle, and supplemented far-red (FR) light), Epp (20/4 h and 26/20°C of light/dark cycle), Epp+FR (20/4 h, 26/20°C of light/dark cycle, and supplemented FR). **(B)** Representative graph depicting the development stages of 'MR' under the treatments (Ctl, Ctl+FR, Epp, and Epp+FR).

presence or absence of FR. In particular, growth and development were promoted the most in the experimental group in which the photoperiod was prolonged and FR was supplemented. To demonstrate that different day lengths and light quality can be a genetic signal for the transition from vegetative to reproductive stage, as well as simply increasing photosynthesis, GWAS analysis was performed to determine the genetic loci associated with flowering time.

The phenotypic distribution of the flowering time using the GWAS population is illustrated in Figure 5A. This figure depicts the average flowering time over three years (2015, 2016, and 2017) for each C. annuum accession. Notably, the 'MR' accession exhibited an average flowering time of 68 ± 1.7 days under the normal greenhouse (traditional sunlight). The GWAS of the flowering-time traits was performed using a compressed MLM model (CMLM) as implemented in FarmCPU program with 221,487 high-quality SNPs and 220 C. *annuum* accessions. We identified 5 genome-wide significant SNPs ($-\log_{10} P > 7.158562$). These SNPs were located on chromosomes 2, 3, 4, 5, and 12 (Figures 5B, C). The boxplots drawn with the genotypes of the 5 significant SNPs against the flowering-time phenotypes revealed that 3 SNPs on chromosomes 2, 4, and 12 were significantly associated with phenotype variation (Figures 5D-H). The gene annotation dataset was investigated to find significant flowering-related genes in the vicinity of the SNPs. The *APETALA2* (*AP2*), *WUSCHEL-RELATED HOMEOBOX4* (*WOX4*), *FLOWERING LOCUS T* (*FT*), and *GIGANTEA* (*GI*) genes were identified and selected as candidate genes for sequencing and expression analysis (Supplementary Tables 3–5).

3.6 Gene expression of *FT* and *GI* showed significant differences due to growth conditions in the pepper-specific speed breeding system

Candidate gene expression patterns were analyzed in young leaves of 'MR' under Ctl and other treatments (Ctl+FR, Epp, and Epp+FR). The gene expression patterns of *AP2* and *WOX4*, which play an important role in floral development in Arabidopsis (Costanzo et al., 2014; Borovsky et al., 2015), did not show significant differences among environments (Supplementary Tables 4, 5). On the other hand, the expression levels of *FT* and *GI* were significantly different depending on growth condition (Supplementary Tables 4, 5). The *FT* gene found near the SNP on chromosome 5 had the highest expression in the Ctl (Figure 6). *GI* was highly expressed in Epp+FR, which extended photoperiod and supplemented FR light, and its expression level was the lowest under Ctl conditions (Figure 6). These gene expression results indicate that not only was flowering accelerated by increasing the amount of photosynthesis and photosynthetic efficiency due to extended photoperiod and adding FR (Zhen and van Iersel, 2017; Watson et al., 2018), but also the prolonged photoperiod or FR light can promote or inhibit the expression of flowering-related genes to change the flowering phenotype.

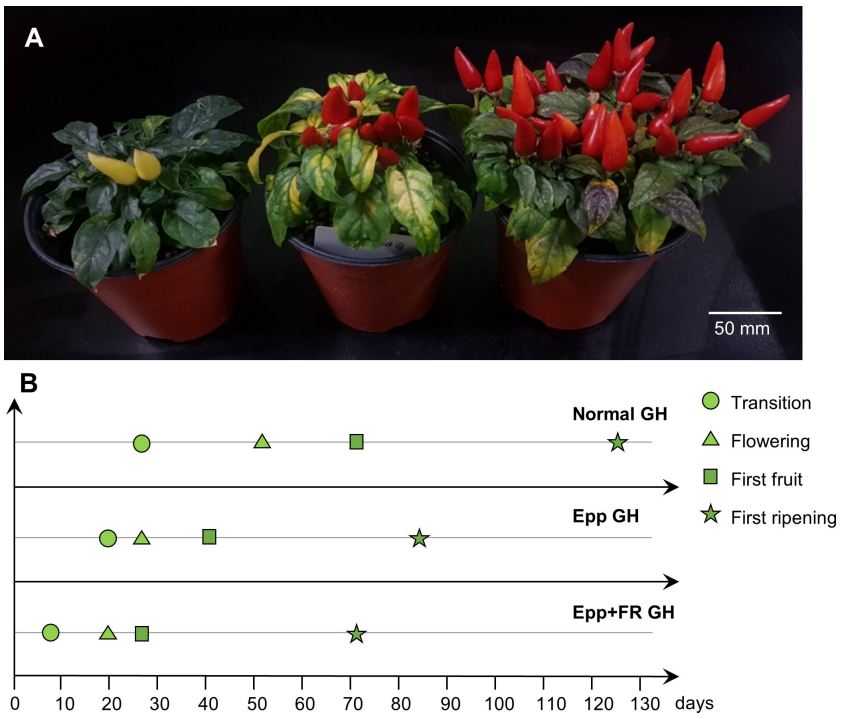

FIGURE 4
Growth and development of *Capsicum annuum* 'MicroPep Red' ('MR') from application of speed breeding in greenhouse (GH). **(A)** 'MR' at 99 days after transplant (DAT) under the Normal GH (Suwon farm condition), Epp GH (20/4 h of light/dark cycle), and Epp+FR GH (20/4 h of light/dark cycle and supplemented FR). **(B)** Representative graph depicting the development stages of 'MR' under the treatments (Normal GH, Epp GH, and Epp+FR GH).

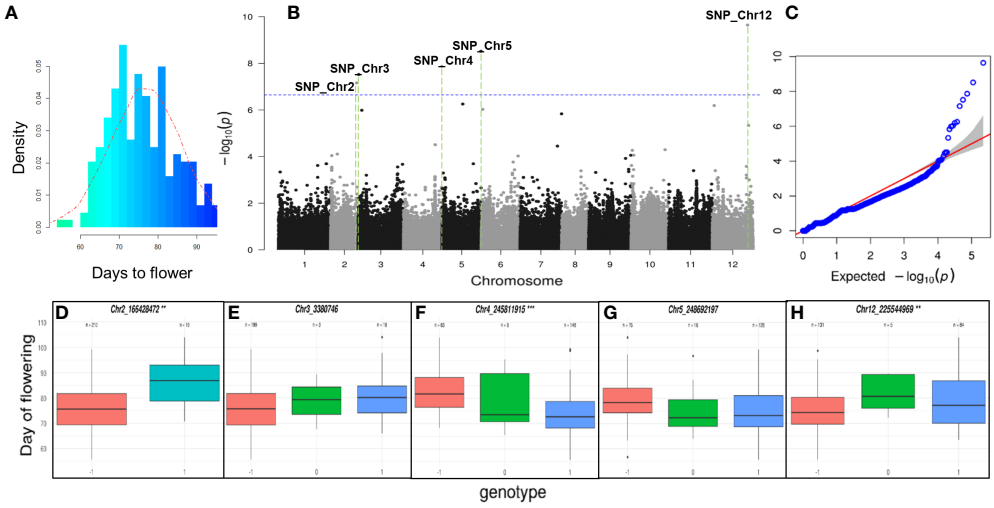

FIGURE 5
Genome-wide association analyses (GWAS) for flowering time. **(A)** Histogram for flowering time phenotype of pepper GWAS population. The histogram was drawn using the average flowering time of three-year data (2015, 2016, and 2017) in GWAS population. The red dotted line is the probability density curve following a normal distribution. **(B)** The 221,487 filtered single nucleotide polymorphisms (SNPs) detected from the 220 individuals of the GWAS population were used for detection of candidate genes. The GWAS were conducted using the R package Genomic Association with default settings. Blue line represents the threshold for GWAS significance after a Bonferroni correction. Five genome-wide significant SNPs ($-\log_{10}P > 7.158562$) were identified on chromosomes 2, 3, 4, 5, and 12. **(C)** Quantile-quantile (QQ) plot of the data derived through FarmCPU model shown in the Manhattan plot. **(D–H)** The box plot showed the phenotypic variation of each SNP in the chromosomes 2, 3, 4, 5, and 12. The boxplot displays the phenotype variation in the GWAS population with the three different genotypes (-1: reference, 0: hetero, 1: alternative genotype) of 5 SNPs over the Bonferroni correction. Statistical significance was determined by ANOVA test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The 3 SNPs in the chromosomes 2 (**), 4 (***), and 12 (**) showed significance in the phenotype variation among the 5 SNPs.

4 Discussion

Developing strategies for the rapid generation of homozygous lines is a dream for plant breeders. Watson et al. (2018) followed a new breeding approach, speed breeding, to shorten the generation time. In this approach, the authors exposed the plants to prolonged photoperiods, which resulted in a significantly shorter generation time for field crops such as wheat, barley, pea, and chickpea. However, this protocol is limited to long-day or day-neutral plants and does not consider other environmental controls such as light quality.

Extended daylight can clearly be beneficial for the flowering of some plant species and confirming the best day length is the key to success in speeding up the flowering. Extending the photoperiod, however, may also have some negative effects, because plants use the photoperiod as a signal for many other physiological processes. For example, tomato plants under continuous light showed leaf chlorosis and epinasty (Pham et al., 2019). These effects are largely on a species-specific basis. Continuous photosynthesis and accumulation of carbohydrates produced by continuous light may also result in negative feedback (Demers and Gosselin, 2000). These responses of plants to extended photoperiod and continuous light are very complex and are largely species or even cultivar specific (Velez-Ramirez et al., 2011). There is still much that is unknown about the mechanisms of plant responses to continuous light and the causes of the negative effects (foliar chlorosis, limited growth, productivity, leaf injury, etc.) (Sysoeva et al., 2010).

Recently, Jähne et al. (2020) proposed a protocol for short-day crops, such as soybean, rice, and amaranth, modulating optimal light conditions for each crop by adjusting blue, green, red, and FR light intensities. FR light did not affect the flowering time of soybean, but early flowering was observed under higher FR light in rice and amaranth. The flowering responses of plants can vary depending on environmental factors, such as various day-length and specific light spectrums and different plant species (Watson et al., 2018; Jähne et al., 2020); thus, species-specific breeding protocols need to be developed for each species, cultivar, and accession.

In this study, a speed breeding protocol for pepper was developed by extending the photoperiod to 20 h, controlling proper temperature, and supplementing FR light. Interestingly, this system demonstrated that prolonged photoperiod with FR lighting was the best condition for shortening the transition, flowering, fruit, and ripening time of pepper. This protocol shortened the flowering time to that of half of the plants grown in normal conditions. The time to first harvest was shortened by 75 DAT, which can shorten the breeding and research cycle of pepper remarkably. The effectiveness of the developed speed breeding system was observed not only in *C. annuum* 'MicroPep Red', the main variety used in this study but also in another species, *C. chinense* 'Habanero'. Supplementary Figure 5 illustrates that the flowering time was significantly promoted in the extended photoperiod+far-red light (Epp+FR) treatment compared to the control and extended photoperiod (Epp) treatments for 'Habanero'. These findings imply that the speed breeding system developed in this study holds potential for application in other species within the *Capsicum* genus.

FR light was the key to this protocol and finding the best ratio of R:FR was essential. In the FR light intensity experiment, the lower R:

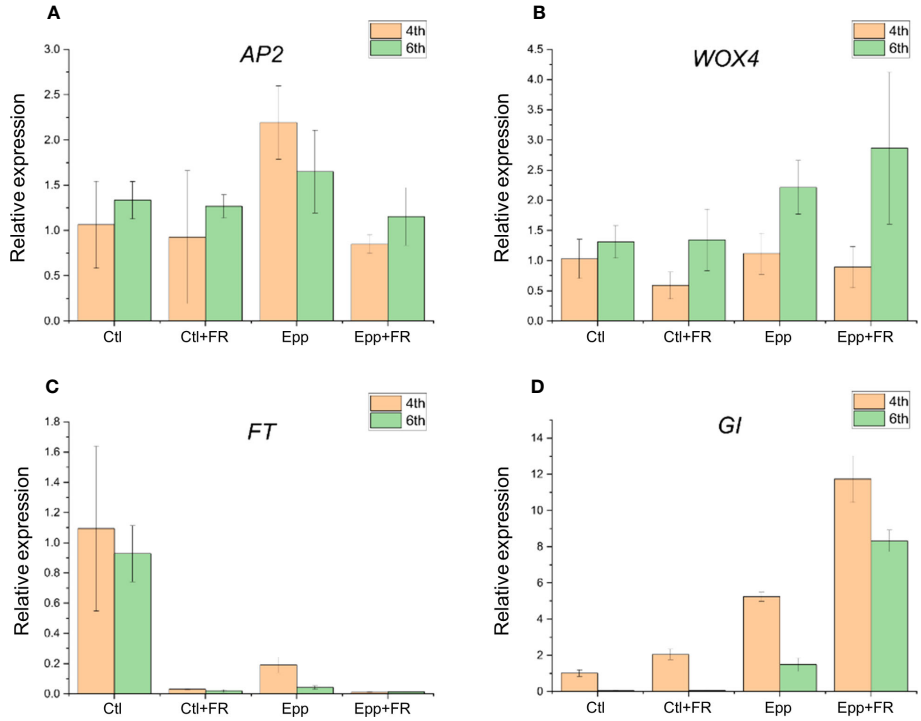

FIGURE 6
Quantitative RT-PCR results of candidate genes. Relative mRNA level of **(A)** *AP2*, **(B)** *WOX4*, **(C)** *FT*, and **(D)** *GI* in the Ctl, Ctl+FR, Epp, and Epp+FR at 4^{th} and 6^{th} leaves. Data for each group are means ± SE of three independent replicates. The *P*-values for significant differences between each treatment are specified in Supplementary Tables 4, 5.

FR ratio was effective for shortening flowering time in pepper, and the best R:FR ratio was 0.3. However, FR light can cause abnormal plant morphology of tall plants (Franklin, 2008). Plants with abnormally elongated stems are not suitable for growing in growth chambers and plant factories that have a limited height. Furthermore, breeders may face difficulties in growing and phenotyping plants due to lodging associated with elongated stems. To obtain an environment that can both promote flowering and maintain optimal stem growth in pepper, light quality and duration should be optimized. It is possible that the use of additional blue light along with Epp and FR light conditions (Epp+FR) could prevent the enhanced shoot growth due to blue light-mediated inhibition of hypocotyl growth regulated by cryptochromes (Franklin, 2008).

For the application of the developed speed breeding system in the greenhouse, we established an LED-controlled system. We set the photoperiod at 20 h and the R:FR ratio at 3.8 for accelerating the flowering time. We shortened the duration of seed to seed by 57 DAT with Epp+FR GH compared with Normal GH. When we applied this Epp+FR GH system in breeding the multiple disease resistant *C. annuum* and producing mature seeds of F_1 plants, it took only 110 days and is predicted to be 880 days for developing BC_5F_3 lines.

The differences in plant responses to light can be explained by the expression of flowering-related genes. For example, in the case of Arabidopsis, FR light can promote *FT* expression (Cerdán and Chory, 2003; Halliday et al., 2003; Valverde et al., 2004) and flowering (Goto et al., 1991; Reed et al., 1993; Bagnall and King, 2001). These studies suggest that flowering-related genes can promote or inhibit flowering depending on the light conditions, therefore it is necessary to confirm gene expression under speed breeding, and relevant studies are lacking to date. To identify the genetic loci associated with flowering responses to day length and light and verify the gene expression patterns in the speed breeding system, GWAS and gene expression analysis were performed.

Multiple GWAS studies have elucidated the genetic structure related to key characteristics of pepper (*Capsicum* spp.), such as capsaicinoid content, fruit weight, and shape (Lozada et al., 2022a). Recently, Lozada et al. (2022b) performed a multi-locus GWAS analysis to investigate quantitative trait loci (QTL) associated with agronomic traits, including flowering time, in pepper (*Capsicum* spp.). Flowering time exhibited a strong correlation with the first fruit date trait, and shared QTL on chromosomes P1, P6, and P7 were identified. Relevant candidate genes were found to be involved in biological functions such as defense response, metabolic processes, oxidation-reduction, phosphorylation, and gene silencing (Lozada et al., 2022b). In this study, we identified five significant SNPs that were located in different positions compared to previous GWAS studies. Among them, *AP2* (located on chromosome 2), *WOX4* (located on chromosome 4), *FT* (located on chromosome 5), and *GI* (located on chromosome 12) were selected as candidate genes. Notably, *AP2* is the same locus as *CaAP2* introduced in a previous paper (Borovsky et al., 2015). As *CaAP2* acts as a flowering repressor in pepper, it was expected that *AP2* gene expression would be lowered in the Epp+FR treatment; however, *CaAP2* expression did not change significantly depending

on the environment. A similar result was observed for the *CaWOX4* gene, a member of the *WOX* gene family. The *WOX4* protein contains the distinct WUS-box motif (van der Graaff et al., 2009), which is essential for shoot stem-cell population maintenance or differentiation, lateral organ formation, floral patterning, and embryogenesis (Kamiya et al., 2003; Haecker et al., 2004).

Contrary to *AP2* and *WOX4*, significant differences were found in the expression of *GI* and *FT* under different treatment conditions. Expression of *GI*, a flowering promoter in Arabidopsis (Mizoguchi et al., 2005), was significantly increased in the experimental conditions in which FR was applied with Epp. It was expected that *FT* gene expression would be enhanced in the FR condition since it is a representative flowering promoter and considered to be a florigen in Arabidopsis (Corbesier et al., 2007); however, expression of *CaFT* was inhibited in Epp+FR. This is because the functions of the flowering gene homologs in different plant species can be very different (Borovsky et al., 2015). Therefore, it can be inferred that the *FT* gene in *C. annuum* functions to delay flowering. To clarify the role of these candidate genes, further functional studies are required to better understand the molecular mechanisms by which these genes are involved in flower transition.

In conclusion, our results demonstrate the usefulness of the new speed breeding protocol developed for pepper. Pepper speed breeding can shorten the crop period to half that normally required for pepper, and thus shorten the crossing and inbreeding phase of breeding programs and enable breeders and scientists to save cost and time in their research programs. The shortened breeding cycle, made possible by speed breeding, leads to decreased resource requirements linked to extended cultivation periods, labor expenditure, and maintenance expenses. Furthermore, the shortened breeding time empowers breeders and scientists to rapidly assess and evaluate different genetic combinations, facilitating prompt decision making and optimal allocation of their research resources. In addition, breeding technologies such as marker-assisted or genomic selection can be incorporated into this speed breeding system to accelerate the development of pepper cultivars. The developed speed breeding protocol exhibits exceptional flexibility, making it highly adaptable for farm implementation. By incorporating this protocol into their farming operations, growers have the opportunity to innovate their cultivation techniques, leading to expedited yields and heightened overall efficiency. Furthermore, we located a new flowering gene locus for *C. annuum* and revealed the changes in gene expression during the speed breeding treatments. The significant associations identified herein provide a basis for further GWAS and mapping efforts to determine causal genetic variants and to clarify how the associated genes affect flowering transition in pepper.

Data availability statement

The datasets presented in the study are deposited in the NABIC repository (https://nabic.rda.go.kr/nolog/NV-0799-000001/snpVcfView.do), accession number NV-0799.

Author contributions

Conceived and designed the experiments: HC, SB, HL, B-CK. Performed the experiments: HC, SB, GK, KL, B-CK. Analyzed the data: HC, GK, B-CK. Wrote the paper: HC, JV, J-KK, B-CK.

Funding

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through Technology Commercialization Support Program (821012-03) and Digital Breeding Transformation Technology Development Program (322062-3), funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA).

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/fpls.2023.1151765/full#supplementary-material

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frontiers | Frontiers in Sustainable Food Systems

TYPE Original Research
PUBLISHED 07 December 2023
DOI 10.3389/fsufs.2023.1253063

OPEN ACCESS

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RECEIVED 04 July 2023
ACCEPTED 17 November 2023
PUBLISHED 07 December 2023

CITATION
Armengot L, Picucci M, Milz J, Hansen JK and Schneider M (2023) Locally-selected cacao clones for improved yield: a case study in different production systems in a long-term trial.
Front. Sustain. Food Syst. 7:1253063.
doi: 10.3389/fsufs.2023.1253063

Locally-selected cacao clones for improved yield: a case study in different production systems in a long-term trial

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Ageing plantations, poor genetic material, soil degradation, pests and diseases are, among other factors, limiting cacao production. To meet the increasing demand for cacao in the absence of productivity gains, forests are cleared and the use of external inputs is generalised, with severe negative impacts on biodiversity and GHG emissions. The use of improved plant genetic material should support a sustainable increase of production. In this study, we evaluate and compare the yield performance of four locally-selected clones with those of four widely-used international clones in South America and four full-sib families (crosses of the same international clones). The research was conducted in a long-term trial in Bolivia with different production systems, including monocultures and agroforestry systems under organic and conventional farming and a successional agroforestry system without external inputs. Their cacao yields and the factors determining productivity (pod index, flowering intensity, pod load, pod losses, aboveground biomass, harvesting period) were assessed during 5 years. The cacao trees grown in the two monocultures had higher yields than those in the agroforestry systems. This was the result of higher aboveground biomass, flowering intensity and pod load, and similar pod losses due to cherelle wilt and fungal diseases in the former when compared with the latter. No differences between conventional and organic management were observed. We did not identify any genotypes performing better in a specific production system. On average, the local clones had twofold and five times higher yields than the international ones and the full-sib families, respectively. This was related to their higher total pod load, bigger pods and higher yield efficiency, i.e., higher yield per unit of tree biomass. However, the local clones had less flowering intensity, more cherelle wilt and similar losses due to fungal diseases to those of the international clones. This study clearly shows the need to invest in selection and breeding programmes using locally-selected genetic material to increase cacao production and support renovation/rehabilitation plans. Breeding genetic material that is adapted to low light intensities is crucial to close the yield gap between monocultures and agroforestry systems, and to further promote the adoption of the latter.

KEYWORDS

agroforestry, monocultures, full-sib families, international clones, flowering intensity, yield–biomass ratio, cherelle wilt, diseases

1 Introduction

The cacao tree (*Theobroma cacao* L.) is an ancient crop native to the lower Amazonian rainforest. Nowadays, cacao is an important cash crop for over 5 million smallholder farmers in many developing countries of Latin America, West Africa, and Southeast Asia (World Cocoa Foundation, 2017). Globally, the popularity of cocoa consumption is increasing rapidly, and the global market of cacao beans is expected to grow at an annual rate of 7.3% until 2025 (Grand View Research, 2019). In the meantime, the productivity of cacao trees is declining due to old plantations, poor genetic material, deficient management, soil degradation, and pests and diseases (Blaser et al., 2018; Effendy Pratama et al., 2019). In the past decades, vast areas of pristine rain forests have been cleared and converted into cacao farms to meet the growing demand (Rice and Greenberg, 2000; Vieira et al., 2008; Vaast and Somarriba, 2014; Orozco-Aguilar et al., 2021). Moreover, the traditional cultivation of cacao in shaded agroforestry systems has gradually shifted towards full-sun production for the purpose of increasing cacao yields in the short term (Franzen and Mulder, 2007; Vaast and Somarriba, 2014; Armengot et al., 2016).

Although the productivity of cacao trees can increase significantly when grown intensively in full-sun monocultures, these systems require more inputs (fertilisers, pest and disease management, irrigation, etc.) than agroforestry systems to maintain their productivity stable over longer periods (Ahenkorah et al., 1987). Together with deforestation, conventionally-managed monocultures are a major cause for the loss of biodiversity and ecosystem services (Klein et al., 2002; Milestad and Darnhofer, 2003; Foley et al., 2005; Morris, 2010). On the contrary, cacao agroforestry systems are more biodiverse than monocultures and can increase food security among farmers, contribute to climate change mitigation and adaptation, and increase energy efficiency (Perfecto et al., 2005; Mbow et al., 2014; Pérez-Neira et al., 2020, 2023).

Given the growing demand for cocoa and the need for massive renovation/rehabilitation plans (Somarriba et al., 2021) the use of improved planting material adapted to specific environmental conditions, with high-yielding profiles and low disease susceptibility, is overall a promising practice to improve productivity sustainably. In comparison with other tropical crops, such as rubber (*Hevea brasiliensis*), the genetic improvement of cacao through breeding has been rather slow due to several reasons, including long selection cycles, misidentification of parents, and lack of financial resources (Gutiérrez et al., 2016; Bekele and Phillips-Mora, 2019). Even though large collections maintain over 30,000 different genotypes only in Ecuador and Brazil (Bekele and Phillips-Mora, 2019), most breeding and selection programmes are based on less than 80 genotypes (Lopes et al., 2011; CacaoNet et al., 2012) and do not fully use the cacao diversity in the respective countries (Ceccarelli et al., 2022). It is estimated that more than 75% of cacao famers are currently using poor genetic material from unknown provenance, which is one of the major factors behind the low yields and high losses caused by fungal diseases (End et al., 2018). Improving the genetic material for agroforestry systems, where cacao yields are usually lower, is even more relevant. However, most breeding and selection of cacao genotypes is nowadays adapted to full-sun production. Breeding in agroforestry systems adds a source of variation mostly unwanted by breeders, and, in addition, the potential for increasing yields is higher in full-sun monocultures compared to agroforestry systems.

The biggest improvement in cacao breeding is represented by the discovery of heterosis around the 60s (Lopes et al., 2011). This led to the development of full-sib families, which are the result of controlled crosses between two selected clones. Compared with local selections, the progenies of hybrid crosses produce higher yields, and present higher resistance to biotic and abiotic stresses (Lopes et al., 2011). However, more recently, in America and Southeast Asia, the recommendation has shifted towards the use of grafted clones, which are highly resistant to specific fungal diseases, and high-quality cacao beans (End et al., 2018). An example of this recent shift towards grafted clones is found in the Alto Beni region of Bolivia, where the production of cacao has been improved through a selection programme carried out by the Bolivian cacao farmers' cooperative El Ceibo.

In this study, we evaluate and compare the yield performance of four local clones from the El Ceibo selection with four international clones widely used in South America and four full-sib families (obtained from crosses of the same international clones) in various cacao monocultures and agroforestry systems in a long-term trial in Bolivia. Through this research, we aim to answer the question of whether cacao trees under full-sun monocultures and conventional management are more productive than under shaded agroforests and organic management, and whether locally-selected clones perform better than international clones and full-sib families. Furthermore, by directly comparing the different production systems on the same site, this study allows us to search for possible interactions between cacao genotypes and production systems, and to identify promising genotypes specifically adapted to shaded low-input systems.

To better understand the behaviour of the different genotypes and production systems, we also evaluate various crucial factors determining cacao productivity, such as the flowering intensity, pod load, pod losses due to cherelle wilt (the premature abortion of young pods) and fungal diseases, and vegetative growth of the cacao trees. Despite the economic relevance of cacao cultivation for millions of smallholders and for the large cocoa value chain, little information is available on the long-term potential of different cacao production systems [but see Somarriba and Beer (2011) and Ramírez-Argueta et al. (2022) for studies in agroforestry systems] and even less on their interaction with different genotypes. This study provides solid data about different production methods and helps understand which factors contribute the most to increasing productivity.

2 Materials and methods

2.1 Study site

The study site is located in Sara Ana, Alto Beni, Bolivia (15° 27′ 36.60" S and 67° 28′ 20.65" W), at an altitude of 380 metres above sea level, where the Research Institute of Organic Agriculture (FiBL, Switzerland) and its Bolivian partners, i.e., Ecotop Foundation, Instituto de Ecologia UMSA and PIAF- El Ceibo, established an experimental long-term trial in 2008/09 to compare conventional and organic cacao production in monoculture (full-sun) and agroforestry (shaded) systems, as well as different cacao genotypes. The region is characterised as tropical humid with dry winters. Between 1964 and 2019, the average annual precipitation was 1,540 mm, the average annual temperature 26.6°C, and the average annual relative humidity

83% at the nearby Sapecho weather station (410 m a.s.l., 15° 33′ 56" S and 67° 19′ 30" W). The soils are characterised as Luvisols and Lixisols.

2.2 Cacao genetic material

In total, 12 different cacao genotypes from three different genetic groups were compared, i.e., four local clones, four international clones, and four international full-sib families. The local genotypes, i.e., IIa-22, IIa-58, III-6, and III-13, were originally selected by the El Ceibo cooperative in areas close to the study site (the numbers refer to the area where they were selected). El Ceibo initially evaluated a total of 340 trees, of which 113 were selected. Thirty of them were prioritised for their high productivity [Trujillo, 2007; Institutio Nacional de Innovación Agropecuaria y Forestal (INIAF), 2016]. Eighty local selections from El Ceibo, including the ones selected for this study, were widely distributed to the farmers (Somarriba and Trujillo, 2005). All four genotypes were chosen because of their high yielding profile and tolerance to the fungal diseases black pod and witches' broom, which caused severe yield losses until the 1990s [Institutio Nacional de Innovación Agropecuaria y Forestal (INIAF), 2016]. An early harvest period was also among the selection criteria, as a potential mechanism to escape from the peak of the black pod infection. In Bolivia, propagation through grafting started already in the 1990s.

The international clones selected were ICS-1, ICS-6, ICS-95, from the Imperial College Selection, and TSH-565, from the Trinidad Selection Hybrids. The choice was based on the yield evaluations of these clones made by Trujillo (2007) in the study region, more specifically, at the research station of the IBTA (Instituto Boliviano de Tecnología Agropecuaria), between the 1960s and the 1980s. ICS and TSH clones are widely used globally, as well as by farmers in the region because of their high production potential and moderate-to-high tolerance to fungal diseases (Phillips-Mora et al., 2005; Trujillo, 2007; Johnson et al., 2009). Except for TSH-565, all clones are self-compatible, i.e., they can be pollinated by the pollen of the same tree.

The full-sib families (henceforth, FS-families) were generated by using the four international clones as female parent and pollinating them with the clone from the Iquitos Mixed Collection IMC-67 (male parent). Hence, the resulting families ICS-1 × IMC-67, ICS-6 × IMC-67, ICS-95 × IMC-67, and TSH-565 × IMC-67. The IMC-67 clone is frequently used in crossings and as rootstock for grafting because of its tolerance to the fungal disease commonly known as *mal de machete* (*Ceratocystis cacaofunesta*) and it is high yielding (Maharaj et al., 2011). Moreover, it is reported to be a good pollen donor due to its high sexual compatibility (Trujillo, 2007; Phillips-Mora et al., 2012).

2.3 Production systems

The 12 genotypes were tested in five different production systems: two monocultures and two agroforestry systems under organic and conventional management, and one successional agroforestry system without external inputs (see Supplementary material 1). The cacao trees were planted at a spacing of 4 m × 4 m (625 trees ha^{-1}) in all production systems, which is common practice in the study area (Quenta et al., 2005) and in other Latin American countries (Cerda et al., 2014). However, planting density in other regions and countries can increase up to 1,600 cacao trees ha^{-1} (Niether et al., 2020). The plantations of the conventional and organic monocultures consisted of cacao trees only. In the two agroforestry systems (under organic and conventional management), banana trees and different shade and timber trees were planted in the inter-row space between the cacao trees. The banana trees were planted at a spacing of 4 m × 4 m. At a lower density, shade trees were planted at 8 m × 8 m, and other fruit and timber species at 16 m × 8 m. The total shade tree density was about 304 trees ha^{-1}. In the successional agroforestry system (SA), the same trees and crops as in the agroforestry systems were planted at the same density, but additional timber and shade trees, as well as understorey crops such as coffee, ginger and curcuma, were included. The total shade tree density was about 1,180 trees ha^{-1}. The average canopy cover (measured monthly in 2018 and 2019 with a GRS densimeter in 84 points in each net plot) was 48, 34 and 37% in the SA, OA and CA, respectively. It changed over the year, with maximum values before the main shade tree pruning (SA: 60%, OA: 41%, OC: 45%) and minimum values just after the pruning (SA: 29%, OA: 22%, OC: 18%). Further details and the complete list of species planted in the trial can be found in Niether et al. (2018).

The herbaceous stratum in the conventional monoculture and agroforestry system plots was controlled with brush cutters and chemical herbicides, which were applied four to five times per year. The cacao trees in the conventional plots received two applications (March and December) of mineral fertiliser per year (Blaukorn BASF, Germany, 12–8–16-3 N-P_2O_5-K_2O-MgO) from 2010 onwards. The monocultures received 112 kg ha^{-1} year^{-1}, and the agroforestry systems received half of the dose, 56 kg ha^{-1} year^{-1}. In the organic system plots, weeds were managed with a leguminous perennial (*Neotonia wigthii*) cover crop and, occasionally, with brush cutters. The organic plots were fertilised with self-made compost from 2010 onwards, 8 t ha^{-1} year^{-1} in the monocultures and 4 t ha^{-1} year^{-1} in the agroforestry systems. From 2017 onwards, the organic agroforestry system did not receive any compost. Half doses were applied to the agroforestry systems because the potential of added nutrients can only be fully exploited at high light intensities (Evans and Murray, 1953).

In all production systems, pests and diseases were fought exclusively by implementing cultural management practices, without the application of chemical pesticides or biological control agents. The practices consisted in harvesting and removing diseased pods every 2 weeks, regularly pruning to maintain canopy density and adjust the shade, and removing branches with visual symptoms of witches' broom.

The field trial was established as a split-plot design with four blocks, five plots (corresponding to the five production systems), and 12 subplots (corresponding to the 12 genotypes; Supplementary material 1). In each block, each of the five production systems was randomly distributed among the plots. Each plot measured 48 m × 48 m, and comprised a net plot for data collection of 24 m × 32 m. The net plots of all production systems contained a total of 48 cacao trees. The genotypes were randomly distributed, but the distribution within each block was the same for all five production systems. In total, 960 trees were planted in the net plots, 80 of each genotype.

The cacao seedlings and grafted clones were grown in the nurseries of Ecotop and El Ceibo. The seeds of the rootstocks and the seeds of the FS-families were planted at the same time, between May and June 2008. The locally selected and the international clones were

grafted onto 4- to 6-month-old seedlings that originated from the two clones IMC-67 and ICS-6 by open pollination. In November 2008, all seedlings of the FS-families and the grafted clones were ready to be transplanted into the field. The planting was completed within 7 weeks. The transplanted trees that died during their first year of growth in 2009 were replaced with the surplus trees of the same age that had been kept in the nurseries. Trees that died in the following years (24 trees) were replaced at the end of each cropping season (between November and March) with surplus young trees (6 to 8 months of age) from the nursery.

2.4 Data collection

The collection of data was performed at tree level over a period of 5 years, from 2015 to 2019. The mature pods were harvested approximately every 15 days all year round, even if the main production period starts in June and ends in September. At each harvest event, the number of healthy pods of each tree was registered and the total fresh bean weight of each tree was measured.

Fungal diseases of pods were recorded 1 day before each harvest event during a phytosanitary inspection. They included frosty pod rot (*Moniliophthora roreri*), black pod rot (*Phytophthora* spp.), and witches' broom (*Cripinellis perniciosa*) and were identified in the field by recognising their visible infection symptoms. The number of pods affected by each disease was recorded and the pods were removed and left on the floor close to the tree trunk. In addition, if additional diseased pods were observed during the harvest event, the same procedure was applied. Pods damaged by insects and animals such as birds or small mammals were not taken into consideration, due to their very low incidence (only 0.36% were damaged) and because these losses are not expected to be related with the cacao genotype.

Furthermore, all pods lost due to cherelle wilt (a thinning mechanism leading to a premature abortion of the small fruits) were recorded following the same procedure as the one used for the diseased pods, i.e., the number of pods affected by cherelle wilt was counted during the phytosanitary inspection (and during the harvest, if any) and they were cut to avoid double counting. In 2017, 2018, and 2019, the number of unripe pods that were lost during the regular maintenance pruning, which usually occurs in early September, shortly after the main harvest period, was also recorded. Across all years, approximately 2% of the overall pod production was lost due to maintenance pruning. In 2015, the diameter of each cacao tree in the net plots was recorded to estimate the aboveground biomass. The diameter was measured 30 cm above ground. In case of ramifications below 30 cm, the diameters of all branches were measured at the height of 30 cm.

The flowering intensity was assessed every 15 days, also during the phytosanitary inspection. For every observation, the number of flowers on each tree was visually estimated and assigned a flowering intensity score ranging from 0 to 4, with 0 assigned if no flowers were present and 4 assigned if almost all branches were covered with a large amount of flowers (Esche et al., 2023). To guarantee an objective and homogeneous ranking, reference photos of the different genotypes representing the respective score were used and the assessments were always performed by the same observers.

2.5 Analysed variables

A detailed summary of all the variables analysed is included in the Supplementary material 2. The number of total pods produced annually was calculated by adding the healthy and the diseased pods of every sampling date. The fresh weight was converted into dry cacao bean weight by applying a factor of 0.33, which is the conversion factor used in previous studies conducted in the same field trial (Armengot et al., 2016, 2020). The cacao pod index, i.e., the number of pods needed to produce 1 kg of dry cacao beans, was calculated for each year by dividing the dry cacao bean weight by the number of healthy pods produced at tree level.

An assessment of the annual distribution of the cacao harvest was performed to identify the length and the starting, peak, and final day of the harvest period. For the sake of simplicity, the "harvest period" was defined as the estimated number of days required to produce 80% of the total annual production. The "first day" defines the estimated calendar day in which the harvest period starts; the "peak day," the day when the harvest is peaking; and the "last day," the day in which the harvest period is completed. The first day, the last day, and the production period of each tree were found by using the macro facility tool of the SAS software (SAS Institute Inc, 2015). The FS-families were excluded from these analyses because of their low production throughout the cropping year (see result section 3.1.1), which made it difficult to define the main production period with a start, an end, and a peak day.

Furthermore, all yield-determining factors were analysed, such as flowering intensity, cherelle wilt, pod load, incidence of fungal diseases, and aboveground biomass production. The flowering intensity scores, ranging from 0 to 4, were transformed into percentages, and the annual mean values of each tree were used for the analysis. The total pod load was calculated for every tree and year by adding the total number of pods lost due to cherelle wilt to the total number of healthy pods and the diseased pods. The percentages of healthy pods, wilted cherelles, and diseased pods were calculated in relation to the total pod load. The aboveground biomass was estimated through the allometric equation of Andrade et al. (2008), based on the trunk diameter measured at 30 cm (see details of the equation in Supplementary material 2). This equation was developed mainly for seed-based cacao plants, which have a distinct architecture than grafted plants. In our study, local and international clones were grafted and FS-families were seed-based. To our best knowledge, an allometric equation for cacao grafted trees has not been developed. For this reason, we used the same equation for the three genetic groups. The estimation of the biomass with this equation may not be fully accurate, but we decided to use it because the biomass values obtained corresponded very well to the observations in the field, i.e., FS-families were the biggest trees and the local selections the smallest trees (see results section). Allometric formulas estimate more accurately woody biomass, which represents almost 90% of the aboveground biomass (Bastide et al., 2006). Furthermore, the aboveground biomass was used to estimate the yield biomass ratio (YBR), i.e., the relationship between cacao yield and vegetative vigour. The YBR was a proxy for the tree yield efficiency (e.g., Daymond et al., 2002; Padi et al., 2012), calculated using the existing allometric equation for seed-based cacao plants.

2.6 Data analyses

The effect of the production system, the genotypes and their interaction across the 5 years of data was tested for all the variables analysed (except for the incidence of fungal diseases and frosty pod rot) using a model for split-plot designs with the MIXED procedure of the SAS 9.2 software and applying the Kenward-Roger approximation (SAS Institute Inc, 2015), according to the following model:

$$Y_{ijklm} = b_i + S_j + bS_{ij} + C_k + SC_{jk} + bSC_{ijk} + y_l + yb_{il} + yS_{jl} + yC_{kl} + year_{ijklm} + e_{ijklm} \quad (1).$$

Where Y_{ijklm} is the trait in question, b_i is the random effect of block i S_j is the fixed effect of production system j, bS_{ij} is the random interaction between block i and production system, C_k is the fixed of cultivar/full-sib family k, CS_{jk} is the fixed interaction between production system j and cultivar/family k, bSC_{ijk} is the interaction between block i, production system j and cultivar k, y_l is the random effect of year *l*; yb_{il} is the random interaction between year *l* and block *i*; yS_{jl} is the random interaction between y_l and production system *j*; yC_{kl} is the random interaction between y_l and genotype *k*; $year_{ijklm}$ is the linear covariate of the planting year nested within year; and e_{ijklm} is the residual. A first-order autoregressive structure between years, with individual trees as subjects, was added in the MIXED procedure of the SAS 9.2 software (SAS Institute Inc, 2015).

Data were square root- or logarithmic-transformed in case of lack of variance homogeneity, examined by plots of studentised residuals as function of predicted values, or in case of skewed distributions of studentised residuals. A generalised linear mixed model for the total incidence of fungal diseases and frosty pod rot was applied due to highly skewed distributions. The procedure GLIMMIX of the SAS software was used for the generalised linear modelling, assuming a binomial distribution and using a logit link (SAS Institute Inc, 2015). Least square means were estimated and inverse logit-transformed values were calculated to obtain frequency estimates.

The plots did not show any outlier. In addition to the across-years analyses (2015–2019), a similar model was repeated for each year to verify the consistency of the results over the 5 years. Least square means were calculated for genotypes and production systems to estimate the correlation between years as a scale-free estimate of their repeatability. High correlations between the least square means of the genotypes between years indicate low interaction between genotype and year. Similarly, high correlations between the least square means of the production systems between years indicate low interaction between production and year, and vice versa. For most variables analysed, the correlations between years were high and significant, showing high consistency in rank for both genotypes and production systems (data not shown). However, for the variables "incidence of fungal diseases," the correlations between years were low and non-significant, suggesting high production system × year and genotype × year interactions.

For all analyses, only trees planted between 2008 and 2012 were used. Generally, breeding programmes start the evaluation of cacao progenies after 5 years of planting (Dos Santos Días and Kageyama, 1998). Trees planted after 2012 were considered too young to provide consistent data throughout the data collection period. To adjust for the effect of different planting years, the planting year was included in the model as a covariate (see above) if it proved to have a significant effect at $\alpha = 0.05$. Trees that died or stopped producing pods during the period of data collection were also excluded (because of their unknown cause of death). All progenies of the FS-families were grafted in 2018 with new genetic material because of their low and unstable yield performance (see the Results section). Therefore, all data collected in 2019 from these trees were not considered.

3 Results

3.1 Cacao production

3.1.1 Dry bean yield, number of pods and pod index

The overall cacao dry bean yield and the number of harvested pods were on average 1.24 kg tree^{-1} year^{-1} and 30.7 per tree^{-1} year^{-1}, respectively.

We found significant differences in the dry bean yield between production systems: the trees under conventional and organic monocultures were the highest yielding trees across all 5 years, followed by the cacao trees in the agroforestry systems, and the ones in the SA (Figure 1A; Table 1; Supplementary material 3). On the other hand, in the SA system, the cacao trees produced the biggest pods with the highest dry bean yield per pod, as indicated by its significantly lower pod index (19.7) compared with the other production systems (the overall average pod index of the other production systems was 23; Table 1; Supplementary material 3). The two monocultures had the highest pod index (conventional: 25.9; organic: 23.4), and therefore produced the smallest pods. No significant differences were found between organic and conventional management in the monocultures or in the agroforestry systems for the variables dry cacao bean yield, number of pods, and pod index (Figure 1A; Supplementary material 3).

Across production systems, the local clones produced the highest yield and number of pods, and differed significantly from the international clones and FS-families (Table 1; Figure 1B). Overall, IIa-22 and III-6 were the highest yielding clones, but there was no significant difference between IIa-58 and III-13 and the best international clone, ICS-1 (Figure 1B; Supplementary material 3). Over the whole period analysed, the FS-families presented not only the lowest yield but also the highest heterogeneity in yield production. For instance, in 2018, the number of pods per tree produced by the FS-families ranged from 4 to 26 pods, and 5 to 40% of their progenies did not produce any pods at all. In addition, the local clones produced bigger pods than the international ones and the FS-families, as indicated by their lower pod indexes (Table 1). In the 5 years analysed, on average, the local clones needed 19.6 pods to produce 1 kg of dry cacao, the international ones 22.3, and the FS-families 25.6.

The interaction between production systems and genotypes was significant for the dry bean yield (Table 1). However, the rank of best-performing genotypes hardly changed, i.e., the best genotypes had good performances in all five systems and the worst ones performed badly in all of them too (Figure 1C).

3.1.2 Harvest period

On average, the main harvest period lasted approximately 80 days, starting on calendar day 161 (around June 10), peaking on day 198

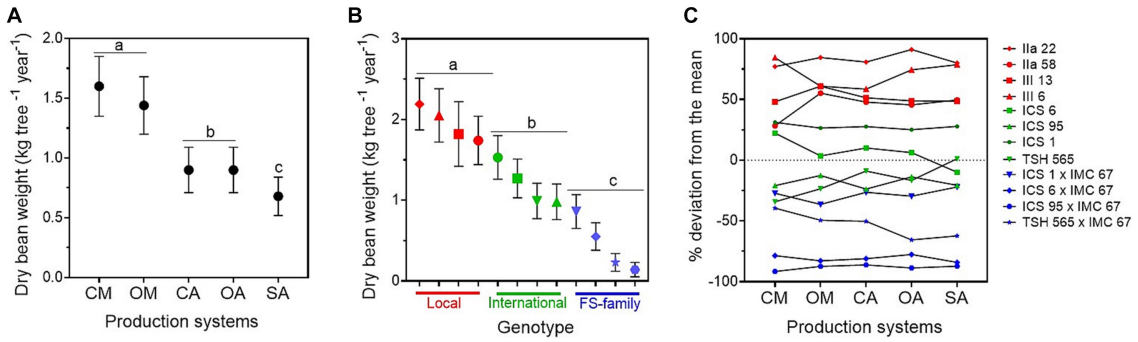

FIGURE 1
Annual mean dry bean yield (kg tree^{-1}) ± standard error (average for the years 2015–2019) estimated for **(A)** the five cacao production systems, i.e., conventional monoculture (CM), organic monoculture (OM), conventional agroforestry (CA), organic agroforestry (OA), and successional agroforestry (SA); and for **(B)** the genetic groups, i.e., local clones in red, international clones in green, and full-sib families in blue. In **(C)**, deviation of the cocoa yield of the different genotypes (in percentage) from the mean. It was estimated as the least square mean for the genotype plus the least square mean for the production system × genotype interaction over the overall mean of the least square mean estimates.

TABLE 1 Anova results testing the effect of the production system, genotype and their interaction on the dry bean weight, number of harvested pods, pod index, and harvest period from 2015 to 2019.

	Dry bean yield (kg tree^{-1})		Harvested pods (ind tree^{-1})		Pod index (number pods kg^{-1})		Length of harvest period (days)	
Fixed effects	F Value	Pr > F	*F* Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Production system	22.0	<0.0001	36.4	<0.0001	16,4	<0.0001	2	0.1931
Genotype	29.9	<0.0001	25.7	<0.0001	61,3	<0.0001	3	0.0129
P. system × Genotype	1.9	0.0026	2.2	0.0002	1,0	0.5056	2,5	<0.0001
Production system	LSM±SE		LSM±SE		LSM±SE		LSM±SE	
CM	1.6 ± 0.3	a	38.6 ± 5.4	a	25.9 ± 0.2	a	83 ± 12	a
OM	1.4 ± 0.2	a	32.3 ± 5.0	a	23.4 ± 0.2	b	84 ± 12	a
CA	0.9 ± 0.2	b	19.2 ± 3.8	b	21.8 ± 0.2	bc	75 ± 11	a
OA	0.9 ± 0.2	b	19.3 ± 3.9	b	21.6 ± 0.2	c	76 ± 11	a
SA	0.7 ± 0.2	c	12.9 ± 3.1	c	19.7 ± 0.2	d	71 ± 11	a
Genetic group	LSM±SE		LSM±SE		LSM±SE		LSM±SE	
Local clones	1.9 ± 0.2	a	39.7 ± 5.5	a	19.6 ± 0.1	c	71 ± 10	b
International clones	1.1 ± 0.2	b	26.5 ± 4.3	b	22.3 ± 0.1	b	85 ± 11	a
Full-sib families	0.3 ± 0.1	c	9.6 ± 2.7	c	25.6 ± 0.1	a	-	-

Least square mean estimates (LSM) ± standard error (SE) of the five production systems and the three different genetic groups. Estimates with the same letter are not significantly different from each other ($p < 0.05$). Cacao production systems, i.e., conventional monoculture (CM), organic monoculture (OM), conventional agroforestry (CA), organic agroforestry (OA), and successional agroforestry (SA). Genotypes, i.e., local clones: IIa22, IIa58, III6, III13; international clones: ICS1, ICS6, ICS95, TSH565; full-sib families: ICS1 × IMC67, ICS6 × IMC67, ICS95 × IMC67, TSH565 × IMC67.

(around July 17), and ending on day 248 (around September 5). Across years, no significant differences were found between production systems concerning the first and the last days, the peak day, and the length of the harvest period. However, a non-significant trend towards shorter length was observed in the agroforestry systems, especially the SA, in comparison with the monocultures (Table 1; Supplementary material 4).

In contrast, we found significant differences between genotypes for all the above-mentioned variables (Table 1; Supplementary material 4). On average, the local clones started their main harvest period 24 days earlier than the international clones, and had a shorter production period by 13 days (Table 1; Figure 2). Moreover, the local clones reached the peak day only 34 days after the harvest period started, 32 days earlier than the international ones, and completed their harvest 39 days before the international clones.

3.2 Factors determining cacao production

3.2.1 Flowering intensity

The average flowering intensity across the years was 32%, with significantly higher values in the monocultures than in the agroforestry systems (Figure 3; Table 2; Supplementary material 5). There was no significant difference between conventional and organic management, neither in the monocultures nor in the agroforestry systems. The lowest flowering intensity was recorded in the SA.

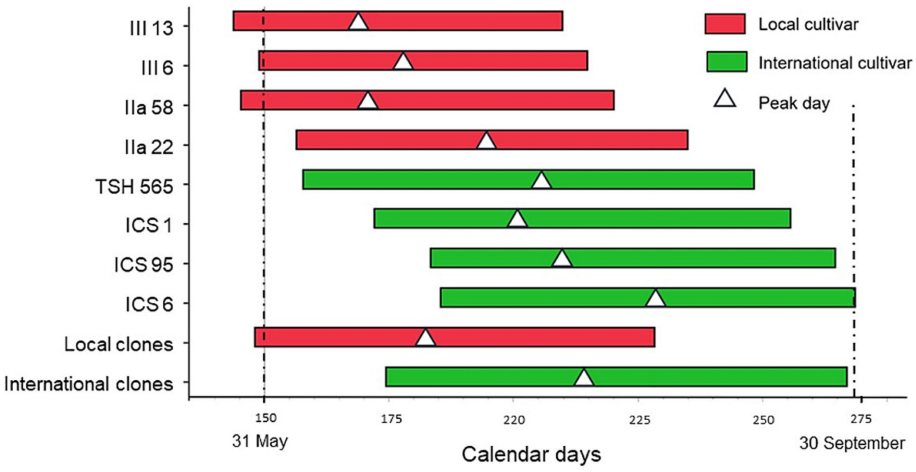

FIGURE 2
Estimated harvest period and peak day (white triangle) for the genetic groups, with local clones in red and international clones in (green).

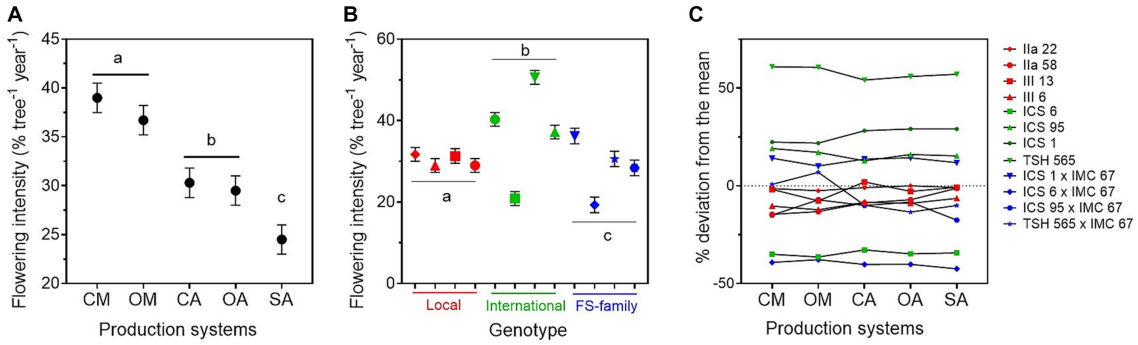

FIGURE 3
Annual flowering intensity ± standard error (average for the years 2015–2019) estimated for **(A)** the five cacao production systems, i.e., conventional monoculture (CM), organic monoculture (OM), conventional agroforestry (CA), organic agroforestry (OA), and successional agroforestry (SA); and for **(B)** the varieties of the three genetic groups, i.e., local clones in red, international clones in green, and full-sib families in blue. In **(C)**, deviation of the flowering of the different genotypes (in percentage) from the mean. It was estimated as the least square mean for the genotype plus the least square mean for the production system × genotype interaction over the overall mean of the least square mean estimates.

We found relevant differences in flowering intensity between genotypes. The international clones had higher flowering intensity than the local clones and the FS-families (Table 2; Figure 3B; Supplementary material 5). TSH-565 was by far the genotype with the highest flowering intensity (51% across years), and ICS6 the one with the lowest. The interaction between genotypes and production systems was significant (Table 2). For instance, we found that the FS-family TSH-565 × IMC-67 had a low flowering intensity in the shaded systems and a much higher one in the monocultures (Figure 3C). Nevertheless, the interaction was small compared to differences between genotypes (Figure 3C).

3.2.2 Pod load: cherelle wilt, diseased and healthy pods

Overall, the trees produced an average pod load of 49 pods tree^{-1} year^{-1} across all years, including harvested pods and pods lost due to cherelle wilt and diseases. On average, 55% of the pod load developed into mature healthy pods, 37% was lost due to cherelle wilt and 7% due to fungal diseases.

Pod load differed between production systems. Conventional monoculture produced the highest pod load, followed by the organic monoculture; in both systems, the pod load was significantly higher than in all agroforestry systems (Table 2). The percentage of losses due to both cherelle wilt and fungal diseases did not significantly differ between production systems, except for the SA, which had more premature abortions (Table 2). This resulted in a higher absolute number of healthy pods in the systems with higher pod load, i.e., monocultures (Table 1).

The group of local clones produced by far the highest pod load, followed by the international clones and the FS-families (Table 2; Supplementary material 5). All genotypes produced higher pod load in the two monocultures, especially in the conventional one, except for TSH-565, which was the only genotype producing the same amount in all five production systems (Supplementary Figure S5; material 5).

The international clones showed significantly lower incidence of cherelle wilt (31.7%) than the local ones (39.3%) and the FS-families (39%). In contrast, losses due to diseased pods were overall very low

TABLE 2 Anova results testing the effect of the production system, cacao genotype and their interaction on flowering intensity, poad lod, percentage of healthy pods, cherelle wilt and pod affected by fungal diseases, aboveground biomass and yield biomass ratio from 2015 to 2019.

	Flowering intensity		Pod load (tree^{-1})		Healthy pods (%)		Cherelle wilt (%)		Fungal diseases (%)		Aboveground biomass (kg tree^{-1})		Yield biomass ratio (x 100)	
Test of fixed effects	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Production system	40.8	<0.0001	29.4	<0.0001	4.0	0.016	4.2	0.012	0.8	0.553	39.5	<0.0001	0.8	0.5597
Genotype	44.7	<0.0001	27.1	<0.0001	3.7	0.001	3.4	0.002	1.4	0.204	33.0	<0.0001	64.2	<0.0001
P. system × Genotype	3.0	<0.0001	2.3	<0.0001	1.7	0.009	1.2	0.211	1.2	0.205	0.7	0.908	2.9	<0.0001
Production system	LSM±SE		LSM±SE		LSM±SE		LSM±SE		LSM±SE		LSM±SE		LSM±SE	
CM	39.0 ± 1.5	a	74 ± 9	a	56 ± 3	a	36 ± 4	b	7 ± 2	a	18 ± 1.4	a	20 ± 2	a
OM	36.7 ± 1.5	a	56 ± 8	b	59 ± 3	a	32 ± 4	b	8 ± 2	a	16.3 ± 1.3	a	21 ± 2	a
CA	30.3 ± 1.5	b	39 ± 7	c	54 ± 3	ab	38 ± 4	ab	6 ± 2	a	11.1 ± 1.1	b	22 ± 2	a
OA	29.5 ± 1.5	b	36 ± 7	cd	57 ± 3	a	34 ± 4	b	7 ± 2	a	11.2 ± 1.1	b	23 ± 2	a
SA	24.5 ± 1.5	c	32 ± 6	d	48 ± 3	b	44 ± 4	a	6 ± 2	a	8.3 ± 1	c	23 ± 2	a
Genetic group	LSM±SE		LSM±SE		LSM±SE		LSM±SE		LSM±SE		LSM±SE		LSM±SE	
Local clones	30. ± 1.4	b	76 ± 9	a	53 ± 3	b	39 ± 3	a	6 ± 2	a	3 ± 0.2	c	39 ± 2	a
International clones	37. ± 1.4	a	46 ± 7	b	60 ± 3	a	32 ± 3	b	7 ± 2	a	3.4 ± 0.1	b	26 ± 2	b
Full-sib families	28. ± 1.4	c	24 ± 5	c	51 ± 3	b	39 ± 4	a	7 ± 2	a	4.3 ± 0.1	a	7 ± 1	c

Least square mean estimates (LSM) ± standard error (SE) of the five production systems and the three different genetic groups. Estimates with the same letter are not significantly different from each other ($p < 0.05$). Cacao production systems, i.e., conventional monoculture (CM), organic monoculture (OM), conventional agroforestry (CA), organic agroforestry (OA), and successional agroforestry (SA). Genotypes, i.e., local clones: IIa22, IIa58, III6, III13; international clones: ICS1, ICS6, ICS95, TSH565; full-sib families: ICS1 × IMC67, ICS6 × IMC67, ICS95 × IMC67, TSH565 × IMC6.

10.3389/fsufs.2023.1253063

(4.2% on average) and did not differ significantly between genotypes (Table 2; Supplementary material 5). Excluding the pods lost due to cherelle wilt, the average incidence of fungal diseases was overall 14% across all years. Frosty pod rot had the highest incidence (9%), followed by black pod rot (3%) and witches' broom (1%). As a result, the percentage of healthy pods was highest among the international clones. However, the absolute number of healthy pods harvested was higher for the local clones due to the higher pod load produced.

3.2.3 Tree aboveground biomass

Tree aboveground biomass, estimated through the allometric equation of Andrade et al. (2008), was highest in the monocultures, followed by the two agroforestry systems and, finally, the SA, and no differences were found between organic and conventional management (Figure 4; Table 2). In contrast, the estimation of the dry bean yield produced per unit of biomass, i.e., the yield biomass ratio (YBR), was similar in all production systems (Figure 4; Table 2).

The tree aboveground biomass also differed between genetic groups, being highest among the FS-families and lowest among the local clones (Supplementary Figure S6; Table 2; Supplementary material 6). However, the lowest value was found for the international clone TSH-565. Contrary to what happened in the production systems, there were differences in the YBR between genotypes. The local genotypes had the highest YBR, followed by the international clones and the FS-families. In the same line, we found a highly significant negative correlation between individual tree biomass and pod load (-0.85, $p = 0.0009$). In other words, the genotypes with higher aboveground biomass per tree produced lower pod load and lower yield than the genotypes with lower aboveground biomass. Interestingly, the international clone THS-565 increased its YBR notably in the agroforestry systems and had the highest YBR in the SA (Supplementary material 6), but with low yield and biomass per tree, as mentioned above.

4 Discussion

4.1 Production systems

The cacao trees grown in the two monocultures showed a higher number of pods and a higher yield than the agroforestry systems. This was the result of their higher flowering intensity, higher total number of pods produced (pod load), similar pod losses due to cherelle wilt and fungal diseases, and higher aboveground biomass compared with the cacao trees in the agroforestry systems.

Even though cacao is a shade-adapted species and is traditionally grown in forest/agroforestry systems, its productivity can significantly increase in full-sun systems (Ahenkorah et al., 1987; de Almeida and Valle, 2007; Blaser et al., 2018). For instance, below certain light intensities, cacao trees suppress their flower production (Asomaning et al., 1971), which causes a significant reduction in pod loading. Furthermore, competition for nutrients and water between cacao trees and agroforestry trees may also limit cacao yields in agroforestry systems (De Almeida and Valle, 2007). In our study, cacao trees in the agroforestry systems received half of the dose of the monocultures, in view of the lower efficiency in the uptake of nutrients at lower light intensities (see "Material and methods"). However some studies have reported increased cacao productivity in the presence of legum trees, even at short distances from the cacao trees (Nygren et al., 2013; Notaro et al., 2021). In addition, a complementary use of water between cacao trees (which have a shallow root system) and agroforestry trees (usually with deeper rooting systems) has been observed (Niether et al., 2017). However, shade in agroforestry systems reduces the transpiration rates of cacao trees (Saavedra et al., 2020; Blaser-Hart et al., 2021), which may lead to a lower soil nutrient uptake and limit the yield. This may also explain the smaller trees found in the agroforestry systems compared with the monocultures, which had an influence on the final yield. Interestingly, the yield produced per unit of aboveground biomass (YBR) was similar in both systems, which resulted in lower yields produced by smaller trees, i.e., cacao trees in the agroforestry systems. The YBR also indicated that the trees allocated similar resources to reproduction per unit of biomass. It is expected that the cacao trees in the agroforestry systems will eventually reach a similar size as the ones in the monocultures, since they grow at a slower pace than those in monocultures (Schneider et al., 2016). This can contribute to increasing the yield and may reduce the yield gap between shaded and full-sun production systems. The methodology for assessing the aboveground biomass using allometric equations estimates more accurately the woody biomass than leaf biomass, since they are based on the trunk dimensions. The relationship between the aboveground biomass and yield found in this study is therefore more directly related to the woody biomass rather than to the leaves, which only account for about 10% of the total biomass (Bastide et al., 2006).

With regard to diseases, the incidences were overall rather low when compared with those found in other studies (Krauss and Soberanis, 2001; Phillips-Mora and Wilkinson, 2007). This could be due to the good implementation of cultural management practices, which consist in regularly pruning and harvesting diseased pods throughout the year, and removing branches with visual infection symptoms (Armengot et al., 2020). This contrasts with some local recommendations to cut shade trees in order to prevent fungal diseases (personal communication, El Ceibo). However, contrasting results concerning the role of agroforestry systems on pests and diseases are also found in the literature (Niether et al., 2020).

Interestingly, when comparing organic and conventional management, we did not find any differences in cacao yield between the monocultures and the agroforestry systems, which shows the potential for organic cacao production. This is not the case for most of the crops: an overall yield gap of about 20% is usually reported (De Ponti et al., 2012), but this seems to be very crop-specific. Lower cacao yields were produced in the organic monoculture, compared with the conventional one, during the first years of the trial (Armengot et al., 2016). This shows the importance of analysing long-term data. In addition to the well-known benefits of organic production for the environment, including diversity conservation and reduced environmental impact, among others (Marconi and Armengot, 2020; Armengot et al., 2021; Pérez-Neira et al., 2023), organic management has lower costs (Armengot et al., 2016; Pérez-Neira, 2016) and is more economically efficient (Caicedo-Vargas et al., 2022) than the conventional production. The potential difference in resource availability between conventional and organic agroforestry systems (the conventional system received mineral fertilisers consistently throughout the period, whereas no compost was applied in the organic systems from 2017 onwards) did not translate into a difference in production or in any of the other

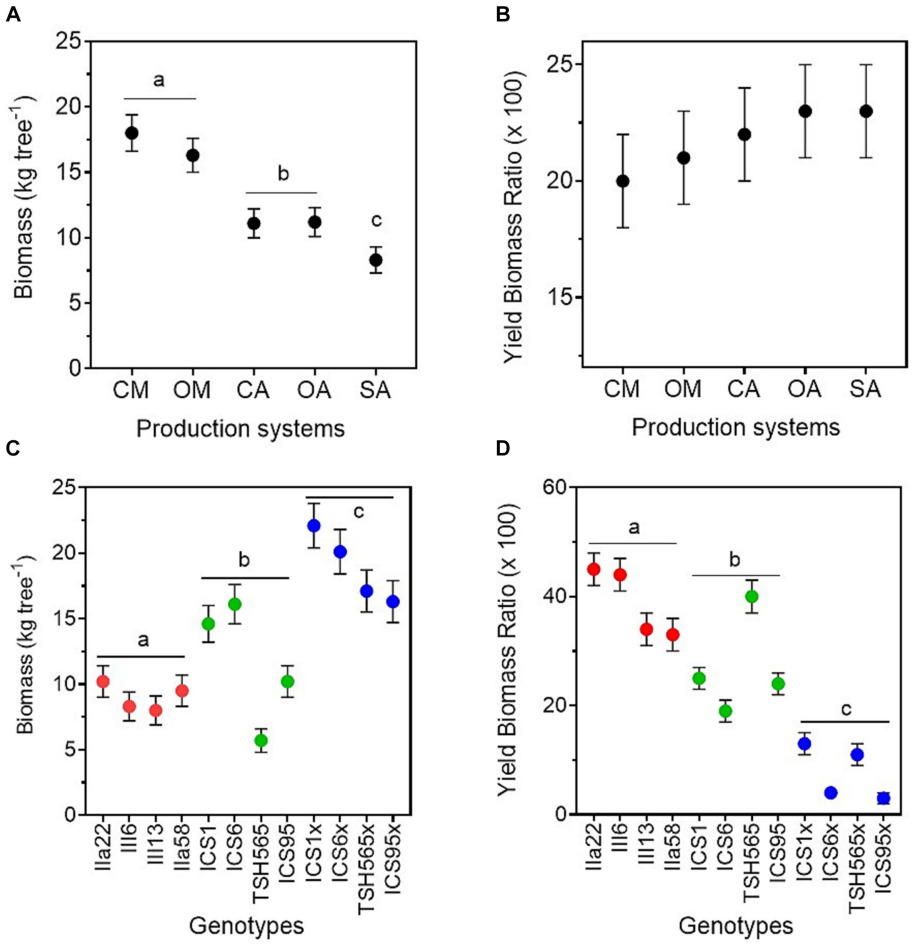

FIGURE 4
Aboveground tree biomass and dry bean weight relation with aboveground tree biomass for the five cacao production systems **(A,B)**, i.e., conventional monoculture (CM), organic monoculture (OM), conventional agroforestry (CA), organic agroforestry (OA), and successional agroforestry (SA), and for the three genetic groups **(C,D)**, i.e., local clones in red, international clones in green, and full-sib families in blue.

parameters analysed (e.g., flowering or pod load). Consequently, the limitation of light (see "Material and methods") could be the reason why the available extra resources were not exploited (Evans and Murray, 1953; Ahenkorah et al., 1987). For the same reason, even though we cannot discount the influence of the reduced fertiliser dose in the lower yields of the trees under agroforestry compared to the monocultures, the limitation of light in the agroforestry systems might have reduced the uptake of nutrients by the trees in the scenario of equal dosage. In the case of the monocultures, we observed that the lower number of pods produced in the organic system was compensated by a higher weight per pod. This explains that, even though the organic monoculture produced less pods than the conventional one, the final yield harvested was similar under both managements. The pod index can be determined by both the number of beans per pod and the bean weight (Lachenaud, 1995). A higher pollination intensity in the organic systems could have increased the dry bean weight per pod. But it could also be related to a competition for resources between the number of pods and the number of seeds per pod (Lachenaud, 1995). This compensation was also observed in relation to the agroforestry systems, i.e., the dry seed weight per pod was higher in the agroforestry systems compared with the monocultures. However, this mechanism was not enough to reach similar values in dry bean yield between monocultures and agroforestry systems. It is important to mention that the dry seed weight is relevant for cacao buyers, i.e., a high seed weight is a positive component of seed quality.

4.2 Genotypes

This study demonstrates the importance of selecting and using local genetic material for cacao production, since the locally-selected genotypes performed better than the recommended international clones and the FS-families in all the parameters assessed.

4.2.1 Yield and productivity

On average, the local clones produced 50% more pods than the international ones and four times as many as the FS-families. Other elite tree-selection programmes in various cacao-producing countries in Latin America and West Africa have reported positive results as

well (Lachenaoud et al., 2007; Ahnert and Eskes, 2018; Feumba de Tchoua et al., 2021). The yield data presented here do not reflect the yield potential of the cacao trees, since the trees were not yet fully grown (data collection included 6 to 10 years after planting).

The yield data for the international genotypes were lower in this study than in the literature (e.g., Johnson et al., 2004). It is also worth mentioning that we expected lower competitive interactions between cacao trees in this study (625 cacao trees ha^{-1}) compared to others with higher cacao planting densities. At higher cacao densities, the competition starts quite early in the production phase (Trebissou et al., 2021). International clones are usually managed at high densities, even twice the density in this trial. Genetic selection of trees based on the performance of individual genotypes does not usually consider the competitive interactions between cacao trees (and even less with other trees in the system) that develop over time (Trebissou et al., 2021), which can be substantial in highly competitive and highly-yielding individuals. Therefore, the performance of the local selections should be evaluated also at higher densities to consider this effect before recommending their use at a different planting scheme.

Overall, the grafted clones, i.e., the local and international clones, performed significantly better than the FS-families, which showed considerably lower yields and high heterogeneity throughout the studied years. The poor production performance of the FS-Families may be partly attributed to a poor breeding value of the common father IMC-67 in terms of reproduction at the study site. Phillips-Mora et al. (2012) reported very low levels of inter-compatibility of IMC-67 as mother and father, which contradicts the widespread belief that this clone acts as a universal pollen donor in the plantations, and it have been found to be incompatible with some clones (Cadavid-Vélez, 2006). Our results cannot definitively exclude the possibility of using seed-propagated seedlings. Actually, until the late 1990s such crosses between Upper Amazon clones (IMC) and Trinitarios (ICS and TSH) were the ones usually recommended to increase yield potential and disease tolerance (CacaoNet et al., 2012). Nevertheless, our study demonstrates that utilising tested clones can lead to earlier, stable, and high productivity. In general, seed-planted trees need more time to reach the reproductive stage than clones, which are grafted with physiologically older material. In our case, even if the FS-families significantly increased their productivity in the following years, they would hardly be able to compensate for the low production of their long juvenile period. Actually, the main motivation for the grafting programme started in the Alto Beni region of Bolivia in the early 1990s was the low performance of the FS-families that were planted during the 1960s and 1970s (Quenta et al., 2005). Initially, international clones were widely distributed and used by farmers in the region, but they did not produce as much as it was expected. The local elite tree-selection programme was then initiated by the El Ceibo cooperative to improve the cacao yields (Somarriba and Trujillo, 2005).

Not only did the local clones produce the highest number of pods, but they also had the lowest pod index (i.e., bigger pods). A low pod index is not only an important breeding and selection criterion (bigger pods with more and bigger beans; Cilias et al., 2010), but it is also of particular interest to farmers, since, with the same yield, a lower pod index reduces the amount of labour needed for harvesting and opening the pods. For instance, the clones IIa-58 and ICS-95 produced a similar number of pods, but the international ICS-95 needed almost twice as many pods to produce the same amount of dry cacao beans as IIa-58 (Supplementary material 3).

The highest pod indexes were found among the FS-families, indicating that they produced not only fewer pods, but considerably smaller ones with a lower dry bean weight. This is in contrast to the results obtained from the production systems, where the trees in the less productive systems (e.g., SA) compensated to some extent their low pod number with bigger pods.

4.2.2 Harvest period

The local clones started and finished their main harvest period significantly earlier than the international ones, and they also had the earliest harvest peak and the shortest harvest period. These parameters are in fact important escape mechanisms against fungal diseases (Maddison et al., 1995). An early harvest period was among the selection criteria of the cacao tree-selection programme of El Ceibo (Trujillo, 2007); the aim was to avoid pod infection by black pod disease, which was the main disease at that time in the study region. However, our results do not show any clear differences in the incidence of fungal diseases.

In addition to the potential advantages of disease avoidance, a shorter harvest period increases labour efficiency by reducing the number of days required for harvesting. It is important to highlight that a shorter harvest period did not indicate a lower pod production in this study; actually, the locally-selected clones produced higher yields. Moreover, a shorter and more compact harvest period facilitated the management of agronomic practices. For instance, we found a high and significant correlation between the last harvest day and the percentage of unripe pods cut during regular maintenance pruning (an average of 1.6% of the total number of pods produced, data not shown).

4.2.3 Yield potential and yield-determining factors

When looking at the individual genotypes, we found that flowering intensity was not related to pod load and yields, contrary to the results obtained for the production systems. For instance, the local clones, which were the highest-yielding ones, showed a significantly lower flowering intensity compared with the international clones. In addition, the highest flowering intensity was registered for TSH-565, the clone with the lowest pod load and yield. Since we did not manipulate pollination in this study, the lack of correlation between pod load (and pod production) and flower intensity indicates that other factors, such as sexual incompatibility for some genotypes and plant resources limitation/allocation, may have been important drivers determining the final pod production. Among the international clones in this study, only TSH-565 is known to be highly self-incompatible (Lopes et al., 2015), for it depends 100% on pollen from other genotypes to achieve successful fertilisation. This might explain why TSH-565 produced the lowest pod load among the tested clones despite its high flowering intensity.

Valle et al. (1990) showed that with an increasing pod production, the number of flowers gradually decreases because they compete for assimilates with the maturing pods. This could explain the significantly higher flowering intensity of the international genotypes TSH-565, ICS-1, and ICS-95, which may have not produced enough pods to suppress the resource allocation that induces flowering.

Vegetative growth is another important sink that competes with the production of flowers and pods (Valle et al., 1990). A strong vegetative growth could potentially reduce pod load, independently of the amount of fertilised flowers. We found that the local clones were more efficient in allocating assimilates to reproduction than the other genotypes, since they showed the highest yield biomass ratio, i.e., a proxy for the tree yield efficiency calculated using an allometric equation for seed-based cacao trees. Several authors consider yield efficiency to be an important trait of the improvement of cacao productivity (Daymond et al., 2002; Padi et al., 2012). Additionally, breeding smaller trees with higher productivity could increase the productivity per cultivated area (Larsen et al., 1992; Lachenaud, 1995) and reduce the time devoted to management tasks, for example, pruning activities. We should consider that the allometric equation used to estimate the cacao tree biomass was developed for seed-based cacao trees but it was applied to both seed-based (FS-families) and grafted (local and internal clones) cacao plants (see "Materials and methods"). This might have overestimated the biomass of the grafted trees. But even in this case, our results showed lower tree biomass and higher yield efficiency of the local clones compared to the international ones and the FS-families.

4.2.4 Pod losses due to cherelle wilt and fungal diseases

The final number of pods reaching maturity is mainly conditioned by the physiological phenomenon known as cherelle wilt, and further limited by pest and diseases.

Losses due to cherelle wilt are usually high, reaching up to 75% (Bos et al., 2007). In this study, the average incidence of cherelle wilt was 37%. This is explained by the fact that the pollinated flowers dropping soon after pollination could not be registered, only the ones where the wilted pod remained attached to the trunk. However, the genotypes tested showed high variability in the percentage of cherelle wilt, and this was not consistently related to the three genetic groups. For instance, the highest percentage of cherelle wilt was observed in the FS-family ICS-95 × IMC-67 and the local clone III-13, which were the genotypes with, respectively, the lowest and highest pod load. The number of aborted pods could be linked to vegetative growth and vegetative events such as leaf flushing (Valle et al., 1990). This would explain the high losses in the low-producing FS-families, which overall had the highest tree biomass. But cherelle wilt is also the physiological mechanism through which trees regulate the number of pods according to nutrient availability (Valle et al., 1990). Therefore, the higher cherelle wilt percentage of the local genotypes compared with the international ones could be explained by the higher pod load of the former.

The total incidence of fungal diseases was quite low considering the high potential for yield losses (Ten Hoopen et al., 2012). Overall, we did not find any differences in the incidence of fungal diseases among the genetic groups. With higher disease pressure we might have observed a clearer pattern in susceptibility and resistance, as described for some of the genotypes tested: for instance, the inherited resistance of ICS-95 to frosty pod rot (Phillips-Mora et al., 2005) but high susceptibility of ICS-1 and TSH-565, which was nevertheless described as resistant in another study (Lopes et al., 2015). The active limitation of spore dispersal through fortnightly removal of infected pods may have been more important in suppressing disease outbreaks than genotype characteristics.

5 Conclusion

The highest yield of the local selections compared to the international clones (ICS-1, ICS-6, ICS-95, and TSH 565) and full sib-families (ICS-1 × IMC-67, ICS-6 × IMC-67, ICS-95 × IMC-67, and TSH-565 × IMC-67) clearly show the need to invest in local selection and breeding programmes using locally-selected genetic material to increase cacao production. To validate the performance and recommend the further propagation of the local selections evaluated in this study, additional evaluations need to be done at farmers' fields. This would allow to explore the capacity and adaptability of these clones to sustain their performance in different planting contexts, e.g., pollen genetic diversity (different neighbouring cacao trees) or nutrients availability. Farmers' organisations should be empowered to select and evaluate their own best-performing local material, which might result in better yield improvements than those of international or national governmental programmes. Farmers should have direct access to the improved genetic material.

We did not identify any genotypes performing better in a specific production system, i.e., the best-performing genotypes (mainly the local IIa-22 and III-6) were so in all production systems. But our results highlight the potential of organically-managed cacao production systems, since they reach similar yields to those of conventional systems. In addition, the lower cacao yields recorded in the agroforestry systems compared with the full-sun monocultures indicate the need to select and breed genetic material that is better adapted to low light intensities. This might help increase cacao production in agroforestry systems and favour a broader adoption of these more sustainable systems, compared to full-sun systems.

Data availability statement

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

Author contributions

LA, MS, and JM contributed to conception and design of the study. MP and LA organised the database and wrote the first draft of the manuscript. MP and JH performed the statistical analysis. All authors contributed to the article and approved the submitted version.

Funding

This study is part of the SysCom Bolivia project (https://systems-comparison.fibl.org/project-sites/bolivia.html), financed by the Swiss Agency for Development and Cooperation (SDC), the Liechtenstein Development Service (LED), the Biovision Foundation for Ecological Development, and the Coop Sustainability Fund. LA was supported by the Ramón y Cajal RYC2021-032601-I fellowship, funded by MCIN/ AEI/10.13039/501100011033 and UE "NextGeneration EU"/PRTR.

Acknowledgments

The authors thank all the staff at Sara Ana Research Station for their support in data collection.

Conflict of interest

JM has been employed by ECOTOP SRL and ECOTOP Suisse GmbH.

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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1253063/full#supplementary-material

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frontiers | Frontiers in Sustainable Food Systems

TYPE Original Research
PUBLISHED 16 February 2024
DOI 10.3389/fsufs.2024.1359486

OPEN ACCESS

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RECEIVED 21 December 2023
ACCEPTED 05 February 2024
PUBLISHED 16 February 2024

CITATION
Sarmah D, Mohapatra PP, Seleiman MF, Mandal T, Mandal N, Pramanik K, Jena C, Sow S, Alhammad BA, Ali N, Ranjan S and Wasonga DO (2024) Efficient regeneration of *in vitro* derived plants and genetic fidelity assessment of *Phalaenopsis* orchid. *Front. Sustain. Food Syst.* 8:1359486. doi: 10.3389/fsufs.2024.1359486

Efficient regeneration of *in vitro* derived plants and genetic fidelity assessment of *Phalaenopsis* orchid

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This study uses inflorescence stalk node as explants to establish an efficient and quick *Phalaenopsis* orchids cloning procedure for the most significant monopodial orchid in floriculture, without callus formation. The current study aimed to develop a rapid and easy regeneration process utilizing flower stalk nodes as explants, while also evaluating the clonal fidelity of the *in vitro* micropropagated plants through the analysis of RAPD markers. The tissue-cultured plantlets were grown on a solidified half-strength Murashig and Skoog (MS) base medium enriched with 15% coconut water (CW), 150 mg L^{-1} activated charcoal, and a mixture of 6-benzylaminopurine, BAP (cytokinins) and α-napthalene acetic acid, NAA and indole 3-butyric acid, IBA (auxins). After 14 weeks of growth, the early production of shoot bud was reported in ½ MS medium enriched with 2.5 mg L^{-1} BAP alone. Maximum shoot bud multiplication was observed in ½ MS fortified with BAP (2.5 mg L^{-1}) + NAA (1.0 mg L^{-1}), while the lowest was observed in 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} IBA after 4 months of culturing. In this investigation, roots emerged simultaneously with shoot elongation from the axil, indicating the absence of a distinct rooting stage. The largest number of roots (3.25) was produced by BAP (2.5 mg L^{-1}) + IBA (1.0 mg L^{-1}) compared to NAA. Control, on the other hand, displayed no signs of root growth. Tissue cultured plantlets with well developed root systems while planted in a potting mixture of brick and charcoal (1: 1) resulted in a 70% survival rate during hardening. The clonal faithfulness of *in vitro* regenerated crop plantlets to the mother plant was demonstrated by the DNA extraction method with ten micropropagated plants' young leaves as well as the mother plant using random amplification of a polymorphic DNA marker.

KEYWORDS

6-benzylaminopurine, indole 3-butyric acid, inflorescence stalk node, Murashig and Skoog medium, *Phalaenopsis*, α-Napthalene acetic acid

1 Introduction

The world's most exquisite flowers are orchids. It belongs to the Orchidaceae family, which has 25,000–35,000 species worldwide and 600–800 genera (Ninawe and Swapna, 2017). Orchids can be found in any ecological circumstance and kind of habitat (Zhang et al., 2022; Xue et al., 2023). Orchids are said to contribute a substantial proportion of the global floriculture trade in both potted plants and cut flowers. De et al. (2014) reported that the worldwide orchid business is valued at US$ 504 million, with over 40 nations exporting and 60 importing orchids. Owing to ease in cultural operations, variation in size and form, bloom color, availability throughout the year, delicateness, and extended shelf life, *Phalaenopsis* orchids is considered as 2nd most favorite flower in both potted plant and cut flower. *Phalaenopsis* orchids is a monopodial orchid species, meaning that it neither branches nor produces new shoots. *Phalaenopsis* orchids is a plant that grows in a monopodial manner on a single stem which grows vertically and produces flowers on lateral branches. *Phalaenopsis* orchids have a 2–3 years growth cycle. Traditionally, *Phalaenopsis* orchids are propagated by cutting or division of offshoots. However, these techniques have a limited rate of multiplication and hinder the growth of the mother plant, making them unsuitable for large-scale production. Their vegetative propagation is so challenging, and the features of their seedlings vary. One of the main issues with commercial *Phalaenopsis* orchid production is that it takes at least three years for it to flower in a greenhouse. Therefore, it is of utmost importance to propagate through tissue culture.

Even though a vast majority of orchids are frequently multiplied using tissue culture, *Phalaenopsis* orchids are one of the few economically significant genera that is reticent and causes difficulties for rapid clonal propagation (Singh et al., 2007; David et al., 2022). In addition to meristem culture, flower stalks, internodal segments of flower stalks, flower stalks with axillary bud, root tip (Park et al., 2002), and leaf segments (Park et al., 1996), numerous tissue culture procedures have been established for *Phalaenopsis* orchids. Despite these challenges, the mentioned approaches have limitations in vegetative proliferation. While many protocorms were produced by some of these techniques, most of these structures matured slowly or poorly into vital plants. The appropriate concentration and combination of the hormones 6-benzylamino purine (BAP) and α-napthalenacetic acid (NAA) in the culture medium is thought to be crucial for the commercial micropropagation of *Phalaenopsis* orchids (Tokuhara and Mii, 1993). Kosir et al. (2004) reported that direct regeneration without callus formation reduces somaclonal variability occurrence and shortens the regeneration time. The genetic preservation of the mother plant is an advantage of this propagation technique, and it's essential that the parent plant itself not be destroyed during the tissue harvesting procedure (Holmes et al., 2021).

Bhatia et al. (2011) stated that synthetic plant growth regulators occasionally cause alterations to the micropropagated plants' genome that could result in DNA methylation, point mutations, and rearrangements. In the context of micropropagation, maintaining genetic homogeneity is crucial for ensuring the uniformity of the propagated plants. To ensure genetic homogeneity, it is of the utmost importance to check the genetic fidelity of *in vitro* micropropagated plants. Chromosome counts and polymerase chain reaction (PCR)-based molecular markers such as inter simplified sequence repeats (ISSR) and random amplified polymorphic DNA (RAPD) can be used to evaluate clonal stability (Li et al., 2019). Molecular techniques are a more reliable and useful tool than other methods to check the genetic stability of *in vitro* micropropagated plants because they are not affected by environmental conditions (Xiang et al., 2021; Huang et al., 2022). As it is very easy to use, quick, profitable, highly discriminative, and authentic, random amplified polymorphic DNA (RAPD) is a commonly used molecular marker for determining the genetic fidelity of *in vitro* micropropagated plantlets (Razaq et al., 2013; Bhattacharyya et al., 2014; Dey et al., 2021). RAPD and ISSR markers are incredibly easy to use, quick, affordable, highly discriminating, and reliable. To design the primer, they only need a minimal amount of DNA material and no prior sequencing information. Since they do not use radioactive probes like restriction fragment length polymorphism (RFLP) does (Lakshmanan et al., 2007), they can be used to evaluate the genetic integrity of clones that were produced *in vitro*.

In a distinctive approach, this study not only focuses on the successful regeneration but also delved into the clonal faithfulness of the *in vitro* micropropagated plants. The objective of the current work was to create an efficient regeneration process using flower stalk nodes as explants. In order to assess the effectiveness of the approach, clonal faithfulness of the *in vitro* micropropagated plants was also investigated using RAPD markers. Consequently, DNA fingerprinting aids in verifying, through RAPD results, that the micropropagated clones generated *in vitro* retained their integrity. The primary advantage of using this propagation technique is the genetic preservation of the mother plant. This study provides a crucial means to verify and ensure the integrity of the *in vitro* micropropagated clones, contributing to the advancement of reliable and efficient micropropagation methods.

2 Materials and methods

2.1 Plant material and processing of explants

For experimentation inflorescence stalks as explants were taken from *Phalaenopsis* orchids (variety: P21-L-34-1033 having white color flower, suitable for both as potted plant and cut flower) which was presented at the orchidarium, AICRP on floriculture, at Mondouri Farm, Bidhan Chandra Krishi Viswavidyalaya, West Bengal. It is one of the most popular commercial potted orchid in West Bengal because of its long-lasting flower, delicate texture and durability. The orchidarium has vast collection of different genera of orchid under All India Coordinated Research Project on Floriculture. When the first flower bud appeared during the first bloom stage, inflorescence stalk explants were gathered. Cotton dipped in 70% alcohol was used to swab the surface of fresh, vigorous, healthy flower stalks. The explant was split into pieces of 2 cm long, each with single node. The segments were then cleaned in distilled water and teepol water. After the removal of the bracts from the buds, fungicidal and antibiotic treatments were applied to the sections. The items were dried with sterile blotting paper, cleaned with cotton dipped in 70% alcohol, and placed in the sterile room. The node was disinfected for 5 min with a 0.1% (v/v) sodium hypochlorite solution, and then for 10 min with a mixture of bavistin (0.5%) and streptomycin (0.1%) as a sterilant (Long et al., 2022).

2.2 Shoot bud induction

In order to initiate the shoot bud, the inflorescence stalk node was placed on ½ MS medium that was supplemented with 30 g sucrose, 150 mg L^{-1} activated charcoal, 15% coconut water (CW), and BAP (1.0, 1.5, 2.0, 2.5, and 3.0 mg L^{-1}) alone or with auxin, i.e., NAA, at a concentration of 0.5–1.0 mg L^{-1}. Even though many plant species grown in tissue culture can withstand a broad pH range of 4.0–7.2, slightly acidic media, typically around pH 5.8 usually give the best growth result. Thus, the pH of the entire medium was brought to 5.6–5.8 using 0.1 N NaOH. For 17 min, all media were autoclaved at 0.1 MPa and 121°C. The cultures were maintained at 26 ± 2°C in a growth chamber with a 16-h light or 8-h dark photoperiod (Piątczak et al., 2015). Every experiment was conducted under the same incubation conditions. The shoot bud induction response was seen after a 14-week culture period.

2.3 Proliferation of shoot buds and root formation

For shoot proliferation and elongation, well-responding shoot buds (2 cm in length) from nodal explants were grown on ½ MS media supplemented with 1.5–2.5 mg L^{-1} BAP and 0.5–1.0 mg L^{-1} NAA and IBA (For specifics, refer Table 1). In this investigation, with the elongation of shoot, roots were found to develop simultaneously from the axil. The number of shoots, leaves, root per explants was recorded after 20 weeks of culture.

2.4 Hardening

After 40 weeks of development, shoots firm, round and silvery white air roots which is called as velamen were taken away from the culture medium and cleaned with sterile water to get removal of agar. Plantlets were then transplanted to a plastic pot with holes in it that held potting medium of uniformly autoclaved bricks and charcoal pieces (1.1), because during the early stages of plant growth, brick and charcoal offer improved moisture and nutrient retention capacity. This process allowed for *in vitro* hardening.

2.5 Genetic fidelity study of the *in vitro* micropropagated plantlets with their parents

Ten micropropagetd plants' young leaves, as well as the mother plant, were used to obtain total genomic DNA. Murray and Thomson (1980) DNA extraction method was followed for standardization of the crop in laboratory condition. A UV spectrophotometer was used to quantify and examine the amount and purity of the extracted DNA at 260 nm. A comparison was made between the absorbance ratios at two wavelengths (A260 and A280) and the standard ratio of pure DNA. It was found that the extracted DNA amounts were optimal for further PCR amplification.

Each 0.2 mL microfuge tube (Dialabs) had 40 ng template of DNA, 2 µM of each of the four dNTPs, 1× PCR buffer (10 mM Tris pH 9.0, 50 mM KCl), 1 U of Taq polymerase (Bangalore Genei, India), 2.5 mM of $MgCl_2$, and 20 pmol of primer. These were used for all PCR processes. In a Veritti Thermal Cycler (Applied Biosystems, USA), the RAPD reaction program was set to 94°C for 3 min. This was followed by 45 cycles of 94°C for 45 s, 36°C for 30 s, 36°C for 45 s, and 72°C for 5 min. After the amplified product was mixed with 2.5 µL of 10 × blue dyes, it was tested on a 1.5% agarose gel in 1× TAE buffer at 65–70 V for 3–4 h. DNA fragments were visible under UV light, and photos were obtained with the Gel Documentation System.

2.6 Data analysis

Each treatment was conducted three times, with the mean ± standard error of the data replicated four times. Version 7.5 of the SPSS (Statistical Package for Social Science) was used to statistically analyze quantitative data at the 5% level. Banding profiles were created based on whether bands were present (1) or absent (0). When scoring the data, only distinct and repeatable amplified bands were taken into account. Non clear, weak and smeared were discarded. Prevost and Wilkison (1999) determined that a primer's resolving power was determined by its capacity to differentiate between individuals.

3 Results

3.1 Axillary shoot bud initiation

For initiating shoot bud, nodal explants were cultured on half strength Murashig and Skoog (MS) basal media supplemented with BAP alone and in combination with NAA and IBA (Table 2). Among all the treatments BAP 2.5 mg L^{-1} showed earliest swelling of node, bud development (26 days) (Figures 1A,B) and multiple bud development (35 days) (Figure 1C). On the same treatment, the earliest observations of leaf initiation (84 days) and shoot induction (60 days) (Figure 1D) were recorded (Figure 1E). Nodal explants cultured on 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} IBA delayed shoot induction (89.33 days) and bud development (78.66 days) as well as leaf initiation (107 days) after 14 weeks of culture (Table 2).

3.2 Proliferation of axillary shoot buds

The shoot buds that were extracted from the nodal segments were placed in half-strength MS medium that was enhanced with BAP along with NAA and IBA (Table 1). The maximum number of shoots (4.15) (Figure 1F) and leaves (7.00) per explant (Figures 1G,H) were produced by shoot buds cultured on 2.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA. Up to a certain point, the number of shoots and leaves increased with increasing concentrations of BAP (1.5–2.5 mg L^{-1}) and NAA (0.5–1.0 mg L^{-1}). At higher concentrations, BAP (3.0 mg L^{-1}) and NAA (1.5 mg L^{-1}) were seen to have an inhibitory response on the number of shoots and leaves.

3.3 Rooting and acclimatization

The current study did not find a distinct rooting stage; instead, roots were found to form concurrently from the axil during the

10.3389/fsufs.2024.1359486

TABLE 1 Effect PGRs on axillary shoot bud proliferation of *Phalaenopsis*.

Treatments			Number of shoots/ explant	Number of leaves/explant	Number of roots/ explant
BAP (mg L^{-1})	NAA (mg L^{-1})	IBA (mg L^{-1})			
1.5	0.5	–	1.67 ^{f} ± 0.14	2.67 ^{g} ± 0.14	0.85 ^{g} ± 0.08
2	0.5	–	2.42 ^{e} ± 0.17	4.15 ^{f} ± 0.08	1.00 ^{fg} ± 0.00
2.5	0.5	–	3.15 ^{cd} ± 0.08	4.92 ^{d} ± 0.14	1.77 ^{bc} ± 0.07
3	0.5	–	2.50 ^{e} ± 0.11	4.40 ^{ef} ± 0.10	0.92 ^{g} ± 0.07
1.5	1	–	2.50 ^{e} ± 0.11	4.50 ^{e} ± 0.11	1.60 ^{cd} ± 0.10
2	1	–	3.40 ^{cb} ± 0.10	5.32 ^{c} ± 0.14	1.92 ^{b} ± 0.07
2.5	1	–	4.15 ^{a} ± 0.08	7.00 ^{a} ± 0.00	2.85 ^{a} ± 0.08
3	1	–	3.15 ^{cb} ± 0.08	5.07 ^{cd} ± 0.07	2.00 ^{b} ± 0.00
1.5	1.5	–	2.00 ^{f} ± 0.12	4.22 ^{ef} ± 0.07	0.92 ^{g} ± 0.07
2	1.5	–	3.00 ^{d} ± 0.12	5.00 ^{d} ± 0.12	1.35 ^{de} ± 0.20
2.5	1.5	–	3.60 ^{b} ± 0.10	6.07 ^{b} ± 0.07	1.92 ^{b} ± 0.07
3	1.5	–	2.85 ^{d} ± 0.08	4.85 ^{d} ± 0.08	1.22 ^{ef} ± 0.07
1.5	–	0.5	1.40 ^{i} ± 0.10	2.60 ^{h} ± 0.10	1.00 ^{g} ± 0.00
2	–	0.5	2.30 ^{g} ± 0.00	4.07 ^{f} ± 0.07	2.07 ^{de} ± 0.07
2.5	–	0.5	2.92 ^{cd} ± 0.07	4.60 ^{de} ± 0.10	2.60 ^{bc} ± 0.10
3	–	0.5	2.60 ^{ef} ± 0.10	3.67 ^{g} ± 0.14	1.85 ^{e} ± 0.08
1.5	–	1	2.50 ^{fg} ± 0.11	4.40 ^{e} ± 0.10	1.85 ^{e} ± 0.08
2	–	1	3.07 ^{c} ± 0.07	4.92 ^{c} ± 0.07	2.75 ^{b} ± 0.16
2.5	–	1	3.92 ^{a} ± 0.07	6.77 ^{a} ± 0.07	3.25 ^{a} ± 0.16
3	–	1	2.85 ^{cde} ± 0.08	4.85 ^{cd} ± 0.08	2.60 ^{bc} ± 0.10
1.5	–	1.5	2.00 ^{h} ± 0.00	4.07 ^{f} ± 0.07	1.50 ^{f} ± 0.11
2	–	1.5	2.77 ^{de} ± 0.07	4.77 ^{cd} ± 0.07	2.07 ^{de} ± 0.07
2.5	–	1.5	3.40 ^{b} ± 0.10	5.85 ^{b} ± 0.08	2.32 ^{cd} ± 0.14
3	–	1.5	2.77 ^{de} ± 0.07	4.60 ^{de} ± 0.10	2.00 ^{de} ± 0.12

Data presented as mean ± standard error. The means with similar letters down the column do not differ significantly at $p \leq 0.05$ by Duncan's Multiple Range Test.

initiation stage (Figure 1I) and development stage (Figure 1J), along with the elongation of a normal shoot. Every examined culture medium reacted favorably to the growth of roots. After 20 weeks of culture, the highest (3.25) and lowest (2.85) number of roots were found on 2.5 mg L^{-1} BAP + 1.0 mg L^{-1} IBA and 2.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA, respectively (Table 1).

A method of acclimatization was developed to increase the survival rate. Individually separated and healthy rooted plants were removed from the media before being placed in the acclimatization chamber. The three to four primary leaves of the marginally acclimated plantlets were moved to the greenhouse. After 2 months of hardening of plantlets using potting medium of brick and charcoal bits (1:1), resulted in more than 70% of success while transplanted (Figure 1K).

3.4 Genetic fidelity of *in vitro* micropropagated plantlets to their parents

The mother plant from which the *in vitro* micropropagated plantlets initially developed showed no differences in morphogenetic and phenotypic expression when compared to the source from which they were derived. Their morphology was true to type, indicating their genetic stability. Afterwards, they underwent molecular analysis using DNA fingerprinting in order to verify genetic fidelity.

3.5 Assay for RAPD markers

OPU 09, 10, 11, and 12 were the only three of the ten RAPD primers that did not amplify when tested in a PCR using *Phalaenopsis* orchids DNA. While OPU 13 and OPU 16 demonstrated positive reproducible bands, the primers OPU 14, OPU 15, P 14 and P 16 exhibited a positive reaction in PCR but were unable to replicate any substantial scorable bands (Table 3). *In vitro* micropropagated plantlets exhibited 55 repeatable monomorphic amplicons, including their mother. Distinctive amplicons were assessed in this study between 220 and 500 base pairs. The same banding patterns displayed by all the primers (Figure 2) suggest that the *in vitro* regenerated clones' purity was preserved.

4 Discussion

Phalaenopsis orchid is ranked 2nd most popular potted plant and cut flower in global market due to its variation in flower color, shape,

TABLE 2 Effect PGRs on shoot bud induction from nodal explants of *Phalaenopsis* orchids.

Treatments			Days required for swelling of nodes	Days required for bud development	Days to first shoot induction	Days to first leaf initiation
BAP (mg L^{-1})	NAA (mgL^{-1})	IBA (mg L^{-1})				
1	–	–	37.00 ^{a} ± 0.40	–	–	–
1.5	–	–	34.33 ^{b} ± 0.23	–	–	–
2	–	–	31.66 ^{c} ± 0.23	42.00 ^{a} ± 0.40	62.33 ^{a} ± 0.23	90.00 ^{a} ± 0.00
2.5	–	–	26.00 ^{e} ± 0.40	35.00 ^{d} ± 0.40	60.66 ^{c} ± 0.23	84.66 ^{c} ± 0.23
3	–	–	29.00 ^{d} ± 0.00	37.00 ^{c} ± 0.40	61.00 ^{c} ± 0.00	85.33 ^{b} ± 0.23
3.5	–	–	32.33 ^{c} ± 0.23	39.00 ^{b} ± 0.40	61.66 ^{b} ± 0.23	-
2	0.5	–	42.33 ^{c} ± 0.33	50.66 ^{bc} ± 0.33	73.66 ^{c} ± 0.33	94.00 ^{c} ± 0.00
2.5	0.5	–	37.66 ^{f} ± 0.33	46.66 ^{e} ± 0.33	69.00 ^{e} ± 0.00	91.33 ^{d} ± 0.33
3	0.5	–	39.66 ^{e} ± 0.33	48.00 ^{d} ± 0.00	71.66 ^{d} ± 0.33	92.33 ^{d} ± 0.66
2	1	–	45.33 ^{a} ± 0.33	54.00 ^{a} ± 0.00	77.66 ^{a} ± 0.33	98.00 ^{a} ± 0.57
2.5	1	–	41.00 ^{d} ± 0.00	50.00 ^{c} ± 0.00	74.00 ^{c} ± 0.00	95.66 ^{b} ± 0.33
3	1	–	43.66 ^{b} ± 0.33	51.33 ^{b} ± 0.33	76.33 ^{b} ± 0.33	96.33 ^{b} ± 0.33
2	–	0.5	55.33 ^{c} ± 0.33	76.00 ^{b} ± 0.57	88.33 ^{ab} ± 0.33	100.00 ^{c} ± 0.57
2.5	–	0.5	51.66 ^{e} ± 0.33	72.33 ^{d} ± 0.33	85.00 ^{c} ± 0.57	96.00 ^{e} ± 0.57
3	–	0.5	54.00 ^{d} ± 0.00	74.66 ^{c} ± 0.33	87.00 ^{b} ± 0.00	98.00 ^{d} ± 0.57
2	–	1	59.33 ^{a} ± 0.33	78.66 ^{a} ± 0.33	89.33 ^{a} ± 0.33	107.00 ^{a} ± 0.57
2.5	–	1	54.00 ^{d} ± 0.57	74.33 ^{c} ± 0.33	85.00 ^{c} ± 0.57	103.00 ^{d} ± 0.57
3	–	1	58.00 ^{b} ± 0.57	76.00 ^{b} ± 0.00	87.00 ^{b} ± 0.57	106.00 ^{b} ± 0.57

Data presented as mean ± standard error. The means with similar letters down the column do not differ significantly at $p \leq 0.05$ by Duncan's Multiple Range Test.

and size, durability. Variety: P21-L-34-1033 is one of the highly demand potted flower in West Bengal because of its durability. The current study has contributed to the development of an improved nodal explant cloning protocol for *Phalaenopsis* orchid. In this experiment, after 14 weeks of culture on ½ MS medium supplemented with BAP alone and in combination with NAA and IAA, shoot bud initiation happened straight from the nodal explants. No protocrom-like bodies (PLB) production or intervening callus was seen during the bud development process. Initially, the nodes began to enlarge and developed green buds that later transformed into shoots. 2.5 mg L^{-1} caused the commencement of early shoot buds. Since BAP is a major cytokinin that stimulates organ development and cell division, this may be the cause. Moreover, Kumari et al. (2013) found that early bud break was promoted in *Dendrobium* Sonia "Earsakul" by culture medium of half-strength MS media supplemented with 4 mg L^{-1} BAP.

According to Asghar et al. (2010), adding cytokinin to the culture media promotes the growth of multiple shoots. The combination that was shown to be most beneficial for shoot reproduction was BAP 2.5 mg L^{-1} + NAA 1.0 mg L^{-1}. While the control group did not respond to the increased concentration of BAP in terms of shoot bud proliferation, the number of shoots, leaves, and roots per explant increased up to a certain point when the concentration of BAP was increased. Shoot growth is inhibited by the external application of cytokinins at levels over the optimum threshold (Roy and Banerjee, 2003; Bhattacharyya et al., 2023). Due to its potent properties, it decreases shoot length by promoting the proliferation of axillary buds. Poor growth with yellow and necrotic shoots was the result of higher concentrations of BAP and KIN (Asghar et al., 2010). Higher ratios of cytokinins are in responsible for enhancing the ethylene synthesis leading to plant tissue senescence (Iqbal et al., 2017). Neither PLB development nor callusing was observed for any combination of treatments. BAP 2.5 mg L^{-1} + NAA 1.0 mg L^{-1} showed the largest number of shoots and leaves per explant after 20 weeks of culture. According to Yakimova et al. (2000) and Ron'zhina (2003), it may be explained by the fact that BAP stimulates rapid cell division, which leads to shoot multiplication. Comparable results were found by in *Dendrobium primulinum* Lindl. (Pant and Thapa, 2012), *Aerides odorata* (Devi et al., 2013), *Dendrobium* orchid (Talukdar et al., 2003), and *Cymbidium aloifolium* (Rajkarnikar, 2011). After 60 days of growth, Suntibala and Rajkumar (2009) counted 7.5 shoots from the nodal part of *in vitro Dendrobium tranparens* L. seedlings grown in half strength MS media supplemented with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA.

When it came to the rooting stage, 2.5 mg L^{-1} BAP + 1.0 mg L^{-1} IBA produced more roots than NAA out of all the treatments. After receiving combinations of 1.5–2.0 mg L^{-1} BAP and 0.5–1.5 mg L^{-1} IBA, the roots showed the greatest number of long, dark green, strong, and healthy roots which are the attributes of physiologically sound plant. By increasing the amount of endogenous enzymes, auxins—an effective plant growth regulator increase the root initiation process. Auxins have a significant influence on the processes of cell division, elongation, and differentiation (He et al., 2023). The processes of root induction and development are accelerated by auxins, which are considered to be effective plant growth regulators that differentiate vascular bundles. They promote rooting by changing the plant biochemical system (Henrique et al., 2006). According to Han et al. (2009), auxin causes shoot buds to sprout, which in turn promotes the growth substances present in

10.3389/fsufs.2024.1359486

FIGURE 1
Effect of BAP (2.5 mg L^{-1}), sucrose (30 g L^{-1}) and + NAA 1.0 mg L^{-1} on shoot bud induction from nodal explants **(A)** Node swelling at advanced stage, **(B)** Bud development, **(C)** Multiple bud development, **(D)** Shoot induction, **(E)** Leaf initiation, **(F)** Shoot induction, **(G)** Shoot development, **(H)** Multiple shoots induction, **(I)** At initiation stage, **(J)** At development stage, and **(K)** Hardening of plantlets.

roots allowing them to expand and elongate (Sherif et al., 2020). Through repeated cell divisions, auxin administration causes the complex processes of lateral root development (Liu et al., 2002). According to George et al. (2008), they are so significant that they are considered to be necessary for the polarity of plants and their organs to be established and maintained (vascular system). Because it functions as a precursor to endogenous IAA, IBA is biologically a more active auxin for root initiation and produces a greater number of roots than NAA and IAA (Liu et al., 2002; Oliya et al., 2020). IAA and IBA developed more roots than NAA in *Dendrobium*

primulinum (Stephin et al., 2020; Pradhan et al., 2023). Compared to IBA, NAA produced fewer roots, most likely because it accumulates and cannot be quickly catabolized, especially at greater levels than IBA (Vuylsteker et al., 1997). IBA is recognized to promote rooting more effectively than other auxins because of its low toxicity and increased stability for root induction (Han et al., 2009). It is very effective at raising endogenous auxin amounts and demonstrating increased stability against catabolism and inactivation through conjugation with growth inhibitors, it produced good root number results (George et al., 2008). Orchid potting media should have good drainage capacity, good water holding capacity and aeration for better growth. Over 70% of the transplanted seedlings survived through the 60-day hardening period in a potting mixer filled with brick and charcoal pieces (1:1). The observation that Dendrobium Sonia "Earsakul" survived 66.67% of its transplantation in a greenhouse with charcoal and brick pieces (1:1) media suggests that the reason for this could be appropriate drainage and aeration of the medium, which is primodial importance in orchid culture. Kumari et al. (2013) corroborates this conclusion. Being an epiphyte, in the natural environment, their exposed roots take up moisture from dew and wet environments. As a result, when growing in artificial conditions, the subsrate's capacity to hold nutrients, water, capillary action, and aeration should all be taken into account. It's also necessary to take into account the medium components' weight and stability, ease of availability, prices, and consistency. Conversely, under greenhouse conditions, a 90% survival rate of *Dendrobium tranparens* L. was demonstrated by a 2:1 potting mixture of brick and charcoal (Vilcherrez-Atoche et al., 2023).

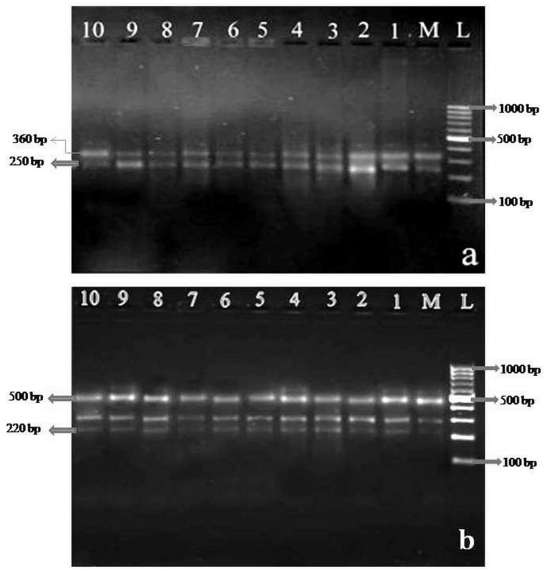

FIGURE 2
Polymerase chain reaction (PCR) amplification products obtained with random amplified polymorphic DNA (RAPD) **(A)** Primer (OPU-13) **(B)** Primer (OPU-16). Lane L represents100-bp ladder, lane M represents the mother plant and lanes 1–10 represent *in vitro* raised clones derived from nodal explant.

One of the most crucial prerequisites for crop species micropropagation is true-to-type clonal fidelity. The broader utility of the micropropagation method may be severely limited by the incidence of cryptic genetic defects resulting via somaclonal variation in the regenerates (Salvi et al., 2001). Therefore, in order to verify the quality of the plantlets for their commercial utility, it is essential to achieve genetic uniformity of micropropagated plants. The RAPD markers in this study exhibit a consistent banding pattern with no variation. All of the amplified bands on the mother and its tissue culture-raised offspring were monomorphic in nature. Several *in vitro* micropropagated plants *viz.* bananas by Alizadeh and Singh (2009), gerberas by Bhatia et al. (2011), *Dendrobium densiflorum* by Mohanty and Das (2013), *Spilanthes calva* by Razaq et al. (2013), and *Rauvolfia hookeri* by Ranjush and Gangaprasad (2014) cleared the genetic fidelity test using RAPD primers. But when clonality tests were performed on the mother and *in vitro* micropropagated seedlings, two of the ten selected RAPD primers showed clear monomorphic bands (Joshi et al., 2023). It was determined that genetic clonality was preserved by taking into consideration the monomorphic banding pattern that the mother and the *in vitro* cloned plant both displayed.

TABLE 3 List of primer code, sequences, reaction to DNA, number, and size of the amplified fragments generated by random amplified polymorphic DNA (RAPD) primers.

Sl. no.	Primer code	Sequence (5′- 3′)	Reaction to DNA	Number of scorable bands/ primer	Total number of bands amplified	Size of amplicons (bp)
1	OPU 09	CCACATCGGT	Negative	—	—	—
2	OPU 10	ACCTCGGCAC	Negative	—	—	—
3	OPU 11	AGACCCAGAG	Negative	—	—	—
4	OPU 12	TCACCAGCCA	Negative	—	—	—
5	OPU 13	GGCTGGTTCC	Positive, reproducible, monomorphic	2	22	250–360
6	OPU 14	TGGGTCCCTC	Positive but not reproducible	—	—	—
7	OPU 15	ACGGGCCAGT	Positive but not reproducible	—	—	—
8	OPU 16	CTGCGCTGGA	Positive, reproducible, monomorphic	3	33	220–500
9	P 14	AGGATACGTG	Positive but not reproducible	—	—	—
10	P 16	GGATCTGAAC	Positive but not reproducible	—	—	—
Total				5	55	

5 Conclusion

Phalaenopsis orchids are commonly known as moth orchid greatly appreciated for its attractive foliar venation which resemblance to moths but is still underexplored for its ornamental potential. The resplendent allure of these orchids lies in their exquisite and intricate floral design, characterized by graceful arching stems adorned with vibrant, delicate blossoms. Beyond their visual appeal, *Phalaenopsis* orchids exhibit a captivating fragrance, filling the surroundings with a delightful scent. Considering the rare and threatened status of this orchid, a protocol for *in vitro* regeneration of *Phalaenopsis* orchids has been successfully established. It avoids the production of callus and can be readily used for commercial micropropagation. In a short amount of time, this approach effectively produced a high frequency of plantlets. It has been found that growing seven well-hardened plants from a single nodal segment would require around 186 days, or nearly 27 weeks (from bud initiation to complete the acclimatization of plantlets). This study aims to underscore the significance of the micropropagation protocol in *Phalaenopsis*, demonstrating its efficacy in expediting plantlet development without the occurrence of callus formation. This protocol has diverse applications and commercial implications, ranging from meeting market demand and ensuring consistent quality to exploring new genetic possibilities and international trade opportunities. Furthermore, this study not only contribute to the specific understanding of *Phalaenopsis* orchid biology but also serve as a valuable model for advancing conservation strategies for endangered orchid biodiversity. The application of these findings to broader conservation efforts underscores the potential of *in vitro* techniques in preserving and restoring threatened orchid species on a global scale.

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 at: https://krishikosh.egranth.ac.in/communities/b2086dee-88ee-4546-90b1-753bcfee495b?spc.page=1&spc.sf=dc.date.accessioned&spc.sd=DESC&scope=b2086dee-88ee-4546-90b1-753bcfee495b.

Author contributions

DS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. PM: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. MS: Data curation, Resources, Supervision, Writing – review & editing. TM: Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Writing – review & editing. NM: Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – review & editing. KP: Writing – review & editing. CJ: Writing – review & editing. SS: Data curation, Writing – review & editing. BA: Data curation, Resources, Formal Analysis, Writing – review & editing. NA: Writing – review & editing. SR: Writing – review & editing. DW: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The authors extend their appreciation to Prince Sattam bin Abdulaziz University for funding this research work through project number (PSAU/2023/01/8920).

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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frontiers | Frontiers in Sustainable Food Systems

TYPE Review
PUBLISHED 08 April 2024
DOI 10.3389/fsufs.2024.1383302

OPEN ACCESS

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RECEIVED 07 February 2024
ACCEPTED 25 March 2024
PUBLISHED 08 April 2024

CITATION
Ćeran M, Miladinović D, Đorđević V, Trkulja D, Radanović A, Glogovac S and Kondić-Špika A (2024) Genomics-assisted speed breeding for crop improvement: present and future.
Front. Sustain. Food Syst. 8:1383302.
doi: 10.3389/fsufs.2024.1383302

Genomics-assisted speed breeding for crop improvement: present and future

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Global agricultural productivity and food security are threatened by climate change, the growing world population, and the difficulties posed by the pandemic era. To overcome these challenges and meet food requirements, breeders have applied and implemented different advanced techniques that accelerate plant development and increase crop selection effectiveness. However, only two or three generations could be advanced annually using these approaches. Speed breeding (SB) is an innovative and promising technology to develop new varieties in a shorter time, utilizing the manipulation of controlled environmental conditions. This strategy can reduce the generation length from 2.5 to 5 times compared to traditional methods and accelerate generation advancement and crop improvement, accommodating multiple generations of crops per year. Beside long breeding cycles, SB can address other challenges related to traditional breeding, such as response to environmental conditions, disease and pest management, genetic uniformity, and improving resource efficiency. Combining genomic approaches such as marker-assisted selection, genomic selection, and genome editing with SB offers the capacity to further enhance breeding efficiency by reducing breeding cycle time, enabling early phenotypic assessment, efficient resource utilization, and increasing selection accuracy and genetic gain per year. Genomics-assisted SB holds the potential to revolutionize plant breeding by significantly accelerating the identification and selection of desirable genetic traits, expediting the development of improved crop varieties crucial for addressing global agricultural challenges.

KEYWORDS

food security, speed breeding, marker-assisted selection, genomic selection, genome editing, genomics-assisted speed breeding (GASB)

1 Introduction

Global climate change is a leading aspect threatening agricultural productivity worldwide, along with the challenges of meeting the food requirements of the world's expanding population. Increased variations in rainfall and raised global temperature, along with increasing unpredictability in growing conditions, have caused the emergence, changed distribution and prevalence of pests and diseases. Abiotic and biotic stresses and loss of agricultural land caused by climate changes have direct or indirect negative impacts on agricultural production, usually causing yield losses (Hoegh-Guldberg et al., 2019; Paul et al.,

2019; Yu et al., 2021). Climate change has likely already affected global food production by reducing the yields of the top ten global crops, including barley, cassava, maize, oil palm, rapeseed, rice, sorghum, soybean, sugarcane and wheat, by 3–12% by mid-century and 11–25% by century's end, under a vigorous warming scenario (Ray et al., 2019). The study also found that impacts are mostly negative in Europe, Southern Africa and Australia (Ray et al., 2019). Thus, efficient systems for delivering improved varieties are crucial for successful adaptation and keeping up with shifting environmental conditions. On the other side, global population growth increased demand for food production and expanded the need for crops with improved nutritional profiles to address malnutrition and dietary deficiencies and to adapt to changing dietary preferences (Fróna et al., 2019). Also, expanding population increased urbanization and put pressure on available agricultural land. Furthermore, in the pandemic era, food security has continued to decline, affecting millions worldwide. The pandemic has affected disruptions in supply chains, labor shortages, and economic challenges, which contributed to rising food prices, affecting affordability for vulnerable populations (FAO, IFAD, UNICEF, WFP and WHO, 2021). Thus, the pandemic underscored the importance of building resilient and sustainable food systems to withstand increased global food insecurity. Therefore, to overcome these challenges and contribute to long-term global food security, the development of superior crop varieties is highly required (Lenaerts et al., 2019; Kondić-Špika et al., 2022b; Cvejić et al., 2023; Potts et al., 2023).

The major bottleneck in the process of breeding is long generation time. For most crop plants, conventional breeding often takes more than a decade to develop and release new cultivars (Jähne et al., 2020). Following the crossing of selected parent plants, 4–6 successive generations are typically required to reach a fixed homozygous state for the identification of a superior genotype. This process also involves multi-year testing in replicated field trials at multiple locations for the detection of candidate genotypes across a wide range of conditions (Voss-Fels et al., 2019). The development of hybrids in cross-pollinated crops follows similar timetables, wherein up to 10 years can pass between parental selection and inbreeding (Shimelis and Laing, 2012). Shortening breeding cycles is crucial to address the challenges of traditional breeding, particularly the prolonged generation times, allowing for faster and more efficient development of crop varieties in response to evolving agricultural needs.

It is considered that shortening the selection cycle's duration has the biggest effect on genetic gain when considering the cost–benefit ratio (Bonnecarrere et al., 2019). Variable agroecological conditions usually allow only one crop cycle per year, while in some tropical conditions, it is possible to obtain two generations per year (Laux et al., 2010). Thus, it has become imperative to develop and exploit new breeding technologies to ensure the rapid production of improved cultivars and accelerate genetic gain for important traits. Over time, various plant breeding approaches were used to advance generation and to fasten the breeding cycle, such as optimization of the traditional selection method - single seed descent (SSD) method utilized during breeding for the development of homozygous lines, use of off-season nursery for growing of two or more generations per year in contrasting environments (shuttle breeding) (Ortiz et al., 2007), *in vitro*/embryo culture (Kondić-Špika et al., 2022a), double haploid (DH) technique (Kondić-Špika et al., 2008; Kondic-Špika et al., 2011; De La Fuente et al., 2020), marker-assisted selection (MAS; Bernardo, 2016), and the use of genetic engineering or genome editing (Gaba et al., 2021; Varshney et al., 2021b). However, even with these methods, only two to three generations per year could be advanced (Fang et al., 2021). Moreover, off-season nurseries are often expensive, and logistically difficult to manage, with possible genetic material loss during transportation and the brief intervals between crop cycles; hence, they do not ensure successful seed production and are not appropriate for large-scale. In addition, the DH technique is not accessible for a variety of crops and often demands highly qualified personnel, specialized equipment and financial resources for *in vitro* culture. Furthermore, transgenic or genome-edited crops could be doubtful options because of political legislation or public safety concerns.

Speed breeding (SB) represents a promising next-generation breeding technology for growing plants under controlled and manipulable conditions to accelerate breeding programs by decreasing generation time and resources and increasing the number of generations per year (Watson et al., 2018; Bhatta et al., 2021). This approach holds significant importance for overcoming traditional breeding limitations, particularly for crops with long breeding cycles. Compared to the usual field and greenhouse conditions, SB can reduce generation length from 2.5 to 5 times. Also, it is assumed that obtaining up to five generations per year in an SB system could approximately double annual genetic gain compared to breeding programs that use off-season nurseries (Jähne et al., 2020). Beside enhancing breeding efficiency, SB rapidly shortens breeding cycles, providing a timely response to emerging challenges such as climate variability and specific agricultural needs. This agility in breeding, achieved through SB, ensures the timely development of resilient and high-yielding crop varieties, contributing to global food security and sustainability (Gudi et al., 2022).

SB promotes rapid growth and development of plants from the vegetative to the reproductive stage, typically in controlled environments such as growth chambers or greenhouses, manipulating the major parameters required by the plants (photoperiod, quality and intensity of light, temperature, and humidity; Figure 1). In general, plants can be categorized into three groups based on how the length of the day influences flowering and reproductive processes of the plant (photoperiodism): short-day plants (SDP) typically flower when the duration of daylight is shorter than a critical length, long-day plants (LDP) flower when the duration of daylight exceeds a critical length, and day-neutral plants (DNP) that are less influenced by the photoperiod and tend to flower independently of day length (Ghosh et al., 2018). SB was first proposed as a strategy for LDP by extending the photoperiod to about 22 h per day (Watson et al., 2018). Extended photoperiods cause early flowering due to the conversion of phytochromes from their active to inactive forms, using specific light intensities. Exposition to prolong daily light reduces the generation time of LDP, while in SDP and photoperiod-sensitive crops, this approach would inhibit flowering. Consequently, the protocols for short-day crops (soybean, rice, and amaranth) were proposed, varying light intensities to create the ideal optimal light conditions for each crop (Jähne et al., 2020). In numerous crops, early flowering and seed formation have been found to be successfully promoted by applying light intensity ranging from 360–650 μmol/m^2/s within the PAR range (Ghosh et al., 2018; Watson et al., 2018), enabling SB procedure. Beside light intensity, light quality is another major element with a direct effect on different plant growth activities such as photosynthetic and transpiration rates, stomatal conductance, and the level of

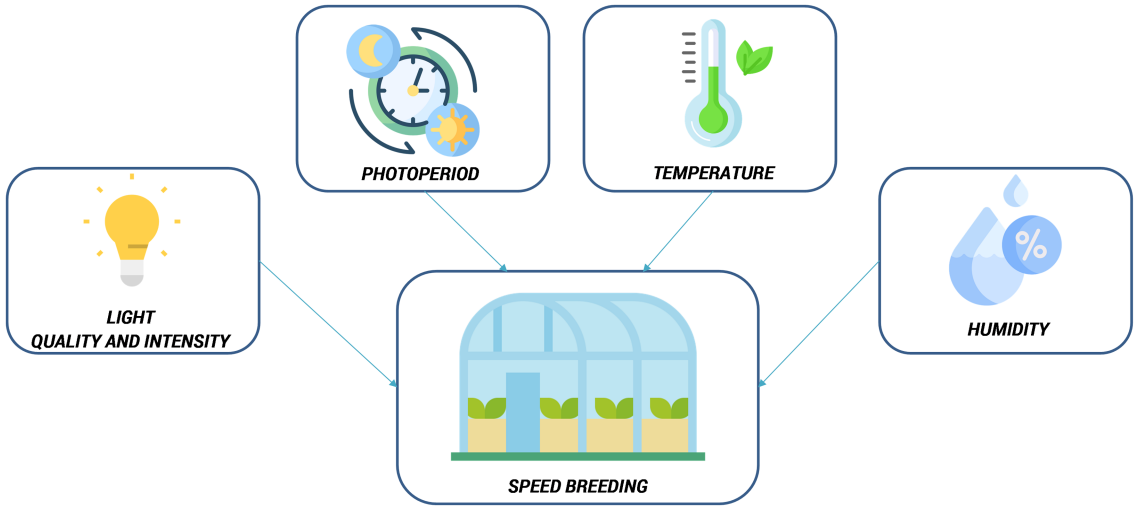

FIGURE 1
Manipulation of different environmental factors in speed breeding (this figure has been designed using icons from Flaticon.com).

intercellular CO_2 (Yang et al., 2017). Considering that the key factor in controlling a plant's ability to flower is the red to far-red (R:FR) ratio, altering this ratio may cause different responses in flowering. Therefore, light spectrum and light quality are essential for the optimization of SB protocols. Different lighting sources can be used in the growth chambers to create PAR (photosynthetically active radiation) range: light-emitting diodes (LEDs), metal halide bulbs combined with LEDs, halogen or sodium vapor lamps (SVLs) (Ghosh et al., 2018). Furthermore, it is important to consider the different reactions of plant species to the wavelength spectra emitted by different lighting sources. It has been shown that early flowering in legumes, including lentil, pea, and chickpea, can be induced by applying blue and far-red light spectrums (Mobini et al., 2015). Photoperiod, light intensity and quality are important factors in flowering regulation, while maintaining consistent temperature and humidity levels can contribute to faster growth. Ideal soil and air temperatures are essential to achieving satisfactory germination, seedling establishment and proper vegetative and reproductive growth (Shendekar et al., 2023). Several main crops, such as soybean, canola, wheat, barley, and maize, have distinct temperature requirements for different phases of growth. For example, they usually need 12–30°C for seed germination and 25–30°C for overall growth and flowering (Wanga et al., 2021). Moreover, winter wheat requires vernalization or cold temperature stress for the transition from vegetative to the reproductive stage (Zheng et al., 2023), while in certain crops, temperatures higher than 33°C can result in reduced pollen viability and increased male-sterility. Also, there has been a considerable variance in the responses of different photoperiod-sensitive crops to temperature regimes that affect their transition from the vegetative to the reproductive stage (Yang et al., 2014). Therefore, the optimal temperature regime, considering both maximum and minimum temperatures, should be used for each crop in SB protocols. Different systems can be used to control temperature, such as foggers, solar air power batteries, fan and pad cooling systems (Wanga et al., 2021). Furthermore, it was also demonstrated that variations in soil moisture can have a significant impact on various aspects of plant growth and development, including plant height, days to flowering, maturity, and seed set (Hussain et al., 2018), which is beneficial for SB. In different crops, such as wheat, barley, canola, and chickpea, grain filling and maturation were accelerated after flowering by gradually lowering the soil's moisture level (Watson et al., 2018). In general, for most of the crops, humidity of 60–70% is suitable, while crops adapted to dry zones require less humidity (Ghosh et al., 2018).

Optimization of all these conditions enhances the rate of photosynthesis and other physiological and metabolic processes, which stimulates early flowering, biomass accumulation and early seed development to reduce generation time, allowing multiple generations to be grown in a shorter period (Ghosh et al., 2018; Shendekar et al., 2023). Understanding the different requirements for photoperiod and light parameters of different crop species is crucial for optimizing growth conditions and achieving rapid generation turnover in SB approaches and could be a limiting factor for successful implementation of SB. Thus, there is a need for protocol optimization on a crop-specific and variety-specific level (Jähne et al., 2020; Pandey et al., 2022) to achieve optimal growth and development, with greater uniformity. Due to genotypic variation in terms of growth characteristics, photoperiod sensitivities, and responses to environmental conditions, optimizing protocols based on genotype ensures that the specific needs and preferences of each plant variety are met. In this way, the full potential of each genotype is harnessed, maximizing the efficiency and success of the approach and enhancing reproducibility. Moreover, optimizing protocols and providing the ideal conditions can maximize resource efficiency, such as energy, space, and time.

Besides optimizing the plant development environment, SB can also be accomplished through other means, such as stress treatments, immature seed harvesting, treatments with plant growth regulators (PGRs), high-density planting, embryo rescue, increasing CO_2 concentrations, genetic engineering targeting the flowering pathway or grafting juvenile plants to mature rootstocks (Richard et al., 2015; Ghosh et al., 2018; Hickey et al., 2019; Pandey et al., 2022). Under conditions of moisture stress, many crop

species exhibit early flowering and seed production (Shavrukov et al., 2017). This approach is used in SB through the application of both drought and flooding stress to cause flowering for early seed sets. In addition, it is considered that immature seed harvesting, avoiding the ripening stage, could significantly shorten the generation time when combined with single-seed descent (SSD) (Chahal and Gosal, 2002). Immature seed harvesting involves picking immature pods and drying seeds under artificial conditions for a few days before shelling and sowing, allowing plants to cycle even faster from seed to seed (Jan et al., 2022). In several crop species, plant nutrition or treatments with plant growth regulators (PGRs) have been effectively employed to expedite development and initiate flowering and seed formation, particularly in techniques like organ tissue culture (Bonea, 2022). Observing different reactions to PGRs can be important in controlled conditions where certain crops, such as lentils and faba beans, have demonstrated the capacity to produce up to eight generations per year (Trnka et al., 2019). High-density planting is a cost-effective strategy for SB that considers planting crops with more plants closer together, which outperforms traditional methods in terms of yield potential. It is a useful technique since it not only stimulates early flowering and maturity, and shortens crop cycles, but also makes it possible to maintain the large population size needed for advanced selections (Sinha et al., 2021). The specific plant species can affect the effectivness of high-density planting. The effect of high-density planting on flowering is not consistently detected across studies and genotype variations considerably alter plant responses (Fukushima et al., 2011). In addition, it has been found that crop plants respond to elevated CO_2 by transitioning from the vegetative to the reproductive phase. Although the reaction may range throughout crop species and within genotypes of the same species, elevated CO_2 levels might expedite the transition from vegetative to reproductive stages and boost plant development (Jagadish et al., 2016).

2 Development of SB protocols for main crop species

The SB approach involves growing plants in controlled environments with specific conditions that influence the acceleration of various physiological processes in plants and quick-generation cycling. This offers flexibility for integration into a larger-scale screening of plant populations in the shortest amount of time and space. The main advantage of SB technology over other breeding methodologies aimed at reducing generation turnover time and accelerating the process of creating new varieties is its suitability for application to diverse germplasm without special requirements regarding *in vitro* culturing.

Different SB approaches have been successfully used to develop and standardize protocols for major crops, including commercially and scientifically significant cereal, legume and oilseed species, which covered SDP, DNP and LDP (Table 1; Ghosh et al., 2018; Watson et al., 2018; Samantara et al., 2022; Shendekar et al., 2023). This enabled the achievement of even 9 generations within a year for some crops. In this way, homozygosity can be reached in a year or two, providing a great opportunity to develop varieties in a short period of time and to deal with challenging food security (Hickey et al., 2019).

2.1 Wheat

As one of the most widely cultivated grain crops that feed approximately 35% of the world's population, wheat has consistently played a key role in global food security strategy programs. Its grain serves as a crucial source of protein, dietary fibres, B vitamins, and minerals in the human diet (Shewry and Hey, 2015). Despite the existence of five domesticated Triticum taxa (diploid *T. monococcum*, tetraploid *T. dicoccon* and *T. durum*, and hexaploid *T. aestivum* and *T. spelta*), global wheat production is predominantly centered on bread and durum wheat, comprising 90–95% and 5–10% of the total, respectively (Shewry, 2009; Shewry and Hey, 2015). Bread wheat is primarily used as flour for various flatbreads and pastries, whereas durum wheat stands out as the key ingredient in the production of pasta, couscous, and bulgur. Traditional cereal breeding methodologies have brought many significant improvements regarding wheat yield and quality (Hristov et al., 2010; Mladenov et al., 2011). Furthermore, the introduction of the new breeding tools relying on the molecular and phenotyping high-throughput approaches enabled accurate pyramidization of genes with the potential to result in varieties with stable production in the environment with different combinations of abiotic and biotic stresses (Kondić-Špika et al., 2023). However, the long breeding cycle presents one of the main constraints in achieving revolutionary progress in wheat production, impeding the timely development and deployment of improved cultivars. Traditional wheat breeding involves a series of steps, with each phase demanding considerable time. The delayed release of resilient and high-yielding wheat varieties hampers the ability to adapt quickly to evolving challenges posed by climate variability, emerging pests, and shifting consumer preferences. In contrast, approaches like SB enable the acceleration of the breeding cycle, allowing rapid progress, facilitating the development of wheat varieties that can address contemporary agricultural demands and contribute significantly to global food security. History of the development of wheat SB protocols, can be traced to the mid-1980s, when joint efforts by NASA and Utah State University to assess the possibility of growing rapid cycling wheat under constant light on space stations, resulted in the creation of the dwarf wheat line USU-Apogee (Bugbee and Koerner, 1997). Later on, at the beginning of the 2000s, researchers at the University of Queensland introduced the term 'speed breeding' for a set of improved methods to hasten wheat breeding (Hickey et al., 2019). So far, several protocols have been developed for the SB of spring wheat. The group of authors developed a very effective protocol for rapid generation cycles based on culturing young embryos and optimizing plant growth conditions that allowed the production of up to eight generations of spring wheat per year (Zheng et al., 2013). Afterwards, to make SB technically simpler and capable of processing a larger number of genotypes, researchers from the United Kingdom and Australia developed two SB protocols for spring bread wheat and durum wheat that involve the use of extended photoperiod and high light levels to accelerate plant development, followed by immature seed harvesting and planting developing kernels (Ghosh et al., 2018; Watson et al., 2018). According to the first protocol, plants grown in a controlled environment room with an extended photoperiod (22 h light/2 h dark) were compared with the plants grown in glasshouses with no supplementary light or heating during the spring and early summer. The authors concluded that plants grown under the SB protocol progressed to flowering in 35–39 days, approximately half the

TABLE 1 List of speed breeding protocols developed in different crops.

Family	Crop species	Type of photoperiod	Techniques	Trait enhanced (goal)	Field generation (days)	Days to flowering	Generation time (days)	Generation per year	Selection method	References
Amaranthaceae	Amaranth (*Amaranthus* spp.)	SD	Photoperiod and temperature	Rapid production of segregating populations	95–100	28		6	SSD	Stetter et al. (2016)
Brassiacceae	Canola (*Brassica napus*)	LD	Photoperiod, light intensity, temperature, immature seed germination and soil moisture	Pod shattering resistance	135–150	73	98.2	4	SSD	Ghosh et al. (2018) and Watson et al. (2018)
Fabacae	Chickpea (*Cicer arietinum*)	LD	Photoperiod and immature seed germination	Rapid generation advance	150–160	33	50–59	6–7	SPD	Samineni et al. (2020)
	Clover (*Trifolium subterraneum*)		*In vitro*	Rapid development of bi-parental and multi-parental populations		32–35		2.7–6.1	SSD	Pazos-Navarro et al. (2017)
	Faba bean (*Vicia faba*)		Plant hormones, photoperiod, temeprature, light intensity and immature seed	Early flowering and seed development (rapid generation advance)	104	29–32	54–80	7	SPD	Mobini et al. (2015, 2020)
	Lentil (*Lens culinaris*)		Plant hormones, photoperiod, light intensity and immature seed	Early flowering and seed development (rapid generation advance)	102–107	31–33	45	8	SPD	Mobini et al. (2015)
	Lupin (*Lupinus angustifolius*)		Photoperiod and *in vitro* immature seed germination	Rapid generation advance				5		Croser et al. (2016)
	Pea (*Pisum sativum*)		Plant hormones, photoperiod, temperature, immature seed harvest and drying, hydroponics	Development of RILs	60–80	33	68.4	4–6		Mobini and Warkentin (2016), Watson et al. (2018), and Cazzola et al. (2020)
	Groundnut (*Arachis hypogaea*)	SD	Photoperiod and temperature	Advancement of early generation breeding material	125–145	25–27	89	3–4	SPD	O'Connor et al. (2013)
	Pigeon pea (*Cajanus cajan*)		Photoperiod, temperature, immature seed germination	Development of photoperiod insensitive lines	120–190	50–56		4	SPD	Saxena et al. (2019)
	Soybean (*Glycine max*)		Photoperiod, temperature and imature seed germination	Development of RILs; effect of light intensity on germination rate	100–140	23	77	5	SSD	Nagatoshi and Fujita (2019) and Jähne et al. (2020)
Poaceae	Barley (*Hordeum vulgare*)	LD	Photoperiod, temperature, soil fertility, immature seed germination and embryo rescue	Leaf rust resistance	130–150	24–36	68	4–9	SSD	Zheng et al. (2013), Hickey et al. (2017), and Watson et al. (2018)
	Oat (*Avena sativa*)		Photoperiod, temperature, and micro-nutrients	Shortening of the generation time and early panicle harvest	114	21	51	5	SSD	González-Barrios et al. (2020)
	Spring wheat (*Triticum aestivum*)		Photoperiod, temperature, immature seed germination and drying, soil fertility,	Rapid production of segregating populations and pure lines; Biotic stress tolerance	145–155	24–41	65	4–8	SSD	Zheng et al. (2013), Riaz et al. (2016), Ghosh et al. (2018), and Watson et al. (2018)
	Durum wheat (*Triticum durum*)		SB with multitrait phenotyping	Resistance to crown rot	150–155		77	6		Alahmad et al. (2018)
	Winter wheat (*Triticum aestivum*)		Photoperiod		160–180		87	4–5		Cha et al. (2022) and Schoen et al. (2023)
	Rice (*Oryza sativa*)	SD	Photoperiod, temperature and high-density planting	Rapid development of high yielding varietiy	90–120	75–85		4	SSD	Collard et al. (2017)
	Sorghum (*Sorghum bicolor*)		Photoperiod, temperature and imature seed germination	Rapid development of high yielding varietiy	100–120	40–50		6	SSD	Forster et al. (2014)

10.3389/fsufs.2024.1383302

time of those grown in glasshouse conditions. Under SB protocol, a slight decrease was observed in seed number per spike, while wheat plants produced a healthy number of spikes per plant, and crosses produced viable seeds. The second protocol included the use of a temperature-controlled glasshouse fitted with high-pressure sodium lamps aiming to extend the photoperiod to a day length of 22 h. Here, control treatment plants were grown in a glasshouse using a natural 12-h control photoperiod. Both control and treatment used the same temperature regime: 22°C day and 17°C night. As a result, flowering was significantly reduced relative to control plants (22 ± 2 days), and wheat plants produced significantly more spikes than those in day-neutral conditions. Further, the authors recorded an additional reduction in the breeding cycle when wheat seeds were harvested before maturity, 14 days post-anthesis, followed by 4-day cold treatment, without the need for labor-intensive embryo rescue (Watson et al., 2018). The development of SB protocols for winter wheat varieties addressed challenges to optimizing controlled environments for accelerated growth due to vernalization requirements for the transition from the vegetative to reproductive phase, making it complicated to synchronize growth cycles in SB. Therefore, SB protocols for winter wheat were modified by adding one step that involved seed exposure to low temperatures and optimal results were obtained at 10°C (Zheng et al., 2023). However, this methodology was not successful when applied to mature seeds because of the postharvest dormancy phenomenon that exists among winter genotypes, while it gave very promising results with the use of the embryo rescue technique when it was possible to produce up to 6 generations of wheat accessions per year.

In conclusion, originating from efforts from more than 40 years ago, SB protocols for spring wheat have evolved, enabling the production of up to eight generations per year. The protocols have been refined for simplicity and efficiency, involving extended photoperiods, resulting in a remarkable reduction in time to flowering and an increase in spike production. Despite challenges in adapting SB to winter wheat, modifications involving vernalization have shown promise.

2.2 Maize

Maize (*Zea mays* L.) represents the major source of food in the world owing to its large growing areas and productivity, and it is also the basic fodder and important raw material for industrial processing (Muntean et al., 2022). On a global level, maize ranks first in terms of production quantities, followed by rice, wheat, barley and sorghum (FAO, 2022). Although it originated in tropical regions from the short-day species Teosinte (*Z. mays* ssp. *parviglumis*), during centuries of cultivation and artificial selection, maize was spread to higher latitudes and adapted to grow under long-day conditions (Brambilla et al., 2017; Mikić et al., 2017; Osnato et al., 2022). While studying maize sensitivity to photoperiod changes under controlled conditions, it was concluded that long photoperiods in photoperiod-sensitive maize lines repressed the development of the tassels, delayed flowering time and increased the period between pollen shed and silking (Chen et al., 2015). Similarly, tropical maize lines showed a delay in flowering transitions under long-day conditions, compared to those adapted to temperate zones for more than 3 weeks (Alter et al., 2016). The same authors observed strong differences among regulated genes between temperate and tropical lines indicating the complexity of flowering time regulation in maize. Thus, traditional breeding cycles for maize can be protracted due to the dependence on specific photoperiod conditions for flowering. Considering the maize requirement of a short day for induction of the reproductive phase, SB may have potential applications for accelerating its vegetative growth (Watson et al., 2018). Although many crop-specific SB protocols in growth chambers or glasshouses have been established for different species, they have not yet been developed for maize (Singh et al., 2021). Besides its photoperiod reaction, there are also other challenges to SB implementation in maize. Being a tall crop with a canopy that can reach a height of 2.5 m, maize needs more space and bigger chambers for growth and development (Singh et al., 2021). Another approach that can shorten the maize breeding cycle is double-haploid (DH) technology (Farooqi et al., 2022). Great success in the application of DHs has been achieved in maize, which was evidenced by the development of parental lines in the majority of the seed companies (Shendekar et al., 2023). When applying DH technology, the time required to develop completely homozygous maize inbred lines is greatly reduced to 2–3 generations (Mitiku, 2022). Coupled with other approaches, like MAS and off-season nurseries, DH technology can enhance breeding efficiency and genetic gain.

To sum up, although SB protocols have been established for other species, maize faces challenges in implementing photoperiod-based growth protocols. So far, DH technology has been successful in shortening the maize breeding cycle to 2–3 generations, reducing the time required to develop homozygous maize inbred lines.

2.3 Rapeseed

Rapeseed (*Brassica napus* L.) is an important oil crop grown in over 50 countries worldwide (Yadava et al., 2012). It is also called oilseed rape or, in the case of varieties with low erucic acid content, canola. It is an allotetraploid crop derived from hybridization between *Brassica rapa* L. (AA) and *Brassica oleracea* L. (CC). Besides its oil and protein-rich meal, it considerably participates in global biofuel production (Zhang et al., 2023). Compared to other major crops, rapeseed yield per hectare is the lowest, reaching ~3,210 kg/ha in Europe in the period between 2015 and 2020 (Zheng et al., 2022). Hence, one of the major challenges in the current unpredictable climates as well as the reduction in the available crop production area is meeting the world's demand for edible oil. In conventional breeding, with the application of greenhouse cultivation, two to three generations per year can be obtained in major oil crops including rapeseed. By achieving multiple generations annually, breeders can expedite the selection and advancement of desirable traits in rapeseed varieties. This enhanced breeding efficiency not only accelerates the overall breeding process but also contributes to the development of improved rapeseed cultivars with enhanced yield, oil quality, and resilience to environmental challenges. The introduction of SB in rapeseed, a LDP, enables speeding up the breeding process by obtaining even four to five generations per year, depending on the genotype and the growth conditions (Song et al., 2022). A detailed protocol for the initiation of setting up SB systems in several crops, including rapeseed, was provided, showing that phenotyping of some traits, such as pod shattering, can be performed in SB systems in adult plants (Ghosh et al., 2018). According to the results, a 22 h photoperiod

led to shortening the time to flowering, duration of flowering, days till drying off, and time to harvest, compared to a 16 h photoperiod. This SB regimen negatively affected the number of seeds per pot and thousand-grain weight, while germination of the harvested seeds was not affected or even was slightly higher in SB conditions. Furthermore, it was reported that seed production (g per plant) was similar between SB conditions in which a 22 h photoperiod was used and day-neutral conditions with a 12 h photoperiod, in 7 rapeseed cultivars (Watson et al., 2018). Recently, a comprehensive SB (CSB) approach was proposed, with the following steps: (1) vernalization of the germinated seeds, (2) high-density seedling culture, and (3) accelerated flowering and maturation with the optimized light regime (Song et al., 2022). For the majority of examined semi-winter and winter canola varieties, the authors observed acceleration in reaching key growth stages, however, a winter canola variety Darmor-bzh did not flower. Thus, the authors had to improve the CSB protocol for this variety by adding 500 µmol/m^2/s far-red light. This showed the importance of optimizing the growth conditions on a genotype-specific level in the SB protocol. Furthermore, the implementation of CSB in combination with marker-assisted and phenotypic selection, as reported (Wang et al., 2023), represents an innovative and comprehensive approach to crop improvement. This hybrid approach harnessed the power of accelerated plant growth through CSB while leveraging molecular markers for precise trait selection and enabled the accelerated creation of six introgression lines with different combinations of genes associated with abiotic and biotic stress resistance, high oleic acid content traits and early maturity. This integrated strategy not only expedites the breeding process but also allows for the targeted incorporation of multiple beneficial characteristics by efficiently addressing diverse stressors and quality traits.

In short, the introduction of SB, particularly in LDP, can accelerate the breeding process by obtaining four to five generations per year in rapeseed, depending on genotypes and growth conditions. Comprehensive SB (CSB) approaches, incorporating vernalization, high-density seedling culture, and optimized light regimes, offer further advancements. The integrated approach of CSB and MAS expedites the breeding process but also enables targeted incorporation of multiple beneficial traits.

2.4 Sunflower

Sunflower (*Helianthus annuus*) is the fourth most important oil crop used mainly for human consumption, and just 10% for biodiesel production and other industrial purposes (Radanović et al., 2023). Sunflowers, characterized by their out-crossing nature, require homozygous parents to produce hybrids, a process that requires demanding backcrossing and selection efforts. Achieving homozygosity in parental lines is crucial to ensuring genetic uniformity and stability in the resulting hybrids. Due to the out-crossing behavior of sunflowers, attaining homozygosity involves multiple generations of backcrossing to eliminate genetic heterogeneity. This process may require up to eight generations of carefully controlled crosses, followed by stringent selection for desired traits. Despite being an important crop, there are no available SB protocols for acceleration of the breeding process of this SDP. To speed up the creation of new, superior sunflower parental lines, a reliable and effective doubled haploid (DH) induction technique would be a useful resource. However, there are a lot of obstacles to creating a successful protocol, such as sunflower's resistance to *in vitro* culture regeneration as well as long fresh seed dormancy, which creates a production bottleneck for DH (Miladinović et al., 2019; Mabuza et al., 2023). Several techniques, including anther culture, microspore culture, interspecific hybridization, and embryo rescue, have been used to create a sunflower DH induction regimen, but none of them have proven effective or dependable. Low crossability, low seed set, low germination and regeneration, and a high albinism rate are the primary obstacles (Kaya, 2014; Mabuza et al., 2023). As a result, different strategies are required to get over these restrictions and raise the production efficiency of sunflower DH. Promising results have been obtained by authors who managed to generate haploid embryos by pollen irradiation and pollination of female flowers with irradiated pollen (Aktaş et al., 2023). Another approach could be using immature embryo culture to shorten the generation time in breeding programs. This technique allows the production of fertile plants from immature embryos and enables the production of three to four generations per year, in contrast to one generation per year with conventional breeding (Vasić and Vasiljević, 1994; Dağüstü et al., 2012). In addition, the development of new breeding techniques, such as genome editing, in combination with different -omics tools could provide new perspectives for speeding up sunflower breeding (Miladinović et al., 2019). Using this technique, multiple genes can be individually engineered at the same time, which in combination with different prediction models, could speed up the modification of linked genes or QTLs that are usually difficult to segregate (Flavell, 2010; Miladinović et al., 2021). The first steps in that direction were made with the recent development and optimization of protocols for genome editing in sunflowers (Yildirim et al., 2022, 2023). Further development of high-throughput and sequence-based genotyping, along with high-throughput and precision phenotyping, should provide a complete set of tools for developing tools for wider application of SB in sunflower improvement programs (Cvejić et al., 2023).

In general, the absence of SB protocols for sunflowers adds to the complexity of their breeding programs. The conventional DH induction techniques face obstacles such as *in vitro* culture resistance and long seed dormancy. Despite attempts with various techniques, significant challenges persist, including low crossability, seed set, germination rates, and high albinism. Recent advancements, including pollen irradiation for haploid embryo generation and immature embryo culture, show promise for overcoming some limitations. Moreover, the integration of genome editing and -omics tools opens new avenues for accelerating sunflower breeding.

2.5 Soybean

Soybean (*Glycine max* L.) is the most important protein cropand is used for animal feed and human nutrition. In addition to the high content of protein and oil, soybeans also contain numerous compounds with a positive effect on health, such as phytoestrogens and antioxidants. The global production of soybean was the fourth highest of all crops and over the past two decades, worldwide soybean production and the area harvested have increased more rapidly

compared to other staple crops (FAO, 2022). Soybean breeding is a long and complex process that involves the crossing, selection, and evaluation of different traits, such as yield, maturity, quality, and stress tolerance. Achieving multiple generations per year for soybean is significant for accelerating genetic improvement to meet the increasing global demand for protein and oil, as well as adapting to the changing climate and environmental conditions. Different strategies were explored to hasten soybean breeding, keeping in mind that uninterrupted periods of darkness and high temperatures are needed for the induction of flowering, accelerating the growth and development of short-day plants (Mao et al., 2017; Miladinović et al., 2018). Approaches, such as immature embryo culture or DH, could shorten one generation time to 65–70 days in soybean (Rosenberg and Rinne, 1988), while the use of off-season nurseries doubled the rate of generation advancement (Gai et al., 2015). On the other hand, SB techniques that use artificial, controlled environments with varying photoperiod, temperature, or CO_2 concentration could produce five generations per year in soybeans, compared to one or two generations in the field (Nagatoshi and Fujita, 2019; Jähne et al., 2020; Jan et al., 2022). Specificly, using CO_2-supplemented growth chambers and fluorescent lamps accelerated soybean breeding of elite Japanese cultivar Enrei by applying a protocol (14 h light, 30°C day/25°C night, CO_2 supplementation 400–600 p.p.m.) that reduced the vegetation period from 102 to 132 days reported in the field to 70 days enabling five generations per year (Nagatoshi and Fujita, 2019). This method also increased the crossing efficiency of soybeans. CO_2 supplementation enhanced the growth and productivity of plants, while specific light and temperature conditions shortened the days to flowering, and the reproductive period was significantly shortened by the reaping and planting of immature seeds. Furthermore, a SB protocol for soybeans based on LEDs providing a high-throughput, rapid SSD system was developed (Jähne et al., 2020). A 10 h photoperiod using a blue-light-enriched, far-red-deprived light spectrum enabled soybean to mature within 77 days after sowing, allowing the development of five generations per year. The authors proposed a possible improvement of the protocol by increasing the CO_2 level, under the condition that a higher photosynthesis rate is achieved through more intense lighting. The additional speed should be weighed against the extra costs of the system setup and operational costs. In the experiments, far-red and blue light treatment at night in most cases caused highly heterogeneous flowering time among soybean genotypes, showing the different responses of cultivars from different maturity groups. Although far-red light (>700 nm) did not affect flowering time, the authors concluded that it caused elongated petioles, which in turn caused lodging. Thus, far-red light should be avoided to grow robust soybean plants suitable for high-throughput systems, while low red/blue light ratio, with green or cool white LEDs (4,000 K) should be included for optimal visual observations. They proposed that light intensity should be ~500 µmol/m^2s at 50 cm distance from the light source to achieve fast generation times using a moderate budget. Other authors also defined the growth conditions for SB system using LED light source, advancing one generation of soybean within 73 days, enabling the growth of five generations per year (Lee et al., 2023). One of the challenges related to SB approaches that use artificial greenhouses is their limited scale and high cost. Thus, a cost-saving SB system established by integrating off-site nurseries and fresh-seeding methods under natural conditions was proposed, accomplishing at least four generations per year, which facilitates and accelerates soybean improvement at a low cost (Fang et al., 2021). This methodology was combined with MAS to predict the optimal adaptation region of the advanced generation lines based on the maturity genes E1-E4 instead of phenotype identification that could facilitate the breeding process for the target region (Fang et al., 2021). Combining SB with MAS using molecular markers for precise adaptation region prediction, streamlined the breeding process by eliminating the need for time-consuming phenotype identification. This not only accelerates breeding cycles but also enhances efficiency and resource utilization.

In conclusion, SB techniques utilizing controlled environments with manipulated photoperiod, temperature, and CO_2 concentration offer a promising avenue for accelerating soybean breeding. Achieving up to five generations per year, in contrast to traditional methods, significantly reduces the vegetation period. While challenges related to artificial greenhouses, including limited scale and high costs, persist, innovative solutions, such as integrating off-site nurseries and fresh-seeding methods under natural conditions, have been proposed. Additionally, combining SB with MAS streamlines the breeding process, eliminating the need for time-consuming phenotype identification and enhancing overall efficiency and resource utilization.

3 Integration of SB with genomic approaches

Standardized SB systems have the potential to significantly reduce the duration of main breeding processes for developing new varieties or to accelerate plant development for other plant research purposes, such as crossing, backcrossing, generation advancement, phenotyping adult plant traits, rapid gene identification, development of mapping populations, pyramiding target traits, mutant studies and genetic transformation experiments (Figure 2; Ghosh et al., 2018; Watson et al., 2018; Hickey et al., 2019; Ahmar et al., 2020; Bhatta et al., 2021; Varshney et al., 2021a). Different breeding selection methods, such as single seed descent (SSD), single pod descent (SPD), single plant selection (SPS), and clonal selection can be integrated into SB to produce a fixed population at a much faster and more affordable rate resulting in accelerated development and release of new cultivars (Hickey et al., 2017; Watson et al., 2018). With these methods, researchers can effectively advance offspring under high-density planting within controlled conditions, while also requiring less labour and cultivation space during initial generations (Table 2). It could also expand the possibilities for maintaining genetic diversity within a crop breeding program and be extended to field environments. In addition to directly accelerating some breeding activities, the rapid development of mapping populations through SB has also stimulated research studies that elucidate associations between genes and traits for breeding purposes (Samantara et al., 2022).

Integration of fast-forward genomic breeding techniques with SB (genomics-assisted speed breeding [GASB]) has a great potential to enable further advancement of crop improvement, by leveraging the benefits of all approaches in terms of improving selection efficiency and reducing generation time. Combining the SB approach with genomics and phenomics may encompass candidate gene discovery, MAS, marker-assisted backcrossing (MABC), genomic selection (GS), genome editing, or metabolic pathway editing for desired traits followed by high-throughput phenotyping (Figure 2). Moreover, GASB can speed up the creation of new variability for crop improvement by quickly manipulating

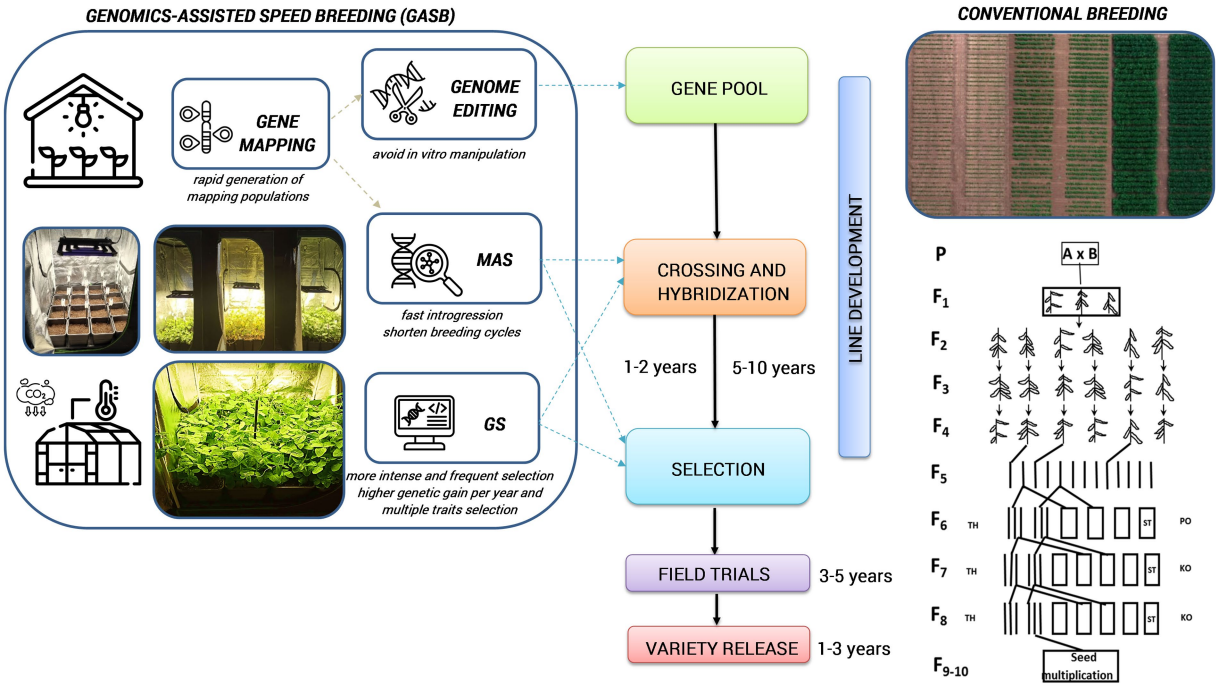

FIGURE 2
Integrated GASB to accelerate variety release compared to the conventional breeding. MAS, marker-assisted selection; GS, genomic selection; (this figure has been designed using icons from Flaticon.com).

the target region in the genome by minimizing the time, field space and overall resources (Varshney et al., 2021b). These genomic tools circumvent laborious phenotyping and facilitate multi-trait selection under growth chambers (Hickey et al., 2019). Furthermore, by combining metabolomic and SB, a robust and quick risk assessment of gene-edited crops can be achieved over several generations (Razzaq et al., 2021).

3.1 Gene mapping and MAS

Gene mapping provides the identification of major genes and quantitative trait loci (QTLs) associated with phenotypic variation, enabling better insight into the genetic control of agronomically important traits and suppling fundamental information for MAS, gene cloning, and genome structure studies (Brbaklić et al., 2015; Dimitrijevic and Horn, 2018; Gupta et al., 2019; Trkulja et al., 2019). The process relies on the comprehensive genotyping and phenotyping of various genetic materials, followed by appropriate statistical analysis for the detection of genes and QTLs. The most common materials used for trait dissection in gene mapping are biparental populations (F_2, doubled haploids, backcross and recombinant inbred lines), multiple parental populations (multi-parent advanced generation inter-cross - MAGIC populations) and nested association mapping (NAM) populations (Li et al., 2010; Huang et al., 2015; Chidzanga et al., 2022). The availability of new sequencing and genotyping technologies together with the development of high-throughput phenotyping approaches, facilitate the evaluation of genetic material for agronomic traits in multiple environments and seasons. However, the production of diverse mapping populations requires substantial amount of time for the selection of parents, their crossing and the evaluation of the variability among obtained lines. All these activities can be performed by SB, producing large and genetically diverse mapping populations with reduced time and overall cost (Watson et al., 2018). This discovery has led to significant advancements in the domains of MAS and QTL analysis (Potts et al., 2023).

Moreover, superior genes or alleles for the traits of interest can be identified through genome-wide association studies or pan-genomics, enabling a reduction in time needed for gene discovery and increasing mapping resolution power (Varshney et al., 2021a). Identified molecular markers that are linked to the gene or QTL, controlling a particular trait of interest, could be used for MAS to increase the efficiency of selection, especially of traits that are under the influence of environmental factors (Collard et al., 2005). MAS is the most effective when targeting a small number of genes with a large effect, such as resistance genes (Chhetri et al., 2017). Identification of target traits and transfer to desirable genotypes by conventional breeding, besides being less efficient than MAS, is also more labour-intensive and time-consuming (Table 2). The advancements and integration of gene mapping techniques and MAS with SB methods could overcome the bottleneck of prolonged crop breeding cycles, providing better exploitation of genetic resources in the development of climate-resilient varieties and general improvement of food security (Watson et al., 2018; Raza et al., 2023). Different MAS approaches, such as MABC, gene pyramiding, marker-assisted recurrent selection (MARS) and genomic selection could be accelerated by GASB which reduces generation turnover time and enables the growth of multiple generations within a single year. Integration of SB techniques with backcrossing and MABC could speed up the transfer of desirable traits from donor to recipient parent. In conventional backcrossing, for the recovery of the recurrent parent, more than six generations are required, while applying DNA markers in MABC can be reduced to

TABLE 2 Advantages and disadvantages of traditional and modern plant breeding approaches.

Approach	Features	Advantages	Disadvantages
Traditional breeding	• Uses natural or induced variation in plant genomes • Phenotypic selection and crossing • Relies on field trials	• Widely accepted and practiced • Preserved natural diversity of crops • Low-cost and simple methods	• Time-consuming and labor-intensive • Limited by the availability of desirable traits • Affected by environmental factors • Limited precision in trait introgression • Slow adaptation to changing conditions
Speed breeding (SB)	• Uses artificial light and temperature regimes to accelerate plant growth • Reduces the generation time and increases the number of cycles per year	• Increased genetic gain per unit time • Rapid introgression of multiple traits into elite backgrounds • Suitable for a wide range of crops and traits • Faster adaptation to environmental changes	• High initial investment in infrastructure and maintenance • May induce physiological and morphological changes in plants • May not capture the full spectrum of phenotypic variation • Faster, but potentially less precise approach
SB and MAS	• Uses molecular markers to select plants with desirable traits • Reduces the need for phenotypic screening and field trials • Enhances the efficiency and accuracy of speed breeding	• Increased selection intensity and precision • Reduced linkage drag and background noise • Pyramiding of multiple genes or quantitative trait loci (QTLs) • Speeds up the release process by focusing on targeted traits identified through molecular markers	• Requires prior knowledge of the genetic basis of the traits • Depends on the availability and quality of markers • May not account for the interactions between genes and environment
SB and GS	• Uses genome-wide markers to predict the breeding values of plants • Exploits the linkage disequilibrium between markers and QTLs • Optimizes the selection response and genetic gain of speed breeding	• Increased prediction accuracy and reliability • Reduced phenotyping cost and time • Selection of complex and novel traits • Accelerated variety release through the use of genomic data for predicting breeding values	• Requires large and representative training populations • Depends on the availability and cost of genotyping platforms • Challenges in the validation and implementation
SB and GE	• Uses nucleases such as CRISPR/Cas to introduce targeted mutations or modifications in plant genomes • Creates novel genetic variation and allelic diversity • Accelerates the development of improved varieties through speed breeding	• Increased flexibility and specificity of trait manipulation • Reduced off-target effects and unwanted transgenes • Creation of complex and novel traits	• Requires high technical skills and expertise • Depends on the availability and quality of genomic data and resources • Ethical and regulatory issues

three generations (Tanksley et al., 1989). By integrating MAS with SB, the *hst1* gene, conferring salt tolerance, was transferred into the high-yielding rice genotype (Rana et al., 2019). In 17 months and six generations, the authors succeeded in developing the BC_3F_3 population with the desired homozygous allele, through three backcrosses, followed by two cycles of self-fertilization. Whole-genome sequencing of advanced progeny showed that 93.5% of the BC_3F_2 genome was similar to the recipient parent, while 2.7% was fixed, being donor parent homozygous alleles.

In addition, integration of SB with gene mapping could be particularly convenient to facilitate the discovery and later on quick insertion of genes for resistance or tolerance to biotic and abiotic stress, which may enable an urgent response to environmental stress by providing the development of resistant genotypes for various crops (Devi et al., 2023). Furthermore, SB could hasten the exploration and utilization of allelic diversity in landraces and wild relatives of crops that present valuable sources of resistance to various abiotic and biotic factors, ultimately contributing to the diversification and strengthening of crop resilience (Anđelković et al., 2020; Pour-Aboughadareh et al., 2021). An integrated approach was also used to introgress some valuable alleles from wild relatives in lentils (Lulsdorf and Banniza, 2018). Also, the discovery of new sources of disease resistance using GASB was well demonstrated in the study of Riaz et al. (2017), who were screening Vavilov wheat collection using SB and molecular markers linked to the known leaf rust resistance genes. The authors did rapid phenotyping under accelerated controlled conditions at the seedling and adult stages. Based on the results, lines carrying known genes for leaf rust resistance and lines with potentially novel sources of resistance were identified. Another successful example of the integration of SB with MAS was introgression of imidazolinone Group 2 herbicide tolerance into chickpeas (Croser et al., 2021). The authors developed KASP markers to discriminate between homozygous tolerant and heterozygous intolerant genotypes in the F_2 generation. Only homozygous tolerant plants were processed for F_4 generation, which led to saving cost and time. Moreover, Australian wheat researchers have implemented a multi-trait approach, including QTL analysis, to improve yield and stability under water and heat stress (Christopher et al., 2015). In this study, SB approach coupled with molecular markers, novel phenotyping techniques, and NAM population, identified valuable traits such as stay-green and root characteristics, validated known QTLs and discovered new ones for these traits.

3.2 Genomic selection

Unlike traditional MAS, which is mostly based on a few major-effect loci to perform selection, genomic selection simultaneously uses genome-wide DNA markers to assess genomic estimated breeding values (GEBVs) for complex traits (Meuwissen et al., 2001). This approach was developed predominantly to understand quantitative traits, controlled by many loci with a small effect. The effects of all loci are estimated in large training populations by establishing prediction models that combine genome-wide DNA markers with extensive phenotypic data. After the development of the prediction model, the breeding values of candidate breeding lines are assessed based on estimated marker effects and genotypic profiles of candidates. The lines with higher GEBV will be selected for the next generation. The advantage of genomic selection over other breeding methods is in rapid screening of elite germplasm, acceleration of crop breeding cycles, and time and resource utilization, especially for traits measured late in the variety development process or that are costly to phenotype, such as yield (Crossa et al., 2017; Đorđević et al., 2019). Lines can be selected and used as parents early in the process of variety development, and multiple breeding cycles based on GEBV can be achieved in the same amount of time as a single cycle of traditional breeding only through assessing the genetic potential of genotypes that have not been tested before.

While genomic prediction contributes to a faster annual rate of genetic gain, its greatest benefit is thought to come from combining it with other technologies that shorten generation cycle times (Hickey et al., 2017; Gorjanc et al., 2018). An integrated approach that combines SB and genomic selection assumes parent selection based on GEBVs followed by a selection of progenies generated by SB. To encourage quick breeding cycling, this process is performed numerous times. Given that SB can significantly shorten generation time (Watson et al., 2018), using genomic selection for choosing the parents in each generation could significantly increase the genetic gain (Pandey et al., 2022). Although the next-generation sequencing technology enabled the practical application of genomic selection, the biggest challenge for implementing this approach is still genome sequencing costs (Bhat et al., 2016). One way to reduce the costs would be to use genomic selection every other generation or to choose candidates only if they meet certain requirements for traits like disease resistance that can be accurately phenotyped throughout SB (Riaz et al., 2016). Moreover, this may also lead to a reduction in inbreeding when compared to phenotypic or genomic selection (Jighly et al., 2019). It is considered that this scheme combined with multiple traits, such as normalized difference vegetation index and canopy temperature and with available high-throughput phenotyping platforms could yield higher gains, enabling early-stage selection (Crain et al., 2018). Also, SB coupled with artificial intelligence (AI) can significantly facilitate understanding of genomic architecture by using a machine learning approach for genomic selection model development (Niazian and Niedbała, 2020). The prospect of combining GS and SB to accelerate genetic gain in crops was recommended in more recent studies (Hickey et al., 2019; Bohra et al., 2020; Krishnappa et al., 2021).

A simulation study suggested that combining genomic selection with SB, known as 'Speed GS', for traits with different genetic architecture and heritability, can maximize genetic gain per unit time and reduce generation time in comparison with conventional breeding (Jighly et al., 2019). This approach has also been demonstrated in spring wheat to increase the genetic gain of complex traits (Voss-Fels et al., 2019). SB was used for the phenotyping of specific traits in the training population of wheat and selection candidate development and phenotyping steps. Speed GS was used to predict grain yield across multiple environments and to shorten the breeding cycle through the rapid generation of inbred lines (Watson et al., 2019). The authors incorporated four speed-breeding traits from a training population into multivariate genomic selection models, which significantly increased the predictive ability of yield compared to univariate GS models.

3.3 Genome editing

Genetic engineering primarily entails the insertion or deletion of a gene or gene segment in the recepient crop using biotechnology, providing several benefits over traditional breeding methods (Rai, 2022). With the further improvement of these technologies, and the emergence of genome editing (GE) technology, crop scientists have the option of making changes at a targeted location. Genome editing techniques exploiting programmable nucleases including transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas-associated nucleases have been used in crop improvement to a certain extent, but there is a significant room for improvement. The most commonly used GE technique is CRISPR-Cas which utilizes non-homologous end joining and homology-directed repair for DNA repair, as well as single-base editing enzymes (Pickar-Oliver and Gersbach, 2019). There are six types of Cas proteins, of which Cas9 is the most exploited one in plant breeding thanks to its accuracy, affordability, and ease of use (Aksoy et al., 2022). CRISPR-Cas9 system relies on guide RNA (gRNA) for guidance and targeting specificity, however, it still has some limitations such as Cas9-related toxicity, possible off-targets, and restrictions in target sites (Zhao et al., 2020; Varshney et al., 2021a). Some other systems such as CRISPR from Prevotella and Francisella 1 (Cpf1) show promise for use in GE as they exhibit higher efficiency rates compared to Cas9.

The advantage of using CRISPR/Cas systems in GE is the option of targeting multiple sites thus enabling simultaneous pyramiding of the desired genes. This fastens up breeding programs and in combination with SB, it can lead to a significant reduction in time to create plant material with improved resistance to pathogens or with improved quality parameters (Table 2). This way edited plants can be grown in SB conditions to quickly produce edited seeds, which can speed up homozygosity and the rate at which genetic gain can occur. Genome editing can produce desirable lines that can be preselected at the T_1 generation, and rigorous evaluation can be done at the T_2 generation to ensure that off-target genotypes are eliminated (Samantara et al., 2022). However, there is still a labour-intensive step in tissue culture laboratories that needs to be performed to obtain edited plants. In a recently proposed ExpressEdit approach that integrates gene editing and SB, plant shoot apical meristems could be directly treated using techniques like particle bombardment with the Cas9 gene and sgRNA (single guide RNA) sequences to get around the bottleneck of traditional tissue culture and plant regeneration in the lab. Plants that lack Cas9 but carry the new trait could be identified by screening their progeny for the new trait (e.g., disease

resistance). On the other hand, Cas9 might stay in "CRISPR-ready" plants, which could undergo additional rounds of editing by using sgRNA to target distinct gene targets (Hickey et al., 2019). ExpressEdit can also be used in conjunction with MAS and high-throughput phenotyping to increase breeding process efficiency. This will facilitate the rapid development of the mutant variety or a variety carrying a mutant allele (i.e., 5–6 years instead of 8–10 years) (Voss-Fels et al., 2019). The integration of SB with genome editing has been proven in *Brassica napus*, *B. oleracea*, tomato and soybean (Yang et al., 2017; Murovec et al., 2018; Bao et al., 2020; Liu et al., 2021).

Another drawback to fully exploiting genome editing and SB integration in breeding programs is the existence of strict GMO (Genetically Modified Organism) regulations in many countries (Table 2). Thus, the exploitation of DNA-free GE approaches may show promising potential. In some countries like the EU, these crops would still fall within the strict GMO regulations. However, there are now countries that do not concentrate on the process but rather on the product, and thus CRISPR/Cas system should have a very bright future in wider application in crop breeding and production.

3.4 Recent high-throughput genotyping based approaches

Current advances in next-generation sequencing technologies and omics methods have made it possible to access the genome sequences and transcriptomes of multiple plant species, significantly transforming plant breeding. With the public availability of plant genomes, there is a chance to identify candidate genes, QTLs, and associated genome-wide molecular markers, enabling high-throughput marker-assisted breeding (Naqvi et al., 2022). Pan-genomic research has been initiated as a result of the development of next-generation sequencing technologies, providing a great platform for studying genetic diversity, examining multiple genomes at once, determining crop evolution and adaptation as well as getting a better understanding of genome functions that may be used in crop breeding (Zhao et al., 2018). The term pan-genome refers to a species' whole gene pool that is present in different individuals. Recently, pan-genomes of some key crops have been constructed and this concept is applied to agricultural plant research (Hurgobin et al., 2018). The integration of pan-genomes with SB and genome editing could accelerate trait-specific breeding. Crop pan-genomes will make better breeding schemes easier to implement, especially for complex genome crops where they will help identify shared or distinct gene combinations for various traits (Naqvi et al., 2022).

The accessibility of whole genome sequencing data for a large number of lines for a given crop enabled haplotype identification. A haplotype represents a group of polymorphic markers that are inherited together in progeny with minimum recombination (Garg, 2021). In rice and numerous other species, a number of haplotypes linked to increased yield have been discovered (Sharma et al., 2023). Haplotype breeding can be achieved by combining desirable haplotypes into the genetic background of crops. This introgression takes a long time due to the requirement of more breeding cycles and long generation time for the crop. However, an integrated approach that combines haplotype breeding and SB and GS has the potential to accelerate genetic gain (Sharma et al., 2023; Shendekar et al., 2023).

4 SB technology: current status, challenges and future perspectives

In the present fast-changing climate and challenging global crises, the traditional slow breeding procedures and programs are not adequate and suitable for quick responses and solutions that the present and future agriculture needs. To be efficient and flexible enough to address all these challenges, plant breeding needs prompt and precise techniques to be incorporated into crop improvement and the development of climate-resilient, adaptive and stable crop varieties. So far, many of these technologies such as shuttle breeding, *in vitro*/embryo culture, DH technology, MAS, genetic engineering, and genome editing are known and have already been introduced into breeding programs worldwide. SB is just one of them and should be integrated with all other approaches and innovations, to offer breeders a contemporary and powerful technology applicable to solving a variety of problems as fast as possible. This results in more effective and expedited plant breeding programs that maintain crop yields and guarantee food security and promote sustainable agriculture. Breeders can quickly create new plant varieties better adapted to environmental challenges like drought, heat or salinity. Moreover, by creating crop varieties with improved resilience to pests and diseases and increased nutrient efficiency, SB lessens dependency on chemical inputs like fertilizers and pesticides.

Currently, the efficient SB protocols are available for a limited number of cereal, legume, vegetable and oilseed crops (Ghosh et al., 2018; Watson et al., 2018). Also, using this technology several varieties have been released so far in wheat, barley, pea, canola, rice and tomato (Shendekar et al., 2023). For wider practical breeding outcomes and realization of the SB potential to substantially accelerate genetic gain, the optimization of SB protocols for all important crop species is necessary, as well as further modifications of the existing protocols based on local needs/innovations. SB is based on the induction of early flowering in photoperiod-responsive crops (Pandey et al., 2022). However, the differences in photoperiodic requirements between different crops could make standardization of SB protocols very difficult (Jackson and Jackson, 2009). Although SB is a valuable tool to accelerate the conventional breeding process, controlled and intensive growth conditions during cycles could lead to limited plant growth and consequently lower seed yield due to the crossover interaction between genotypes and growth systems (Tanaka et al., 2016; Sharma et al., 2019; González-Barrios et al., 2020). These variations may have an impact on the stability and uniformity of crops, raising concerns about the consistency of crop performance across various environments. Hence, an additional effort should be made to determine optimal growth conditions for both, crop species and sometimes also different genotypes within the species, to mitigate the negative effect that intensive growth conditions could have on plants (Pandey et al., 2022). For example, excessive photoperiod can slow down plant growth and elevate stress hormone levels. Thus, it is required to precisely balance the need to accelerate growth with the need to prevent stress-induced responses. The limited seed yields could also lead to the loss of some breeding populations and a decrease in genetic variability, which could pose a problem, especially in crops where the SSD method is used in breeding (Saxena et al., 2019). This issue could be partially overcome by preserving backup seeds from each individual through each generation (Pandey et al., 2022). Additionally, some of the major challenges in SB include

disease and pest control, as well as tracking individuals for gene discovery (Potts et al., 2023).

Moreover, rigorous testing and evaluation of new varieties in field conditions are still necessary to ensure suitability for commercial cultivation. Thus, SB technology needs to be combined with effective field trials or high-throughput phenotyping platforms (HTPP) to enhance the crop improvement process. For accurate measurement, the SB facility should be supplied with automated platforms, hyperspectral sensors, high-end cameras, lasers, thermometers, lux meters, humidity meters, etc., (Sharma et al., 2023). Thanks to the availability of enhanced phenotyping tools such as thermal imaging, 3D imaging, magnetic resonance imaging, computed tomography, and imaging spectroscopy, almost any plant trait can be measured (Shendekar et al., 2023). Accurate phenotyping can facilitate faster improvement of target traits involved in biotic and abiotic stress, addressing the problem of global food security in the future. A combination of multi trait phenotyping and SB was applied for the promotion of root adaptation in water limited environments in wheat (Christopher et al., 2015). Moreover, high-throughput phenotyping and SB technology were used together in barley for introgression of disease resistance to leaf rust, net and spot forms of blotch (Hickey et al., 2017). Possible future directions for SB include exploiting the genetic diversity and novel alleles present in the wild relatives and landraces of crop plants by using SB and molecular methods to introduce them into elite cultivars. This will enhance the genetic variation and adaptability of crop plants to changing environments. Furthermore, high-throughput phenotyping platforms that can capture the dynamic responses of plants to different stress factors under SB conditions should be developed. This will enable the identification of novel traits and genes associated with stress tolerance and yield potential. In this way, dynamic SB programs that can adapt to the changing climate and consumer preferences by incorporating diverse germplasm and novel traits should be developed. Also, Artificial Intelligence (AI) and machine learning have opened up new possibilities for SB, including the ability to make precise decisions and handle large and complex datasets, which could facilitate the discovery of new patterns, relationships, and insights that can guide the breeding decisions and strategies and could provide new insights into how plants function in extreme climates (Rai, 2022).

Finally, SB technology as such requires specific expertise, effective plant phenotyping facilities, appropriate infrastructure and continuous financial support for research and development (Shimelis et al., 2019). Training personnel for specific knowledge needed for SB is one of the aspects that needs to be focused on in the future. The introduction of SB into the breeding programs requires investment in growth chambers with adequate light and temperature conditions, which could be relatively high (Pandey et al., 2022). Maintaining controlled environmental conditions also requires higher energy consumption, which further increases the total cost of SB applications. A potential solution to this problem could be the use of sustainable energy sources, such as solar panels, and the use of energy-efficient LEDs in growth chambers (Yao et al., 2017). With the help of LED lighting systems, precise control of the length and intensity of the light, which is beneficial for photosynthesis, growth and crop development is possible (Jähne et al., 2020). Therefore, further technological innovations are needed to reduce prices for the facilities and to make it affordable for a wider community, small breeding companies and research institutions for better exploitation of the technology. Although these requirements are not limiting factors for the technology application in developed countries, they seriously limit the use of SB, remaining a challenge in developing countries due to their limited infrastructure, poor expertise and insufficient governmental support, as described by several authors (Chiurugwi et al., 2019; Wanga et al., 2021; Samantara et al., 2022). Thus, international collaborations and partnerships of multi-disciplinary teams are needed for faster knowledge and SB technology transfer not only into basic and applied research but also into breeding companies to accelerate the application of SB technology worldwide.

5 Conclusion

Recent advances in SB techniques provide a potential alternative for reducing the amount of time, space, and resources needed to develop and release high-performing cultivars. SB can be achieved by distinct approaches, impacting different phases of plant breeding by accelerating the breeding cycle. SB protocols for numerous economically significant species have been developed. However, further optimization and application of SB should be broadened to other crops with lengthy generation times or that are challenging to breed. The further acceleration of the rate of genetic gain in breeding programs and the development of stable and high-performing crop varieties for addressing global challenges such as changing climate and food security could be achieved through GASB. This integrated approach maximizes the benefits of each technique and produces a comprehensive framework for modern plant breeding. However, future evaluation and research should be focused on the potential detrimental effects of SB conditions on phenotypic and genetic changes and agronomic performance and stability of speed-bred lines under field conditions. Other challenges, such as the lack of qualified personnel, necessary infrastructure, consistent water and electricity supplies, and high costs have limited the application of SB, especially in developing countries, and particularly in public plant breeding programs. Therefore, efforts should be redirected to capacity building, technology transfer, and financing of SB coupled with modern breeding approaches to facilitate crop improvement programs.

Author contributions

MĆ: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. DM: Writing – original draft, Writing – review & editing, Funding acquisition. VĐ: Writing – original draft, Writing – review & editing, Funding acquisition. DT: Writing – original draft, Writing – review & editing. AR: Writing – original draft, Writing – review & editing. SG: Writing – original draft, Writing – review & editing. AK-Š: Writing – review & editing, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work is supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant number 451-03-66/2024-03/200032, by the Science Fund of the Republic of Serbia through IDEAS project "Creating Climate Smart Sunflower for Future

Challenges" (SMARTSUN), grant number 7732457, by the Science Fund of the Republic of Serbia through PRISMA project "Soybean Yield Prediction Using Multi-omics Data Integration" (SoyPredict), grant number 6788, and by the European Commission through Twinning Western Balkans project CROPINNO, grant number 101059784, by Center of Excellence for Innovations in Breeding of Climate-Resilient Crops - Climate Crops and Center of Excellence for Legumes, Institute of Field and Vegetable Crops, Novi Sad, Serbia.

Acknowledgments

We acknowledge MSc Predrag Ranđelović from the Institute of Field and Vegetable Crops, National Institute of the Republic of Serbia for providing the photos in Figure 2.

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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frontiers | Frontiers in Sustainable Food Systems

TYPE Original Research
PUBLISHED 07 May 2024
DOI 10.3389/fsufs.2024.1322562

OPEN ACCESS

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RECEIVED 03 November 2023
ACCEPTED 15 March 2024
PUBLISHED 07 May 2024

CITATION
Egesi C, Mbanjo EGN, Kawuki R, Teeken B, Rabbi IY, Prempeh R, Jiwuba L, Njoku D, Kulembeka H, Gwandu F, Woyengo V, Parkes E, Ofei R, Banda VR, Ntawuruhunga P, Derera J, Weber S and Kulakow P (2024) Development of portfolio management tools in crop breeding programs: a case study of cassava in sub-Saharan Africa. *Front. Sustain. Food Syst.* 8:1322562. doi: 10.3389/fsufs.2024.1322562

Development of portfolio management tools in crop breeding programs: a case study of cassava in sub-Saharan Africa

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The response to the diverse needs along the cassava value chain, the urge to increase genetic gain, and the need for rapid varietal turnover will necessitate not only technological innovations but also transformation of public breeding programs in sub-Saharan Africa (SSA). We developed guiding, flexible and adaptative tools for portfolio management of cassava breeding. The cassava breeding and product development pipeline process was mapped to illustrate activities of each stage, as well as to clarify key decision points. Stakeholders involved at all stages of breeding were identified. This allowed for identification of gaps and new crucial functions. To clarify accountability and reduce complexity in the decision-making at key decision points, the roles were mapped against decision-rights at each stage-gate. Cassava crop calendars for the different regions in SSA were developed to facilitate better planning. A product advancement template was developed to guide product advancement. The tools that have been developed and stage-gate mapping, will support regional efforts to establish more structured, transparent, participatory, efficient, inclusive, and demand-driven cassava breeding in the region. These approaches could be customized to other commodities.

KEYWORDS

cassava, product advancement, stage-gate, crop calendar, decision rights, breeding processes

1 Introduction

In the next 27 years, by 2050, the world population is estimated to reach 9.9 billion, with more than 25 percent of the people estimated to live in Africa. This will be an increase of the African population by around 90 percent compared to 2020 (World Population Data Sheet, 2020). This reality underscores food and nutrition demands in sub-Saharan Africa (SSA) where mismatch in scientific innovation tailored for agricultural growth and population growth rates is highest. Concerted efforts are, therefore, urgently needed to sustainably increase food quantities and quality, productivity per unit area, and incomes (Fraval et al., 2019; Bjornlund et al., 2022). This is especially true in the current context of climate change, depletion of natural resources such as biodiversity, land and water, rapid social changes and demographic growth, changes in nutrition, and need for quality food (IPCC, 2022; Olaosebikan et al., 2023). The performance of the agricultural sector is interlinked with how well the above-mentioned challenges will be addressed (Bhavani and Rampal, 2020). As agriculture is a crucial force of economic development, the hard nut to crack is how to address all the issues that face agricultural production while also delivering a breeding product that is tailored to the needs of the end-users (Tiffin and Irz, 2006; Aboyitungiye and Prasetyani, 2021; Dufour et al., 2021; Polar et al., 2022).

Plant breeding plays an important role in building and sustaining resilient food systems (CGIAR, 2021). Indeed, use of modern technological innovations and proven approaches has greatly enhanced breeding operations; thus, timely development and deployment of end-user preferred varieties in farmers' fields. It is, therefore, important that breeding programs continue to operate optimally to remain relevant and responsive to societal needs, whims, and evolving funding landscape (Renkow and Byerlee, 2010; Wossen et al., 2017; Mbanjo et al., 2021).

It has been shown that more structured breeding programs characterized by optimal stepwise process management, improve breeding operations efficiency, maximize genetic gain, and thus impact agricultural value chain actors, their families, and society (Cobb et al., 2019a). An example is the global wheat breeding program that resulted in the release of thousands of wheat varieties between 1970 and 1990 for both favorable and marginal environments covering well over 50 million hectares (Reynolds and Borlaug, 2006).

Accelerating technology adoption in plant breeding drives increasing specialization of the expertise required along a breeding pipeline. Breeding program efficacy would, therefore, largely benefit from improving the ability of teams to work in interdisciplinary contexts (Morris et al., 2006). This is especially important for public breeding programs that target environmental, nutritional, and social impact in addition to profitability. As public breeding programs are influenced by various stakeholders representing prioritizations of national or regional interests, as well as international organizations, strategies are frequently unarticulated or not even discussed and agreed among stakeholders. Consequently, breeding programs and networks, which are often project-based, and thus ending with project timelines are not sufficiently tied to the adoption and impact of their breeding products. Therefore, public breeding networks would largely benefit from raising the bar on transdisciplinary team management, discipline formalizing coordination and management, definition and assignment of accountabilities while simultaneously promoting transparency. This would fulfill the necessary shift articulated by Cobb et al. (2019b) to move "plant breeding towards a data-rich evidence-based and team-oriented process, and away from the romantic tradition of an individual breeder, "as an artist" stressing the problem of the breeder being the sole decision maker. This fundamental change would, in addition to system effectiveness, also provide for system stability; thus, safeguard return-on-investment.

Innovation, customized product development, and systematic last-mile product delivery in a transdisciplinary manner, as well as continuous re-evaluation of entire workflow is required for success (Cooper, 2018). Implementing a value-creating process and risk model (e.g., a stage-gate system) can successfully and efficiently accelerate superior product development (Cooper, 2008; Edgett, 2015). Accordingly, stage-gate systems, now widely promoted within CGIAR and some National Agricultural Research Systems (NARS), have been widely implemented in industry. An example is in the domain of production (manufacturing and assembly), as well as by service-based firms (Wuest et al., 2014; Sommer et al., 2015; Schultz et al., 2018). Aside from allowing for more focus, a stage-gate strategy provides for more systematic planning, and control all tailored toward driving process efficiencies. While these approaches have been adopted in other breeding programs such as beans, through the Pan-African Bean Research Alliance (Chirwa, 2017), they are still to be introduced, adapted, and scaled in cassava breeding in the region.

Documentation of key players, defining their roles and responsibilities at every step of the breeding process is critical to create focus and eliminate redundancy and waste. It is equally important to document interactions between various actors throughout the processes (Van der Werf, 2000). Role clarity also fosters cross-functional team culture, enhances team efficiency, and also helps to develop skills of individual team members (Van der Werf, 2000; Barke and Prechelt, 2019; Kholová et al., 2021). Therefore, a shift toward an evidence-based and inter-disciplinary organizational model is needed in breeding programs (Cobb et al., 2019a; Ceballos et al., 2021; Kholová et al., 2021). Crop breeding programs in Africa will need this transformative shift to enable them cope with societal needs (Mbanjo et al., 2021).

The success of product development is hinged on effective and high-quality decisions (Akdere, 2011; Ghadir et al., 2021). At each product development level, evidence-based decisions are made to inform next actions, as advancing deficient products has immediate and negative knock-on effects notable of which include resource wastage, investment losses, and poor market share of newly developed varieties and/or products (Schippers and Rus, 2021). Indeed, decision quality is correlated with involvement of relevant actors in terms of roles and disciplines (Ozer, 2005). Today, decision-making practice in cassava breeding operations is fragmented with sole responsibility designated to individual breeders. This setup is no longer adequate to extract the full value of increasingly inter-disciplinary product development processes; thus, needs to be revised to exploit the full innovative potential of public breeding programs and networks.

In industry, healthcare, and information technology process, mapping has been widely used to represent and evaluate business operations (Singh et al., 2011; Antonacci et al., 2018; Andriani et al., 2019; Johansson and Nafisi, 2020; Antonacci et al., 2021). Development of tools that logically enable product development in a graphical way not only allows visualization and control of core activities, but also ensures development of quality products (Klotz et al., 2008). In fact, a process-oriented management strategy has been viewed as a communication tool and has shown to enhance transparency,

visibility, and understanding of current procedures (Antonacci et al., 2018, 2021). It is also in this light that we see such management system, not as a rigid inflexible structure as this could imply increase bureaucracy and inefficiency.

Accordingly, this paper aims to introduce and implement fundamental changes in cassava breeding programs across SSA. Specifically, the paper focused on establishing a scalable system for product advancement and management. In pursuit of this aim, the following strategic actions were undertaken: (1) a cassava stage-gate process with well-defined actions per stage was designed; (2) roles and disciplines of the extended breeding team involved in all stage-gate process beginning with product development and ending with launch were standardized; (3) decision rights at each stage-gate and people involved were mapped; (4) existing cassava breeding pipelines, as well as current competency levels, were documented; (5) tools, including crop calendar and product advancement guide were developed; and (6) stakeholder perceptions of the proposed breeding operation changes were assessed.

2 Methods

2.1 Cassava breeding programs involved in process improvement

The process analysis and improvement in cassava breeding operations was conducted by the International Institute of Tropical Agriculture (IITA), in collaboration with five African NARS breeding programs, including the Kenya Agricultural and Livestock Research Organization (KALRO) in Kenya, the Council for Scientific and Industrial Research—Crop Research Institute (CSIR—CRI) in Ghana, the National Crop Resources Research Institute (NaCRRI) in Uganda, the National Root Crops Research Institute (NRCRI) in Nigeria, and the Tanzania Agriculture Research Institute (TARI) in Tanzania.

These breeding programs were all partners in the Next Generation Cassava Breeding Project,[1] which enabled the establishment of an inclusive governance structure composed of (a) a core team that set the direction, defined the road map, managed deliverables and implementation; (b) an extended team that provided support to the core team and contributed to the task force activities by lending their expertise and assisting in the advancement of the project; and (c) a steering committee that provided strategic oversight and ensured that deliverables were timely attained. This governance structure ensured adequate representation of stakeholders relevant to the subject matter at various levels of operations (Kähkönen et al., 2013; Mosavi, 2014; Harrin, 2023).

2.2 Cassava crop calendar

The purpose of the cassava crop calendar was to capture variations in the schedule of cassava growing season across agroclimatic regions of SSA to enable identification of windows for cross-functional decision-making events. Accordingly, the cassava calendars were designed with representatives from regional IITA hubs and NARS representing four geographies, namely Southern Africa (Zambia, Malawi, Mozambique), Central Africa (Democratic Republic of Congo), East Africa (Uganda, Kenya, Tanzania, Rwanda, Burundi, South Sudan), and West Africa (Nigeria, Ghana, Benin, Togo, Cote d'Ivoire, and Sierra Leone). Consolidated inputs were consequently validated by stakeholders from local teams utilizing the team and governance structure described above. We mapped out when the various activities (i.e., planting, harvesting, and advancement decisions) occur in each of the studies' agroclimatic regions. The regional calendars were then consolidated into a single cassava advancement calendar.

2.3 Role standardization and stakeholder information

Role standardization refers to the procedure that simplifies a highly diverse collection of job titles of stakeholders across the institutions into a reduced set of roles. It is based on similarities in responsibilities and subject-matter expertise of the original job titles. Herein, stakeholders refer to individuals or groups of people holding said job titles whose consent to a simplified set of roles is required to ensure change success.

Core team members were requested to list all individual job titles and their incumbents involved in the product development process from product design to product launch. The information was collated, and each job title's responsibilities specified. The job titles involved in cassava breeding were then first associated to entities (referring to organizational units that participate in the product development process) and then categorized into disciplines (referring to specific fields of subject-matter expertise). The identified disciplines were then further differentiated into limited sets of roles critical to the product development process. This was achieved through a series of iterations in collaboration with NARS representatives on the Core—and Extended Teams. In the final step, individuals from IITA and the five NARS involved in cassava breeding program were mapped to the identified roles, disciplines, and entities.

2.4 Cassava breeding pipeline process analysis

Here, a pipeline is defined as the concatenation of different development stages from product concept design to the final product delivery to end-users. Accordingly, we mapped the cassava breeding pipeline to the most recent CGIAR stage gate available at the time to illustrate activities of each stage as well as to clarify key decisions to be taken between stages. A generic framework process for all stages was designed, annotated, and then challenged by a cross-functional and cross-organizational team during an implementation workshop where the initial workflows were commented, amended, and adjusted. We articulated the activities and procedures, activity duration, and people involved. Four types of process documents were designed: (a) a high-level map, which gives a quick and simple overview of the process without going into details of how it is done; (b) a Six Sigma concept to map Suppliers, Inputs, Process, Outputs, and Customers (SIPOC) of distinct processes. SIPOC help to visualize the processes at a high level, understand the overall picture, who the customers are

1 https://www.nextgencassava.org/

of a major process, the outputs from those processes, the inputs to those processes and who supply them; (c) a Swimlane flowchart that displays the steps in the process and specifies which function, department, or person is performing them and in what sequence. The components or teams are grouped into distinct sequences or lanes the flow of activities are connected between those components; and (d) a simplified process map showing the detailed process at each stage (Landel and Snyder, 2010; Heher and Chen, 2017; Barrera, 2020).

2.5 Analysis and assignment of decision-making rights

Herein, a decision is defined as the agreement by a diverse group of experts on operational, tactical or strategic actions to be taken to ensure the best possible outcome for distinct stages of cassava product development from analysis of market, research, and production data. To ensure a decision-making process that is inclusive of all relevant expertise and stakeholder interests, while still effective and efficient (as time windows to make such decisions are often very small), decision authority and mandate must be adequately distributed among experts. Therefore, decision rights were mapped across the stage plan according to the RAPID (R = Recommends, A = Agrees, P = Performs, I = Inputs, and D = Decides) model (Rogers and Blenko, 2006) to establish clarity on mandate, accountabilities, and responsibilities for decision-making in cassava product development (Table 1). This was done at IITA and NARS with the support and interaction of the core team representatives from both institutions. The decision rights were mapped for each stage and at all levels, including entity (defined as the highest-level grouping), disciplines (defined as domain of specialization), and roles (defined as responsibilities and expectations of each team member). Decision rights mapping was initiated at the core team level followed by a series of iterations with disciplinary representatives from IITA and NARS in the Extended Team and other institution members.

TABLE 1 Decision right definition modified from Robert and Blenko, 2006.

Mapping	Decision-making role	Description
R	Recommends	- Assess and make judgments of relevant facts and data, consults people giving input, develop decision-making options - No veto right
A	Agrees	- Negotiate agreements on recommendations and options, consult with recommenders and performers - **Has a veto right**
P	Performs	- The implementer /doer might also give input as to feasibility and execution implications - Responsible for follow up and implementation of decisions made within allotted time
I	Gives Input	- Deliver facts, no judgments, stand-by for but not necessarily participating in final decision-making - No veto right
D	Decides	- Calls and leads decision-making events, ensures timely input, resolves disagreements, ensures decision communication - **Accountable for decision outcome**

2.6 Template development

Templates to facilitate transparent communication of data and facts in a comparable manner were developed by representatives of the core team to improve information that informs advancement decision-making for variety selection by cross-functional and inter-disciplinary teams. The templates were developed and reviewed utilizing the project's organization and governance structure, as well as consulting additional stakeholders.

2.7 Implementation workshop

We undertook a consultative stakeholder workshop (referred to as implementation workshop) aimed at operationalizing the assets created during the project (i.e., Stage-Gate process, crop and advancement calendar, standardized roles, and decision rights maps). The workshop exposed preliminary deliverables, captured the problems that participants anticipate, and provided participants with useful information and learning. Competencies of staff considered critical to operate effectively in cross-functional and cross-institutional decision-making on the sub-Saharan cassava breeding pipeline were identified across IITA and the five NARS partners using a 1 (i have no or very little competence) to 5 (I am highly proficient expert) Likert scale (Sullivan and Artino, 2013; Joshi et al., 2015).

3 Results

3.1 Cassava crop calendar

Cassava crop calendars show that for each region of SSA (Supplementary Figures 1A–D), peak intensity varies as a consequence of differences in rain commencement. Therefore, there is no single calendar that can be used across the different regions. It was also observed that across SSA, breeding operation decisions are continuously made throughout the year. The gathered information allowed us to define the most appropriate time when important decision-making meetings (e.g., product advancement meeting) could be suggested. For example, in east Africa, the month of April to May would be ideal for planting, while in central and west Africa, the months of May to July and September to October would be most ideal. With the unimodal rainfall pattern of the southern Africa region, the planting would be concentrated from December to mid-January. Merging the calendars from the different regions allowed to identify timing windows when geography-wide, cross-institution advancement, and decision meetings could be held. Thus, the crop calendar is a planning tool to enhance efficiencies of breeding programs (Figure 1; Supplementary Table 1).

3.2 Standardized roles across IITA and NARS

Across both IITA and NARS, an array of 150 individual job titles were mapped to 27 harmonized roles, which were grouped into 12 disciplines in reference to decision-making in cassava advancement (Table 2). A further level of grouping was added by associating the 12 disciplines to three entities: (1) marketing, outreach, and social impact; (2) research and development; and (3) seed supply chain. For example, under marketing, outreach, and social impact entity, four disciplines (market/socioeconomics, product management, social/gender science, sale and extension) and five roles were defined (Table 2). In the end, this analysis revealed common patterns of organization between IITA and the different NARS partners, and how individual experts are proportionally spread across roles, disciplines, and entities in each organization. Evidently, a very strong focus on research and development was noticed. However, marketing, outreach, and social impact, as well as seed supply chain disciplines, were underrepresented. The resource gap was more apparent among the NARS (Table 2). A crucial resource gap was identified for product management wherein only one product manager is available for the whole region and across institutions, who, in addition, also provides support to other commodities. Communication was also identified as a crucial need for most of the breeding programs.

3.3 Stage-gate mapping of cassava breeding pipelines

Cassava breeding pipelines were mapped along the stage-gates to highlight decision points as advancement is being made from one stage to the next (Figure 2). A summary of which decisions are to be made was also highlighted. For example, Stage 0 is where the target product profile (TPP) is updated. At this stage, the fully functional transdisciplinary team, as well as funders and/or development partners, are part of the decision team. Stages 1 to 5 are components of various technical breeding processes. Stages 5 to 6, the last-stage delivery, comprises of final performance and/or registration trials as per the country's varietal release guidelines. Again, decision-making for this stage needs the involvement of a large and diverse group of experts. Finally, what gets advanced to official releases equally needs a full cross functional team.

3.4 Decision-making

Introduction of stage-gates require establishing clear roles and accountability beginning at Stage 0 up to Stage 7. However, within stages 1 to 5, decision-making is more confined to the technical part of the team, although other disciplines are also involved but to a lesser extent (Supplementary Tables 2–5; Figure 3). Stages 1 to 5 are the technically dominated stages where varieties are crossed and selected following the "recipe," the product profile, determined at Stage 0. From Stage 5, there is a clear institutional change, especially with respect to IITA who mostly has a recommending role or agreeing one (seed supply chain) because it is the NARS organizations that have the mandate to access the relevant variety release agency (decision role) to release varieties. What gets advanced to national performance trial and on-farm trials will be decided by the national programs. What gets recommended for release and delivery to the market is largely a responsibility of market/socio economic, gender, and social inclusion science teams, with CGIAR partner (IITA) playing a more supportive role. The seed supply chain both at IITA and NARS would agree with regards to the potential of the varieties to be delivered to users.

3.5 Process maps

We mapped out all the processes in the cassava breeding pipeline, from product conception and design (Stage 0) through trialing and

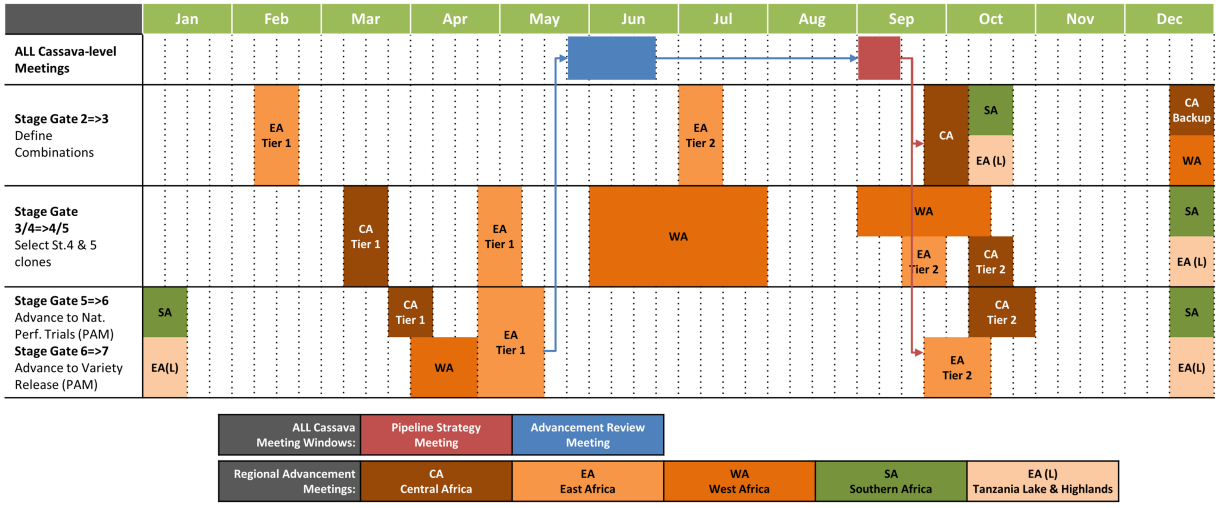


FIGURE 1
All cassava stage-gate advancement calendar. Regional calendars were merged into a single cassava advancement calendar. Crop calendar is a crucial tool for crop management and activity planning for example, key periods for trialing, planting, and harvesting as well as when to schedule critical meeting such as product advancement.

TABLE 2 Detailed overview of the roles and disciplines involved in advancement decision-making in cassava product developement across IITA and the five national agricultural research systems.

Entity	Standardized discipline	Standardized role	IITA	CSIR-CRI	KARLO	NaCRRI	NRCRI	TARI	Grand total
Marketing, outreach & social impact	Market-/Socio Economics	Socio Economist	2	1	1	1	2	2	**9**
		Value chain specialist	2		1				**3**
	Product Management	Product Manager	1						**1**
	Social-/Gender Science	Gender Scientist	1	1		1	1	1	**5**
	Sales and Extension	Dissemination Specialist						1	**1**
									0
Research & development	Breeding	Breeder	7	1	4	2	5	1	**20**
		Breeding Manager	1						**1**
		Lead Breeder	1	1	1	1	1		**5**
		National Program Lead						1	**1**
	Breeding Operations Services	Breeding Operations Manager	1						**1**
		Phenotyping Specialist	1						**1**
		Trait Development Specialist	2	1	2	1	1	1	**8**
		Trial Manager	3				2		**5**
		Field and Laboratory Technical Support		6			1		**7**
	Data Science	Biometrician	1	2					**3**
		Data Analyst	2	1					**3**
		Data manager	1			2	1		**4**
	Disciplinary Expert	Agronomist	2	3		2	2	2	**11**
		Entomologist	1	1		1		1	**4**
		Food Scientist	2	4	1	1	1	2	**11**
		Pathologist	1	5	4	1	2	1	**14**
	Management Oversight	Administrative Manager	5		3	1	2		**11**
		National Program Lead				1			**1**
	Project Management	Project Management Resource Person	1						**1**
		Project Team Lead	1						**1**
	Communication	Communication Expert				1	1	1	**3**
									0
Seed supply chain	Production & Logistics	Seed System specialist	2	5	2	1	2	3	**15**
Grand Total			**41**	**32**	**19**	**17**	**24**	**17**	**150**

selection to launch (Stage 7), as well as all the key intermediate steps, including trait discovery and deployment, population improvement, and candidate selection. Example maps with descriptive captions are provided in Figures 4, 5 and Table 3 and all maps (illustrating the process from stage 0 to 7) are available in Supplementary Figures 2A–Y. The process map could be categorized into three different phases, target product profile, trait discovery and deployment, and breeding pipelines. The process map shows what activities are performed, the flow of activities, who does it, and when activities should be done.

3.6 Template development

A collection of templates to guide advancement process and guide the decision team was developed (Supplementary Files). Two areas were covered with regards to informing the product profile (Stage 0) and with regards to late-stage advancement (Stages 5 to 6). The first is from the breeder's technical perspective and sums up how clones performed in breeding trials with regards to the traits in the product profile and in relation to the current breeder's and commercial checks. It also includes a strengths, weakness, opportunities, and threats

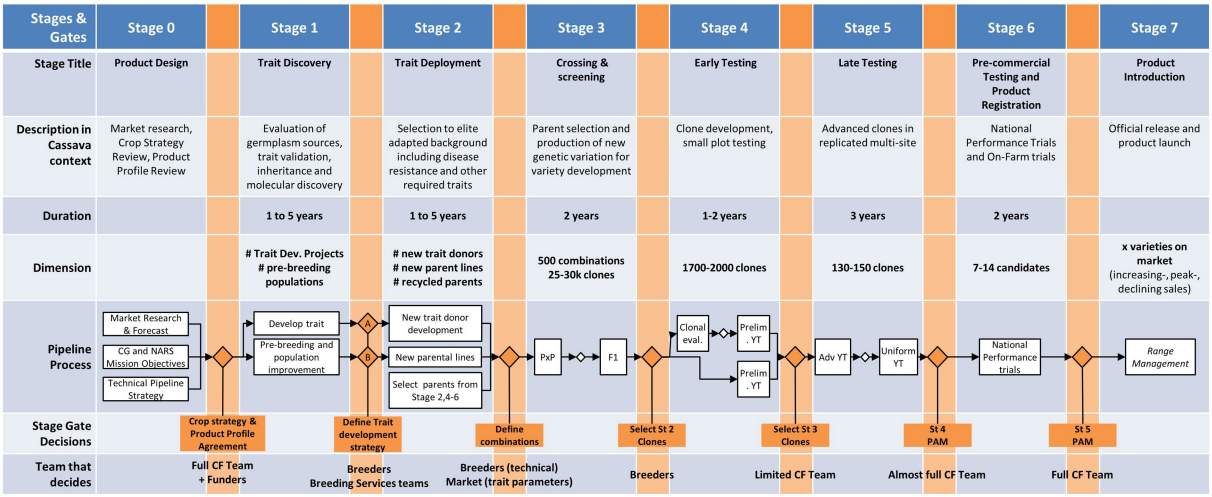
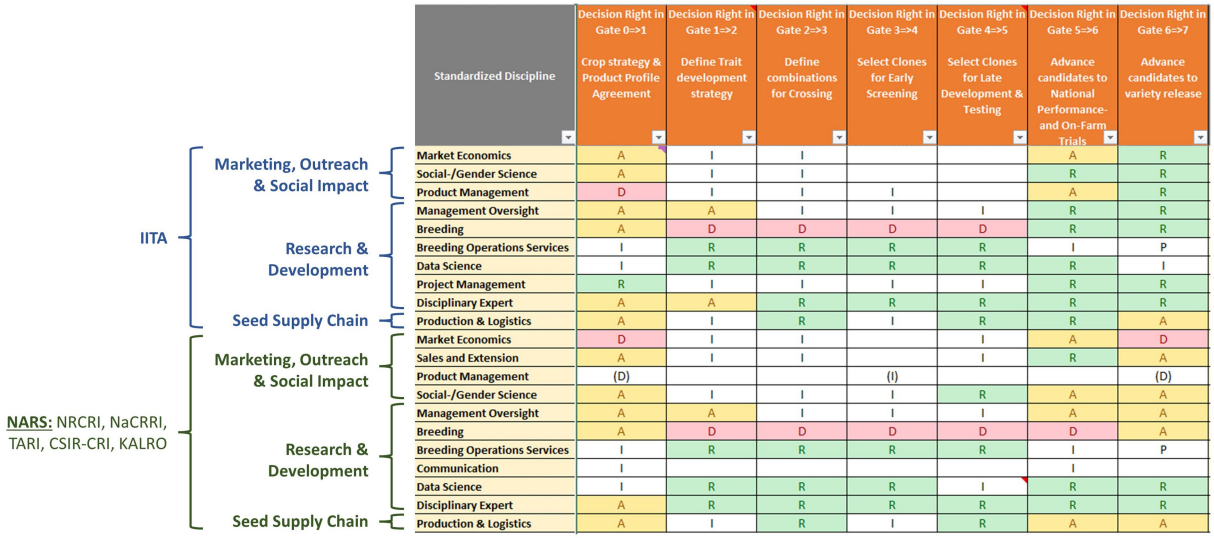


FIGURE 2
Cassava stage-gate process. Cassava product development is mapped into distinct stages and gates, each with a specific objective. The cassava stage-gate process is divided into seven stages, starting with product design (stage 0) and ending with product release (Stage 7). A brief description of what happens at each stage, which include target product profile, trait discovery and deployment, and population development is defined in the blue column, followed by decision points (amber column with diamond symbols) where decisions are taken. An overview of what decisions are made and who made them is presented. Stage 0 entails deciding on crop strategy and establishing or updating product profile. A full functional team, including donors, and funders are involved. Stage 1: Trait discovery and pre-breeding; Stage 2: Trait deployment. It is an intersection to the standard breeding process; stage 3: one makes combinations (decide which parents to put in nursery) and make crosses. Stage 4: clones' selection. Stage 5: A late-stage yield trial within the research organization. Candidate clones are selected to advance to national performance trials (Stage 6). Stage 7: Official release and product launch.



		Standardized Discipline	Decision Right in Gate 0=>1 Crop strategy & Product Profile Agreement	Decision Right in Gate 1=>2 Define Trait development strategy	Decision Right in Gate 2=>3 Define combinations for Crossing	Decision Right in Gate 3=>4 Select Clones for Early Screening	Decision Right in Gate 4=>5 Select Clones for Late Development & Testing	Decision Right in Gate 5=>6 Advance candidates to National Performance- and On-Farm Trials	Decision Right in Gate 6=>7 Advance candidates to variety release
IITA	Marketing, Outreach & Social Impact	Market Economics	A	I	I			A	R
		Social-/Gender Science	A	I	I			R	R
		Product Management	D	I	I	I		A	R
	Research & Development	Management Oversight	A	A	I	I	I	R	R
		Breeding	A	D	D	D	D	R	R
		Breeding Operations Services	I	R	R	R	R	I	P
		Data Science	I	R	R	R	R	R	I
		Project Management	R	I	I	I	I	R	R
		Disciplinary Expert	A	A	R	R	R	R	R
	Seed Supply Chain	Production & Logistics	A	I	R	I	R	R	A
NARS: NRCRI, NaCRRI, TARI, CSIR-CRI, KALRO	Marketing, Outreach & Social Impact	Market Economics	D	I	I		I	A	D
		Sales and Extension	A	I	I		I	R	A
		Product Management	(D)			(I)			(D)
		Social-/Gender Science	A	I	I	I	R	A	A
	Research & Development	Management Oversight	A	A	I	I	I	A	A
		Breeding	A	D	D	D	D	D	A
		Breeding Operations Services	I	R	R	R	R	I	P
		Communication	I					I	
		Data Science	I	R	R	R	I	R	R
		Disciplinary Expert	A	R	R	R	R	R	R
	Seed Supply Chain	Production & Logistics	A	I	R	I	R	A	A

FIGURE 3
Decision-making rights mapping at discipline level. Facts and/or data are needed for decision-making. Depending on the stage, these inputs must be reviewed, suggestions for possible change made, or, when dealt with data, they must be analyzed and summarized for team assessment. The appropriate disciplines should be involved at each step and since each will contribute differently, their decision-right should be well-defined. There are some who recommend (R), agree (A), provide inputs (I), and they ought to be someone who resolve any disagreements and decide (D). Any decision must be followed by an action, known as implementation (P).

(SWOT) analysis and a determination of the unique selling points of varieties, and if this differs per region given the genotype by environment results.

The second is from the social and gender segmentation perspective within the largest breeding pipeline: processed granulated and paste products. Results are based on market intelligence and information, including participatory research and consumer testing (Wossen et al., 2017; Teeken et al., 2018, 2021a,b; Ndjouenkeu et al., 2021), which highlighted the need to consider preferences of specific crop users (i.e., small- and medium-scale men and women farmers and women

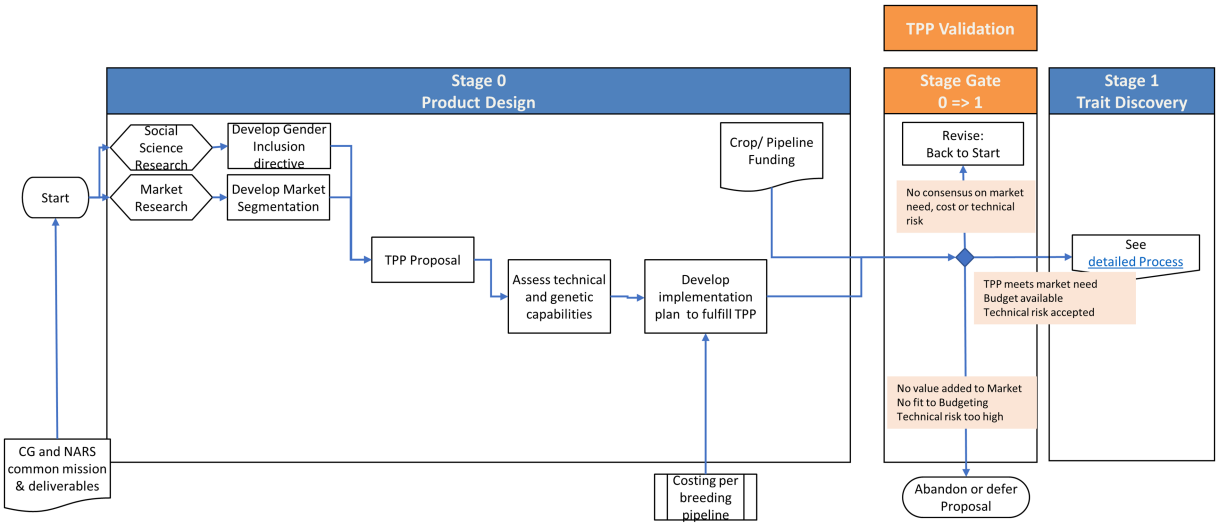


FIGURE 4
Stage 0 (product design) high-level map The crop strategy is defined and reviewed using information provided by market intelligence and social scientists that capture the need of the market. This information is used to develop gender inclusive directives, market segmentations, and, ultimately, the target product profile (TPP). The technical feasibility of the proposed TPP is evaluated by breeders and trait-development breeders, and an implementation plan is developed based on crop/pipeline funding information. A decision point is indicated by a diamond. It is possible that there is no consensus and the TPP must be revised, or if there is no value added to the market, no fit with budgets, or the technical risks are too high or the technical feasibility unsatisfactory; in this situation, the proposed TPP will be abandoned or deferred. However, if the TPP meets market demand, there is adequate money, and technical risk is accepted. The TTP will be validated and proceed to the next stage.

processors). Results pointed to the need to consider consumers of cassava food products in the rural and urban areas, as well as identified priority traits that must be included in the product profile (Stage 0). Variety preferences by intersectional groups of farmers and processors are presented, as well as the outcome of consumer testing in rural and urban areas segmented by relevant social dimensions. Data are then triangulated, and a variety of recommendation based on the late-stage testing is provided. It must be highlighted that late-stage participatory processing reveals information on which varieties to advance (Stages 5 to 6) but at the same time, such participatory work allows to inform trait prioritization among users. This is especially the case for Tricot-scaled participatory variety evaluation of which data are systematized, stored, and analyzed in ClimMob[2] and stored in Breedbase[3] (de Sousa et al., 2024).

3.7 Implementation workshop and outputs

The purpose of the implementation workshop was to disseminate, review, and revise the findings and outputs, assess the current gaps, and to articulate the way forward toward implementation. Accordingly, a total of 64 participants from both IITA and five NARS organizations attended the workshop. Of these, 75 percent were from research and development, 17 percent from marketing, outreach, and social impact, and 8 percent from seed supply chain. The implementation workshop delivered 20 competencies (knowledge, skills, values and behaviors need to execute specified functions) critical for cassava advancement decision-making (Table 4) and brought more clarifications on the stage-gate system and roles and responsibilities.

Participants recognized the importance of developing a clear crop strategy, adopting a structure, and formalizing and standardizing product management and development. They also acknowledged the benefits and advantages of using appropriate tools (i.e., process maps, product advancement template), as well as forwarded suggestions and feedback for further improvement and actions to be taken. The participants additionally recognized the necessity to move from competition to collaboration among the cassava community and across disciplines to reinvigorate partnerships within the community. Other crop representatives also requested the scaling of the tools and concepts. Some of the identified challenges included lack of competence and resources, hindrances related to the achievement of deliverables, insufficient dedication, focus, and commitment to carry the approach forward, and inadequate clarity on criteria and concepts.

4 Discussion

The overarching goal of this project was to develop a scalable system for efficient cassava product advancement and management. This undertaking was done jointly between the IITA and selected NARS. Accordingly, we undertook activities with the hope to develop a more systematic and organized approach that optimize focus; thus, efficiency of product development. Rapid breeding and variety turnover necessitate the establishment of an efficient system to manage product development. Realigning and repackaging breeding and standardization of product management and advancement will have considerable benefits, including a significant improvement in efficiency and effective market-driven variety development. Successful

2 www.climmob.net
3 https://breedbase.org/

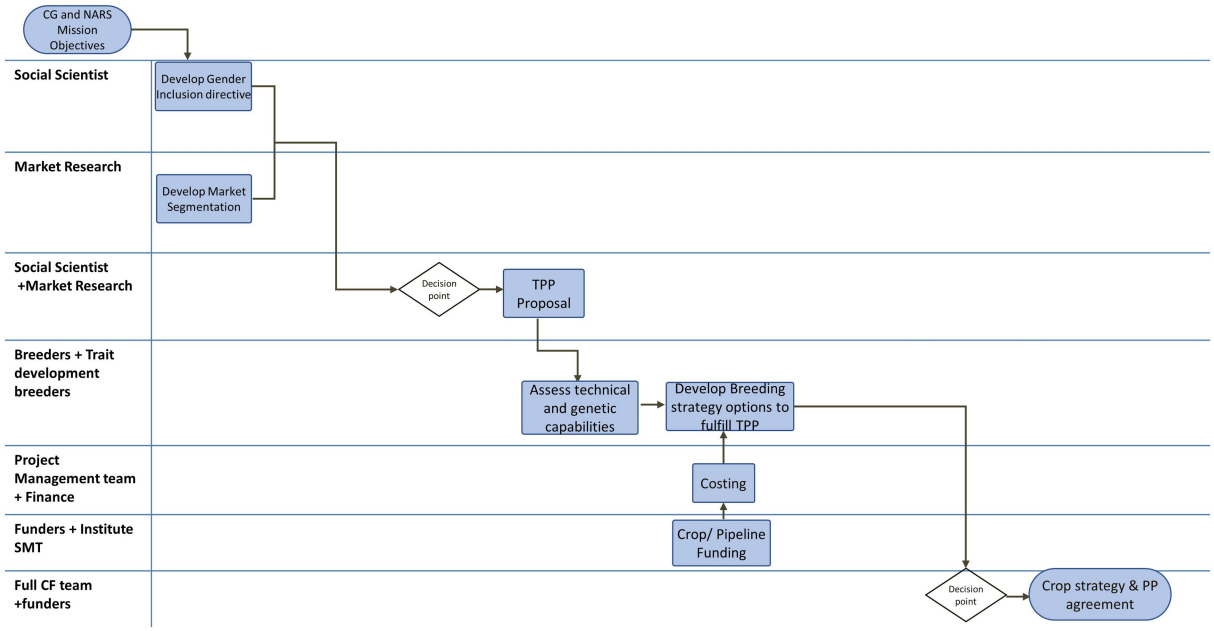


FIGURE 5
Product design swimlane flowchart. On the left side, the various teams involved in Stage 0 are listed and each has its horizontal "swimlane." The diamonds represent decision points. The teams are grouped into distinct lanes. The flow of activities is connected between the teams. While social scientist and market research specialist are involved in the development of target product profile (TPP), its feasibility is assessed by breeders and trait development breeders, who also develop the breeding options to fulfill the TPP. A full cross-functional (*CF*) team should validate the crop strategy and final product profile agreement, and funders should be involved as well.

structure and reorganization require careful planning and development of management tools and models.

We have developed and shared cassava calendars that have been customized to different regions. Such tools have been shown as a critical management tool and may aid in planning (Bauer et al., 1992; Franch et al., 2022). The primary purpose of the crop calendar is for planning breeding activities, including crossing, planting, evaluation, harvesting, and selection; for scheduling critical events (i.e., product advancement meetings) and to inform the public about what we do. We designed a stage-gate system for managing cassava products from design to delivery. The stage-gate approach is widely used in private companies to improve breeding operations and thus impact when released varieties are accessed and utilized by society (Cooper, 2008). Despite being widely acknowledged as a potent instrument for managing product development, some have expressed concern over the approach, which they believe to be too linear, rigid, and bureaucratic; hence, not adaptative enough as it does not encourage innovation and experimentation (Cooper, 2017). In cassava, we are optimistic about its integration and widescale adoption by breeding programs as we see the stage-gate as a guiding flexible principle that provides a thorough understanding of the process and necessary activities among stakeholders to increase focus. Elsewhere, reluctance to adopt it has been overcome by incorporating elements of adaptivity and agility into the original stage-gate approach (Smolnik and Bergmann, 2020). More transparency, control over the breeding pipeline, and the delivery of customs-tailed products will all be made possible by the implementation of such a system for managing cassava breeding pipelines.

Implementation of the stage-gate approach requires specification of activities that must be performed at each stage, and providing a criterion that must be met at each gate, inputs for each stage, as well as the involvement of specific members that are assigned specific tasks, roles, and decision rights. Herein, we described and defined the entire cassava breeding program through process mapping across IITA and its NARS partners. We mapped vital activities at each stage. In the workflow diagrams, we described individual steps and actors in cassava product development. This mapping will help to increase activity transparency while guaranteeing adequate output control at each stage. This mapping is not meant as a rigid inflexible bureaucratic grit but as a flexible and living tool that clarifies the breeding process for all: the whole extended breeding team and other stakeholders. Such management tools are needed and help with operations management and better coordination of tasks, as well as identifying wastes, inefficiencies, blockages, and improvement opportunities in the current processes. Prior to allocating costs for activities, process mapping is a requirement, and it is the first step toward improving processes (Klotz et al., 2008; Abreu et al., 2017). Such a tool will promote inclusive and participatory processes, collaboration across teams and within cassava network, and could be an effective instrument for resource mobilization.

Advancement and management of cassava products are transdisciplinary undertakings as evidenced by the varied roles and disciplines documented in our study. Stakeholder mapping underscored resource gaps (i.e., the requirement for product managers to support and/or justify breeding pipeline investments). We also found overlap between many functions and we identified new roles. Stakeholder mapping highlighted the need to prioritize not only research and development but also other higher-level entities (i.e., marketing, outreach, social impact, and seed supply chain) all of which are currently underrepresented. A limitation to the stakeholder mapping was that it did not capture (external) crop users in the crops value chain (farmers, processors, marketers, and consumers). In our mapping, they were represented by the disciplines and people mapped under Marketing, Outreach and Social Impact. Effective

TABLE 3 SIPOC diagram that illustrates high-level overview of the trial process for stage 3 (crossing and screening).

Supplier	Input	Process	Output	Customer
• Breeder	• Product Profile • Phenotypic data • Genomic data • Pedigree information • Analysis strategy (Selection index) • Selected candidates	•Parental selection	•Crossing plan •Seeds list	Hybridization team
• Hybridization team	• Seed list • Crossing plan	•creation of genetic variation (Intercrossing)	•Botanical seeds	• Seedling nursery team
• Seedling nursery team	• Botanical seeds	•Seedling transplant bed • seedlings evaluation	•Established seedling nursery •Selection of vigorous seedlings	• Seedling nursery team
• Seedling nursery team	• Selection of vigorous seedlings	•Evaluate F_1 seedling in the field • Collect phenotypic data	• Phenotypic data	•Data analyst
• Data analyst	• Phenotypic data	•Conduct data analyses •Select candidate for advancement	• Preselected planting list •Data analysis summaries	•Breeder
• Data analyst	• Preselected planting list • Data analysis summaries	Final selection	• Final planting list and planting material for early testing (CET, PYT)	•Field operation team

A high-level overview of the trial process for stage 3 and its key components is provided by the SIPOC. The relevant inputs and outputs required at each step, who supplies them, the key activities, and who are the customers are captured.

TABLE 4 Competencies for advancement decision-making in a cross functional and cross-organization context.

Product development	Effectiveness	Cross-functional management	Leadership	Transformation
•Product Management	• Data management (Analysis and visualization)	• Competency to integrate various disciplines in decision- making	• Leadership and mediation in a cross-functional team	• Communication to different stakeholders
• Marketing	• Science knowledge management	• Capacity to address gender, diversity, and inclusion issues	• Negotiation and conflict resolution	• Flexibility and openness to change
• Market research	• Project management (Planning, monitoring, evaluation)		• Resource mobilization	• Meeting facilitation
• End-to-end variety development process	• Process optimization		• Mentoring and coaching	• Change management
• Country specific variety release processes	• Continuous improvement			

stakeholders' representation and engaging diverse source of knowledge is key to success. As a result, it will be crucial to engage stakeholders outside the breeding team (i.e., farmers, processors, and marketers) to provide useful perspectives. This information will be integrated with known facts to further enrich and make the system more relevant and practical. For example, new scaled and systematized participatory citizen science approaches to participatory variety selection have been identified as a way to create a network of users to socially inclusively engage value chain actors as citizen scientists (van Etten et al., 2020; de Sousa et al., 2024) as well as feedback from seed businesses. Product development success has been linked to team effectiveness, which can be connected to team composition, participation of relevant stakeholders, and effective communication and coordination across various entities, roles, and disciplines (Edmondson and Nembhard, 2009; Majava et al., 2015). An overview of the stakeholder landscape and mapping of roles offers an opportunity to consider how the various partners could complement one another equally. It also offers a good understanding of the strengths and weaknesses of each organization.

Decisions are made throughout different stages of the product development in breeding programs. Making poor or incorrect decisions can have a negative impact on the overall product development, product performance, and on the achievement of desired outcomes and impacts. We established role clarity and accountability among the stakeholders using the RAPID decision-making model (Rogers and Blenko, 2006). This provides a clear delineation of responsibility. It was shown that the different stakeholders contribute differently at various product development stages. Demarcating each stakeholder's responsibility at various stages can prevent disagreements, conflicts and ensure a more effective, efficient, and inclusive decision-making process. It is crucial to emphasize the need to widen the decision-making group. Indeed, it has been demonstrated that effective stakeholder representation and participation, involvement of relevant actors, and the right team composition throughout the different stages and gate could result in a high-quality information input; thus, a high-quality decision, and a more impactful and durable outcome. Similarly, effective communication and coordination across the different entities, roles, and disciplines is essential (Edmondson and Nembhard, 2009; Majava and Haapasalo, 2015; Reed

and Curzon, 2015). An operational roadmap featuring multiple checkpoints and well-informed decisions supported by diverse perspectives will ensure that the right strategy will be designed, the right parents will be crossed, the right clones will be selected and advanced, the developed product will be in line with the predefined product profile, the right product will be delivered to the end-users and a high rate of genetic gain for key traits will be achieved.

Expertise gaps can impede the product's development and advancement. We highlighted critical competencies that would be required for collaborative advancement decisions, as well as the gap in competencies and required resources. A critical need identified is the need for product management that effectively represents and brings together all the relevant information from marketing, outreach and social impact to inform the product profile. It will be critical to leverage expertise and knowledge within each entity across processes. Among the marketing, outreach and social impact, clear capacity building is necessary on product development from product profile to varietal release. Furthermore, across IITA and NARS, people realized the need for capacity development on cross-functional management competencies and transformation competencies, and with a relatively greater need among the NARS. This could be achieved using classical solutions such as training, workshops, mentoring, participation at technical conferences, and content repositories. The already existing cassava community of practice and partnership (CoPP) initiated by the Next Generation Cassava Breeding Project (see "Footnote 1") could be exploited for this purpose to connect cassava stakeholders, encourage knowledge transfer, and bridge expertise gaps within and between organizations (O'Dell and Trees, 2014). Effective partnerships and interorganizational collaboration within the cassava network will close the existing gap (Bröring and Cloutier, 2008). It will also be crucial to set up a system that continuously support learning and leveraging of newcomers' skills.

The currently developed templates encompass social and gender information, information obtained from crop users along the food chain through participative research, and the technical breeding results. Other aspects of the template will have to be further developed with food science and other relevant disciplinary experts, as well as with the seed supply chain entity, to assure an inclusive and complete input from all the relevant entities and their disciplines. For effective product management and advancement, the concepts and tools developed must be put into practice. Although these tools and concepts are widely used in the private sector, public breeding programs have not yet adopted them. We anticipate slow adoption at the start, which will eventually increase owing to the publicity and relevance that have been emphasized during design and the traction it is gaining at higher CGIAR and government levels as well as among donors who stress the need for adoption of new technologies and equally realizing social impact (CGIAR, 2021; Donovan et al., 2022; Polar et al., 2022). Transdisciplinary mapping stakeholders and their role and decision-making rights do not necessarily assure an inclusive non-disciplinary biased outcome. This is the reason why learnings from studies of power dynamics (Tarjem, 2023; Tarjem et al., 2023) related to the asymmetries between natural and social sciences that are rooted in different epistemological traditions and unequal funding will have to support effective implementation. Awareness must be raised through socialization and communication within the cassava community to acquaint stakeholders with the developed assets and provide them the opportunity to give their perspectives, which may be a source of innovation in the change and/or improvement process. Leadership support and effective communication at all levels could be other essential conditions for these changes to take place. A team culture must be developed, and champions need to be empowered (Waddell and Sohal, 1998; Gesme and Wiseman, 2010; Kuzhda, 2016).

5 Conclusion

The management of product development is complex and requires alignment and effective collaboration between a broad range of stakeholders and technical experts in various disciplines. Therefore, workflow structuring, and management are essential for an end-user-driven, product-oriented variety development that is efficient, effective, and destined to deliver genetic gain in farmers' fields. In this light, we developed tools for portfolio management of cassava breeding, including a cassava calendar for planning and managing activities and templates to guide product advancement. We designed a clear stage-gate system within which we mapped cassava breeding processes to control outputs at each stage, ensure that the relevant inputs are supplied, and the outputs optimized. Successful product development being transdisciplinary, depends on the stakeholders participating, the clarity of stakeholder roles, who has what right to do what and who is accountable for decision-making, as well as coordination between the many actors at various levels. This information is essential to develop and organize cross-functional teams and provide them with highly effective collaborative structures. Capturing the stakeholder landscape has made it possible to find gaps and overlaps, as well as opportunities for team reconfiguration. The integration of the many skill sets will be necessary for the transdisciplinary of product management and advancement. Team effectiveness, being one of the factors that affects how efficiently products are developed, it is crucial to evaluate the available competencies and upskill them as needed. Finding the initial resources required for a such committed transdisciplinary team, routine operationalization of the developed tools, the reluctance in accepting change, and the power dynamics between natural and social sciences are some of the anticipated challenges. The present pilot work done in cassava currently serves as a model for cross-organizational collaboration and is being scaled to other CGIAR crops.

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

The individual(s) provided their written informed consent for the publication of any identifiable images or data presented in this article.

Author contributions

CE: Conceptualization, Funding acquisition, Writing – review & editing. EM: Conceptualization, Investigation, Methodology, Project

administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RK: Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. BT: Investigation, Methodology, Resources, Writing – review & editing. IR: Investigation, Methodology, Resources, Writing – review & editing. RP: Resources, Writing – review & editing. LJ: Resources, Writing – review & editing. DN: Resources, Writing – review & editing. HK: Resources, Writing – review & editing. FG: Resources, Writing – review & editing. VW: Resources, Writing – review & editing. EP: Resources, Writing – review & editing. RO: Resources, Writing – review & editing. VB: Resources, Writing – review & editing. PN: Investigation, Methodology, Resources, Writing – review & editing. JD: Resources, Writing – review & editing. SW: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. PK: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the NextGen Cassava project, through a grant by the Bill & Melinda Gates Foundation (Grant INV-007637 http://www.gatesfoundation.org) and the UK's Foreign, Commonwealth & Development Office (FCDO).

Acknowledgments

We acknowledge and thank the IITA and NARS representatives and the Steering Committee for their contributions and support to the successful implementation of the project.

Conflict of interest

SW is employed by Weber & Fritz Consulting.

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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2024.1322562/full#supplementary-material

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frontiers | Frontiers in Sustainable Food Systems

TYPE Original Research
PUBLISHED 19 July 2024
DOI 10.3389/fsufs.2024.1288579

OPEN ACCESS

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RECEIVED 04 September 2023
ACCEPTED 03 July 2024
PUBLISHED 19 July 2024

CITATION
Anshori MF, Musa Y, Dungga NE, Widiayani N, Arifin AS, Masniawati A, Firmansyah F, Farid M, Dirpan A and Amas ANK (2024) A new approach for selection of transgressive segregants in F3 populations based on selection index and anthocyanin content in cayenne pepper.
Front. Sustain. Food Syst. 8:1288579.
doi: 10.3389/fsufs.2024.1288579

A new approach for selection of transgressive segregants in F3 populations based on selection index and anthocyanin content in cayenne pepper

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The development of cayenne pepper varieties can be optimized by multiple crossings, transgressive segregant selection based on the selection index, and identification of potential anthocyanins. The study objectives were (1) to develop a transgressive segregation index, and (2) to select transgressive segregation cayenne peppers with high productivity and anthocyanins from F3 multiple cross-generation. The study conducted two experiments at the experimental field, Hasanuddin University, from November 2022 to November 2023. The first experiment implemented an augmented design with a randomized complete block design (RCBD) as an environmental design. The genotypes as treatment consisted of two types: 110 lines of cayenne pepper were not repeated, and the 4 older chili varieties as controls were repeated in each block. All genotypes were categorized and divided into five blocks. The second experiment was the validation of the first trial. There were 13 genotypes tested with RCBD design one factor and repeated three times. Based on the study, developing a semi-objective-based selection index with canopy width, fruit weight, and yield was an innovative and effective approach to selecting F3 transgressive segregants of cayenne pepper. High-yielding transgressive lines were identified as G3-2-7-3, G2.6.9–10, G5-12–1-8, and G4.5.2–12. The G3-2-7-3 line was suggested due to its high yield potential and anthocyanin content. However, the anthocyanin content must be examined more deeply, such as using an omics approach. Nevertheless, these lines are still recommended to be continued in yield testing or crossing to produce hybrid lines that have high yield potential and anthocyanin content.

KEYWORDS

antioxidant, *Capsicum frutescens* L., genetic parameters, transgressive segregation, *Z*-value

1 Introduction

Cayenne pepper (*Capsicum frutescens* L.) is a horticultural commodity commonly cultivated in various parts of the world. It is an integral commodity frequently incorporated to enhance the peppery flavor of the dish (Andrade et al., 2020; Kusumiyati et al., 2022; Johnson et al., 2023). The high capsaicin content in cayenne pepper makes this product effective as a spicy seasoning for cooking (Rajametov et al., 2021; Stan et al., 2021; Bu et al., 2022; Kusumiyati et al., 2022). Additionally, cayenne pepper is predominantly preferred in various dishes as it is a rich source of nutrients, vitamins, antimicrobials and antioxidants (Yashin et al., 2017; Hernández-Pérez et al., 2020; Olasupo et al., 2021; Bu et al., 2022; Johnson et al., 2023). Therefore, the demand for cayenne pepper will continue to advance with the increasing world population.

The necessity for cayenne pepper in Indonesia is unresolved (Hanani et al., 2020). According to Hanani et al. (2020), Sundari et al. (2021), and Surya and Tedjakusuma (2022), the urgency for cayenne pepper in Indonesia is relatively high and will persistently advance with the increasing population. In a study by Sundari et al. (2021), it was reported that Indonesian chili consumption reached 5 kg/capita/year in 2019, ceaselessly expanding yearly. However, the cayenne pepper production has considerably proliferated to 1.55 tons in 2022 or an increase of 11.5% (Statistic Indonesia, 2023). However, due to production inconsistencies, the price fluctuates dynamically (Jayanti et al., 2021; Diansari et al., 2023). Therefore, developing adaptive and high-yielding cayenne pepper varieties is imperative to alleviate the challenges of the supply deficit.

Adaptive and high-yielding cayenne pepper varieties can be developed through plant breeding programs. There are various concepts in forming essential populations in plant breeding, one of which is multiple cross (Bandillo et al., 2013; Pathy et al., 2018; Sekine et al., 2021). This crossing technique is carried out by hierarchically crossing more than two pure parental lines (Acquaah, 2012; Syukur et al., 2015). Multiple crosses aim to combine various parental traits into a primary population so that the basic population has a high diversity (Bandillo et al., 2013; Pathy et al., 2018; Sekine et al., 2021). This diversity is very effectively incorporated in the selection process for plotting, for both characters with a unidirectional or opposite orientation. Several studies have reported this concept's effectiveness in establishing essential populations, including hot peppers (Pathy et al., 2018; Arrones et al., 2020). Multiple cross-based varieties must be developed to assemble high-productivity cayenne pepper varieties in that context.

The assembly of a variety cannot be segregated from the process and stages of distribution. This stage takes time and costs to increase line stability (Acquaah, 2012; Ridzuan et al., 2018). There are several approaches to minimizing the straining process, one of which is transgressive segregation (TS) selection (de los Reyes, 2019; Koide et al., 2019; Pabuayon et al., 2021). TS is a collection of lines in the outer region of the distribution of variance in a population (Maryono Yuniawati et al., 2019; Nascimento et al., 2019). The breeding process is swifter as the selection of TS lines can be identified in the early generations of F3 and F4 (Koide et al., 2019; Pabuayon et al., 2021; Shwetha et al., 2022). The selected TS lines have a high level of homozygosity with better stability and adaptation than other test lines or varieties. The application of this concept has also been reported by Nascimento et al. (2019) on ornamental chilies, Alimi et al. (2013), Yunandra et al. (2018), and Rostini et al. (2019) on *Capsicum annum* L. red chilies. However, the effectiveness of TS requires further development. This selection concept is still focused on the main character and does not involve the accumulative role of secondary characters. Therefore, the development of TS selection requires maximal optimization to support the effective and precise assembly of cayenne pepper. One of the concepts that can be offered is the selection index approach.

The selection index is a multiple linear equation for various selection criteria (Lopez-Cruz and de los Campos, 2021; Cerón-Rojas and Crossa, 2022). Each selection criterion is weighted in the equation by the priority value, economics, and the genetic role of each selection criterion (Anshori et al., 2022; Cerón-Rojas and Crossa, 2022; Chung and Liao, 2022). The results of the accumulated weighting multiplication and absolute scores for each selection criterion serve as a ranking reference in the selection process (Lopez-Cruz and de los Campos, 2021; Anshori et al., 2022; Cerón-Rojas and Crossa, 2022; Farid et al., 2022). Several studies have reported the effectiveness of using the selection index in the grafting process (Padjung et al., 2021; Anshori et al., 2022; Chung and Liao, 2022; Fadhilah et al., 2022; Farid et al., 2022). The concept of this selection index can be integrated into selecting transgressive lines in the F3 generation. This combination has yet to be widely reported, especially in the selection of cayenne pepper distribution. Therefore, a transgressive segregated selection index can effectively develop cayenne pepper lines in multiple cross-F3 populations.

The development of cayenne pepper also postulates concentration on the potential quality of the fruit (Johnson et al., 2023). One of the qualities that can strengthen the added value of cayenne pepper is its anthocyanin content. Anthocyanin content is an effective antioxidant substance in the eradication of free radicals and fortification of body immunity (Marszałek et al., 2017; Mahendradatta et al., 2021; Meng et al., 2022). This is relatively beneficial in the current era, where the focus is on healthy living trends that alleviate various disease pandemics, such as COVID-19. Anthocyanins are abundantly found in fruits with red, blue, purple, and black colors (Aza-González et al., 2012; Zhang et al., 2015; Liu et al., 2022; Meng et al., 2022), so these substances can be in high amounts of cayenne pepper. Several studies have reported the effectiveness of the anthocyanin content in chilies (Lahbib et al., 2021; Meng et al., 2022; Zhou et al., 2022). Therefore, it is equivalently crucial to identify the anthocyanin content of the selected F3 cayenne pepper transgressive lines. This makes cayenne pepper F3 transgressive as a food biofortification product in encouraging the healthy living trend (Olasupo et al., 2021). In this framework, the research had various objectives, namely, (1) to develop an effective transgressive segregation index in the F3 multiple-cross population of cayenne pepper; (2) to select transgressive segregation of cayenne pepper with high productivity in the multiple-cross F3 population; and (3) selecting a transgressive group of cayenne pepper with high productivity as well as substantial anthocyanins.

2 Materials and methods

The study had two experiments conducted at the experimental garden of the Faculty of Agriculture, Hasanuddin University, Tamalanrea District, Makassar City, South Sulawesi Province, Indonesia (5°7′40" South Latitude, 119°28′59″ Longitude, and 14 m

above seawater (asw) altitude). The first experiment was from November 2022 to April 2023 and June 2023 to November 2023. The climate parameters in this study are shown in Supplementary 1, where F3 was mostly the rainfall season, and F4 was the mostly dry season. Meanwhile, soil characteristics in this study showed that the soil texture was clay, the percentage of clay was 44%, percentage of dust was 47%, percentage of sand was 10%, C-Organic was 1.23%, total nitrogen was 0.1%, C/N ratio was 13 ppm, pH(H_2O) was 5.85, P Olsen was 10.34 ppm, K content was 0.46 cmol (+) kg^{-1}, Ca content was 4.91 cmol (+) kg^{-1}, Mg content was 0.93 cmol (+) kg^{-1}, Na content was cmol (+) kg^{-1}, and cation exchange capacity was 22.29 me 100 g^{-1}.

2.1 Experimental design

The study implemented an augmented design with a randomized group design as an environmental design. The genotypes as treatment consisted of 2 types, namely 110 lines of cayenne pepper, which were not repeated, and the 4 older chili varieties as controls, which were repeated in each block. All genotypes were categorized into five blocks. The lines planted were the result of selection from 10 combinations of multiple cross populations originating from 4 elders, namely, Dewata F1 (D), Ungara (U), Bara (B), and Katokkon (K) (Table 1 and Figure 1). The four are unrelated, and the selection was based on their genetic background and specific characteristics. Dewata F1 (D) is a hybrid F1 variety with a high yield and is spicy. Ungara (U) is an ornamental pure line variety with a purple fruit color. Bara (B) is a common pure line variety and is pungent. The last, Katokkon (K), is a local pure line variety, and it is very spicy. The different phenotype fruits among parents are shown in Figure 2. Meanwhile, each line consisted of 12 plants, while the older variety in each block was planted with eight plants.

The second trial was the validation of the first trial; 10 genotypes were taken out of the 20 best genotypes in the first experiment. These 10 genotypes were based on various considerations, such as rank line spread, seed growth capacity, and seed quality for transplantation. In addition, there were 3 check varieties, Dewata F1, Bara, and Ungara, so 13 genotypes were tested. As for the experimental design, it used RCBD one factor and repeated three times. So, there were 39 experimental units. Each experimental unit planted as many plants as in the first experiment.

TABLE 1 Details of multiple cross-hybridization in this cayenne breeding study.

Label	Detail of multiple-cross hybridizations	Abbreviation
G1	Ungara/Bara//Dewata F1/Katokkon	U/B//D/K
G2	Ungara/Dewata F1//Bara/Ungara	U/D//B/U
G3	Ungara/Dewata F1//Katokkon F1/Katokkon	U/D//D/K
G4	Ungara/Katokkon//Dewata F1/Bara	U/K// D/B
G5	Ungara/Dewata F1//Dewata F1/Bara	U/D//D/B
G6	Ungara/Bara//Dewata F1/Ungara	U/B//D/U
G7	Ungara/Bara//Dewata F1/Bara	U/B//D/B
G8	Ungara/Dewata F1//Bara	U/D//B
G9	Ungara/Bara//Dewata F1	U/B//D
G10	Dewata F1/Ungara//Bara	D/U//B

2.2 Research procedure

All experiments had the same research procedure. The implementation of this research consisted of several steps, namely, (1) tillage, (2) nursery, (3) transplanting, (4) maintenance, and (5) harvesting. (1) Soil preparation was carried out by perfect tillage using a tractor, followed by making beds. Beds were made with a length of 6 m and a width of 1 m with a distance between beds of 50 cm. The beds were then given black silver mulch, which was perforated using a mulch punch tool in the form of a can with a diameter of 10 cm as a place to plant the cayenne pepper seeds. Seeding (2) comprised a planting medium consisting of soil, compost, and burnt husks with a volume ratio of 1:1:1. Furthermore, the planting medium was saturated with water until it was evenly distributed. The cayenne pepper seeds were transferred to the seedling tray while sprinkled with Furadan to avoid disturbance or pest attack. Applying AB Mix of hydroponic nutrition at 5 mL/L of water was given when the seedlings were 10 days after sowing (DAS) by watering around the plant roots. Then, the cayenne pepper seedlings were maintained until they were 21 DAS and eventually transferred to the beds (transplanting). Transplanting (3) was done with a spacing of 50 cm x 60 cm between rows in a zig-zag manner. So, in F3 planting, there were 23 plants in each bed. Each cayenne pepper plant was supported with a stake so that the plant did not collapse or break. (4) Cayenne pepper plant maintenance consisted of several procedures: watering, replanting, fertilizing, pruning, weeding, and pest and disease control. Watering was done twice daily, in the morning and the evening, using a water hose until the soil looked moist. Stitching was done 2 weeks after planting (WAP) for plants that experienced abnormal growth, withered, and were attacked by pests or diseases based on the variety code and the same age. Fertilization was performed from 1 WAP using AB Mix at a dose of 5 mL/L of water. Subsequent fertilization was carried out using the Mutiara NPK fertilizer (16–16–16), and KNO_3 was given at a dose of 5 g at 3 WAP and 6 WAP around the plant root area. Pruning was done by removing small shoots on the lower stem to focus the growth of chilies on the main stem (central stem) and was conducted at least once a week. Weeding was done to remove weeds that interfere with growth around the plant. Weeding was carried out manually and chemically using the herbicide Gramoxone 276SL at two g/L of water. Pest and disease control used the insecticide Curacron 500 EC with a concentration of 2 cc/L of water and the fungicide Antracol 70 WP with 2 g/L of water. These insecticides and fungicides were applied by surface spraying using a hand sprayer. Meanwhile, (5) harvesting was started when the fruit had entered physiological maturity, marked by reddish fruit in each line or approximately 70 days after planting (DAP), and continued six times, with a weekly frequency.

2.3 Observation parameters and data analysis

The observation parameters were recorded quantitatively. These parameters included vegetative and generative growth parameters.

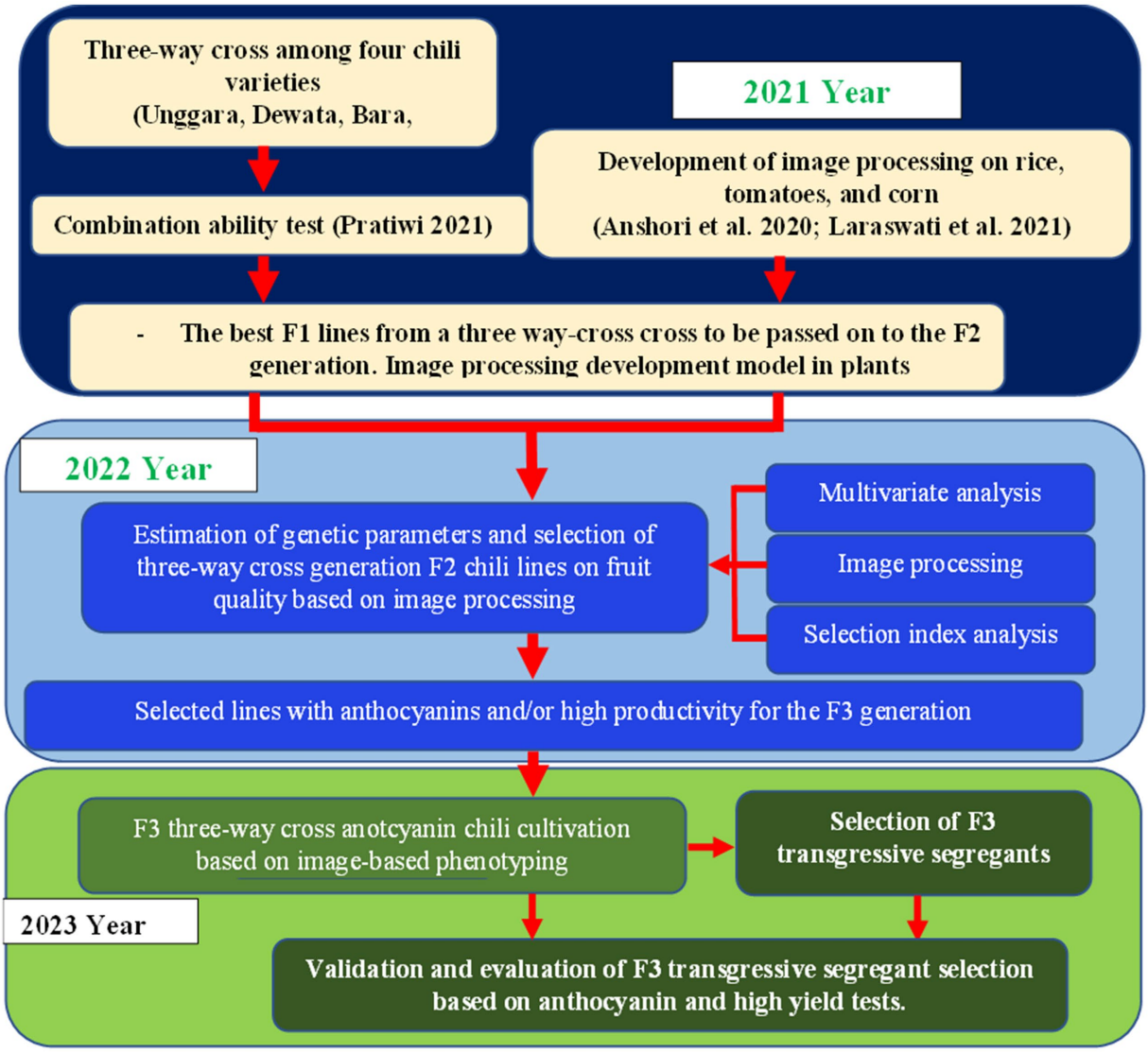

FIGURE 1
Diagrammatic representation of the breeding scheme of cayenne F3 multiple crosses.

Vegetative growth parameters consisted of plant height (cm) measured from the stem basal to the highest shoot, dichotomous height (cm) measured from the stem basal to the dichotomous branch, canopy width (cm) measured diagonally to the widest canopy, and stem diameter (mm) measured 15 cm from the stem basal. Meanwhile, the generative growth parameters consisted of the number of productive branches (branches) calculated from the branches that produce fruit, flowering age (DAP) measured at the time of first flowering, harvest time (DAP) measured when fruit starts the first time, fruit length (cm), stalk length (cm), fruit diameter (mm) measured at the center of the fruit, weight per fruit (g), production per plant (g), and anthocyanin content (mg). All data were tabulated and analyzed.

The performed analysis comprised various analytical concepts. Analysis of variance becomes the fundamental basis for further analysis in developing the basis. The analysis was performed using SAS 9.1 software. Significant parameters at the 5% level were subjected to correlation analysis. The correlation results were followed by path analysis to determine the selection criteria (Anshori et al., 2022). The selected selection criteria were combined into a selection index. Determination of the selection index weight was carried out using several approaches, namely, the value of direct influence, realized narrow-sense heritability, transgressive segregation ratio, and the z-value. The transgressive segregation selection index results were ranked. The lines with the best transgressive segregation level were categorically selected according to the criteria. Each selected F3 transgressive line was tested again for its fruit anthocyanin properties, leading to several transgressive segregated lines with high productivity and anthocyanin. Meanwhile, an explanation of the concepts from various analyses was presented as follows:

2.3.1 Heritability

Heritability uses the concept of comparing selection progress with selection differential. This was explained in the formulation below (Acquaah, 2012):

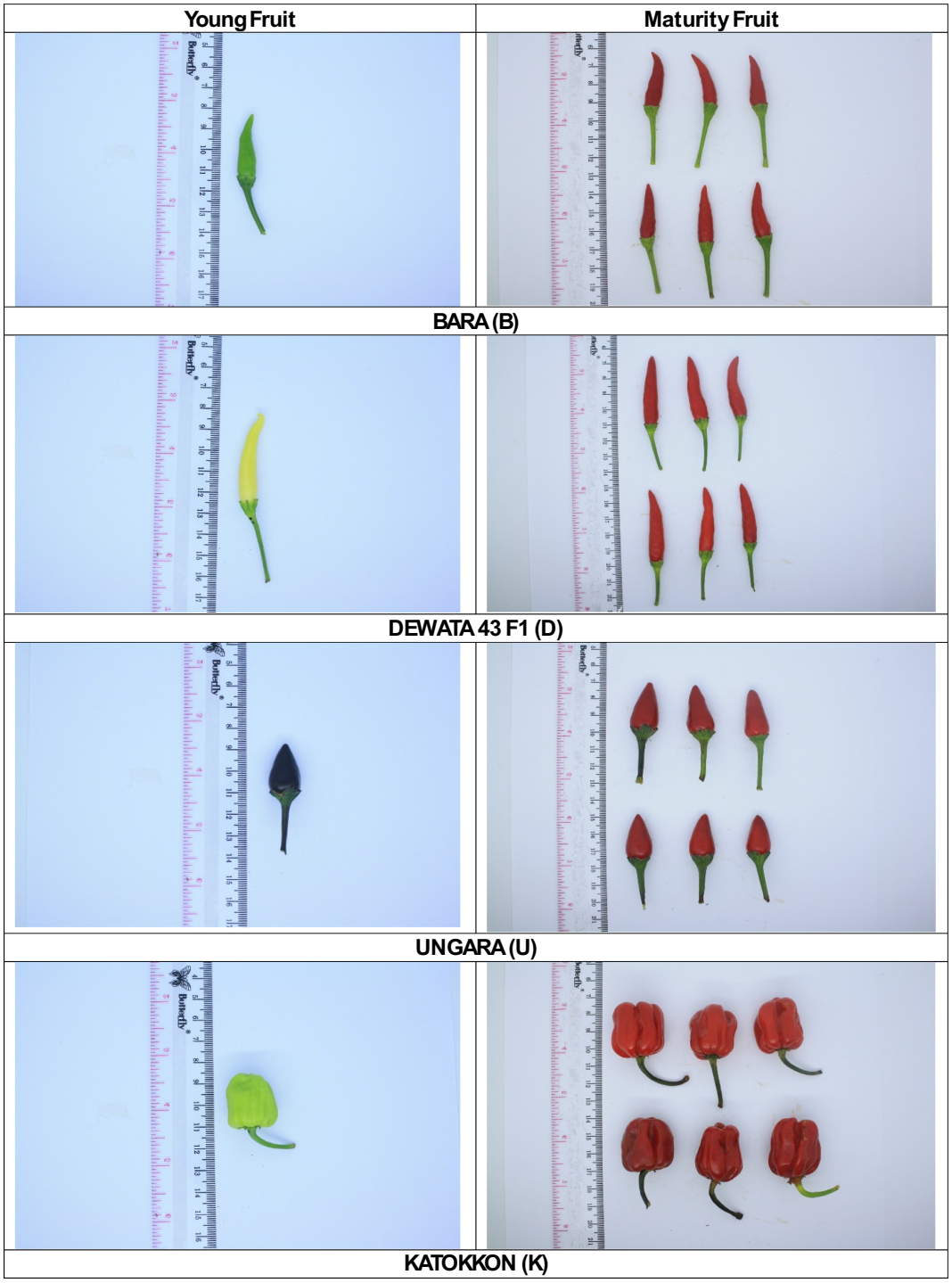

FIGURE 2
Phenotype of young and maturity fruits among parents.

$$G = h_{ns}^2 \times S \quad (1)$$

$$h_{ns}^2 = \frac{G}{S} x\,100\% \quad (2)$$

where, G = selection gain, S = selection differential, h_{ns}^2 = narrow-sense heritability. However, heritability also considers differences in variance or standard deviation between populations, so progress and differential selection were first converted into z-values for two populations with the independence concept. The formula for the z-value was as follows:

$$Z\ value\ for\ independent\ population = \frac{\left(X_i - X_j\right)}{\sqrt{\frac{\sigma_i^2}{n_i} + \frac{\sigma_j^2}{n_j}}} \quad (3)$$

where X_i = selected genotype means, for both gain selection and differential selection, X_j = the general means of the F2 population, σ_i^2 = selected genotype variance, σ_j^2 = the general variance of the F2 population, n_i = number of selected genotypes, n_j = number of all genotypes in the F2 populations.

2.3.2 Ratio of transgressive segregation

The transgressive segregation ratio was the *z*-value of the lines to the best parents on a specific selection criterion. The *z*-value followed the concept of the *z*-value formula in general. However, the difference lies in the standard deviation or variance used. The standard deviation was not the general population total standard deviation but the within-row deviation of each genotype. Meanwhile, the details of the analysis formula used in this study are as follows:

$$Z\ value = \frac{(X - \mu)}{\sigma\ \text{genotype}} \times \sqrt{n} \quad (4)$$

$$\text{Ratio of transgressive segregation} = \frac{z\ lines}{\text{z best parent}} \quad (5)$$

Note: X = means of genotype (lines or check variety).
μ = means of all population.
σ = standard deviation.
n = number of samples.
z = standardization value.

2.3.3 Anthocyanin analysis

Five young fruits were selected for each line to analyze their anthocyanin properties. The analysis was conducted by the method reported by Lee et al. (2005) and Teng et al. (2020). Anthocyanin analysis was performed by extracting anthocyanin pigments. This activity was carried out by crushing the flesh of the cayenne pepper and centrifuging it for 10 min. The juice or water from the extraction of the cayenne pepper fruit was retrieved in 10 mL for anthocyanin analysis. Determination of total anthocyanins using the differential pH method at pH 1.0 and pH 4.5. At pH 1.0, anthocyanins are oxonium compounds, and at pH 4.5, they are colorless carbinols. This was performed by making anthocyanin solutions in water with a pH of 1.0 and 4.5 and then measuring the anthocyanin content. The process involved dual stages of stock solution preparation, namely:

- pH 1.0 solution, prepared by dissolving 1.213 g of K2CrO4 (potassium chloride) in 250 mL of distilled water in a volumetric tube. Add HCl until the pH reaches 1.0.
- pH 4.5 solution, prepared by dissolving 10.599 g of Na2CO3 (sodium acetate) in 250 mL of distilled water in a volumetric tube. HCl was added until the pH reached 4.5.

The anthocyanin test solution was prepared by dissolving 5 mL of cayenne pepper fruit extract in 50 mL of each stock solution. Then, each test solution was measured with a spectrophotometer at the A510 and A700 wavelengths. Then, the results of the spectrophotometer analysis were calculated using the formula (Lee et al., 2005; Teng et al., 2020):

$$A = (A_{520} - A_{700})\,pH1.0 - (A_{520} - A_{700})\,pH4.5 \quad (6)$$

$$\text{Anthocyanin pigment}\left(\text{mg } L^{-1}\right) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1} \quad (7)$$

where MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in D; l = path length in cm; ε = 26,900 molar extinction coefficients, in L mol^{-1} cm^{-1}, for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

3 Results

The results of the ANOVA demonstrated that all sources of variation significantly affected almost all agronomic characters of cayenne pepper with a low coefficient of variation (below 20%) (Table 2). The variance source of variety in the control did not have a significant effect, only on the length of the fruit stalk. The source of line variation had no significant effect on flowering time and fruit stalk length. Meanwhile, the interaction of differences between control varieties and lines had no significant impact on fruit stalk length and stem diameter.

The results of the correlation analysis focused on the yield characteristics and several important characteristics that correlated with the yield (Figure 3). Based on the figure, the characters that were significantly correlated with the yield were canopy width (0.38), plant height (0.20), fruit diameter (0.27), fruit weight (0.47), and flowering age (0.24). Fruit weight character also significantly correlated with plant height (0.26), fruit length (0.36), fruit diameter (0.57), and flowering time (0.23). However, these characteristics did not correlate with plant height and canopy width. Canopy width character also significantly correlated with plant height (0.65), dichotomous height (0.46), flowering age (0.21), harvest age (0.32), and number of productive books (0.54). However, this character also did not correlate with fruit diameter. Fruit diameter also had a significant positive correlation with fruit length (0.28) but did not correlate with plant height. The character of flowering age was also significantly correlated with plant height (0.20), dichotomous height (0.28), and number of productive nodes (0.22). However, this character was not correlated with flowering age. Meanwhile, the character of plant height also demonstrated a significant correlation with harvesting age (0.31), dichotomous height (0.75), and number of productive nodes (0.51).

The path analysis results exhibited that the canopy width (CW) and fruit weight (FW) character had a substantially significant positive direct effect on the yield (0.46 and 0.44, respectively) (Table 3). Both characters also had the most significant indirect effect compared to the others (0.48 and 0.52, respectively). Conversely, the character of plant height (0.24) significantly negatively affected the yield. This character also had a negative impact (0.30) on the correlation of other characters on productivity. Meanwhile, the characteristics of flowering age and fruit diameter had a shallow direct effect on the yield (0.09 and 0.03, respectively).

The narrow-sense heritability analysis results focused on the yield and characters that had a significant direct effect (Table 4). Based on this analysis, the yield had a high and stable broad-meaning

TABLE 2 Population variance of F3 cayenne pepper on agronomic characters.

Characters	Mean square				CV (%)
	Control (C)	Lines (L)	C*L	Error	
Plant height	650.61**	160.15**	6698.72**	32.11	9.47
Dichotomous height	353.47**	63.74**	1059.00**	11.16	10.56
Canopy width	411.19**	188.47*	6920.62**	67.61	16.04
Stem diameter	5.29*	2.82*	1.12	1.11	12.13
Number of productive branches	42.96*	71.44**	324.95**	12.23	9.09
Flowering days	96.47**	0.90	314.17**	0.42	1.64
Harvest days	170.99**	4.17**	172.02**	0.35	0.79
Fruit length	0.48**	0.26**	0.10*	0.02	4.19
Fruit stalk length	0.24	0.19	0.05	0.09	12.84
Fruit diameter	39.14**	0.98**	39.42**	0.20	5.92
Fruit weight	2.15**	0.09**	0.90**	0.011	7.23
Yield	27322.78**	2329.44**	29267.66**	260.56	8.86

CV, coefficient of variation; *, significant effect at 5% error level; **, significant effect at 1% error level.

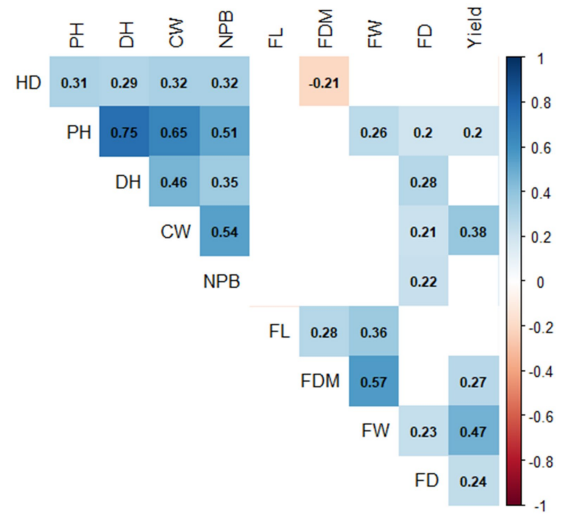

FIGURE 3
Correlation of agronomic characters in populations of cayenne F3 multiple crosses. (PH, plant height; DH, dichotomous height; CW, canopy width; SD, stem diameter; NPB, number of productive branches; FD, flowering days; HD, harvest days; FL, fruit length; FDM, fruit diameter; FW, fruit weight).

heritability, namely, 95.85%. The fruit weight character also had high heritability but was not stable at 177.27%, or 100%. In contrast, the canopy width character had a low. The narrow-sense heritability reached a negative value of −24.71 or can be considered with a value of (0).

Information from heritability and path analysis was considered in forming a selection index called the transgressive selection index (TSI). This TSI consisted of three selection criteria: yield, CW, and FW. These three criteria were weighted based on direct influence from path analysis with subjective heritability weights. The yield was given a subjective weight of 3, fruit weight of 2, and finally, canopy width was given a weight of 1. The combination of these two considerations formed an index formula:

$$\mathrm{TSI} = (3\times 1)\ \text{the yield} + (2\times 0.44)\ \text{fruit weight} + 0.46\ \text{canopy width}$$

Or

$$\mathrm{TSI} = 3\ \text{the yield} + 0.88\ \text{fruit weight} + 0.46\ \text{canopy width}. \quad (8)$$

The TSI results demonstrated that 41 lines had better index values than the best control varieties (Dewata F1 = 1.07) (Table 5). Among these lines, seven lines had an index value above 6. In addition, six lines have a transgressive selectivity ratio value above 1 for the yielding character and positive for other selection criteria. The 13 lines were continued for anthocyanin analysis in Table 6. Based on the table, G3.2.7 (34.09) and G2.6.9 (64.01) had higher anthocyanins than the best parent, Dewata F1 (25.07). In addition, lines G2.6.5 (16.54), G1.12.9 (22.59), G10.5.8 (12.53), and G5.12.1 (12.03) had anthocyanins under the control of Dewata F1 but were above 10. On the other hand, lines G3.1.5, G7.12.3, G6.5.10, G4.7.2, G7.12.2, G7.7.5, and G4.5.2 had low anthocyanins below a value of 10.

The results of the validation analysis are shown in Table 7 and Figure 4. Based on the table, G1-9-2-10, G10-9-6-11, G4-5-2-12, G5-12–1-8, G2-6-9-10, G3-2-7-3, G9-5-4-7, Bara and Dewata F1 were genotypes that had a positive transgressive segregant index value in validation test (F4 Population). G3-2-7-3 (8.80) and G4-5-2-12 (5.25) were genotypes whose index values were better than the check variety Dewata F1 (4.20). On the other hand, the genotype with the lowest index was the Ungara variety (−9.70). In addition to the transgressive segregation index, Table 7 also shows the anthocyanin content. Based on anthocyanin content, lines G1.9.2–10 (58.00), G10.5.8–5 (35.00), G10.9.6–11 (63.00), G3.2.7–3 (27.50), and G9.5.4–7 (63.50) were better lines than the Ungara variety (24.00). Apart from that, G1.9.2–10, G10.9.6–11, and G9.5.4–7 are varieties with higher anthocyanin content than Dewata. (56.00).

Figure 4 validation results showed that the 13 genotypes were divided into 4 quadrants. There were seven genotypes in quadrant I (F3-F4 positive). The second quadrant (F3 negative-F4 positive) and the third quadrant (F3-F4 negative) each comprised one genotype. Finally, the fourth quadrant (F3 positive-F4 negative) consisted of

TABLE 3 Path analysis of several agronomic characters on the yield.

Character	Direct effect	Indirect effect					Correlation
		PH	CW	FD	FDM	FW	
PH	−0.24*		0.30	0.02	0.01	0.11	0.22
CW	0.46**	−0.15		0.02	0.00	0.05	0.38
FD	0.09	−0.05	0.10		0.00	0.10	0.24
FDM	0.03	−0.04	0.02	0.01		0.25	0.27
FW	0.44**	−0.06	0.06	0.02	0.02		0.47
Total		−0.30	0.48	0.06	0.03	0.52	

PH, plant height; CW, canopy width; FD, flowering days; FDM, fruit diameter; FW, fruit weight; * is a significant effect at a 5% error to the yield variance; ** is a significant effect at a 1% error to the yield variance.

TABLE 4 Realized of narrow-sense heritability on F3 chili multiple-cross population.

Population	Statistic	Canopy width	Fruit weight	Yield
F2	Mean	55.19	1.18	117.88
	Var E	18.05	0.002	101.21
F2 selected	Mean	67.74	1.28	182.29
F3	Mean	51.73	1.40	182.04
	Var E	13.52	0.00	52.11
Selection differential (S)	Mean	26.74	20.70	57.98
Selection responds (R)	Mean	−6.61	36.70	55.57
h^2(ns) (R/Sx100%) (%)	%	−24.71 (0)	177.27 (100)	95.85

h^2(ns), narrow-sense heritability.

three genotypes. Meanwhile, the results of this mapping had a linear regression determination value of 0.44.

4 Discussion

The ANOVA results illustrate that almost all characters are influenced by the three sources of diversity. However, in augmented design-based plotting, the assessment of sources of diversity is more focused on the effect of line diversity and the diversity of differences between lines and controls (Fadhilah et al., 2022; Amas et al., 2023). This indicates that the line diversity functions as the locomotive or basis for selection. Without wide diversity, selection will stagnate for development (Litrico and Violle, 2015; Carena, 2021; Anshori et al., 2022). In addition, the difference in response between lines and controls is also an important indicator in mapping the potential of lines. So, the selected lines have better agronomic potential than commercial varieties in general (Fadhilah et al., 2022; Amas et al., 2023). Accounting on this basis, stem diameter and petiole length are two characteristics that do not meet these standards. This indicates that the two lines need to be more effective to be continued in analyzing selection criteria. Effective selection criteria are an important factor in the breeding process and correlate with the expected breeding goals (Marulanda et al., 2021; Rutkoski et al., 2022). In cultivated plants, productivity is the principal character in the evaluation. However, this character is strongly influenced by multiple production component factors; hence, production components are often included with productivity to enhance selection effectiveness (Kassahun et al., 2013; Fellahi et al., 2018; Brinton and Uau, 2019). One of the basic steps in estimating selection criteria is the significant influence of diversity on both sources of diversity (Anshori et al., 2022; Fadhilah et al., 2022; Amas et al., 2023). Therefore, all agronomic characteristics of the F3 cayenne population can be utilized as candidate selection criteria, except stem diameter and fruit stalk length.

Determination of the selection criteria to strengthen the yield can be done with correlation and path analysis. Correlation analysis can distinguish the relationship between one character and another (Oladosu et al., 2018; Sahid et al., 2020; Saleh et al., 2020; Khan et al., 2022; Uhlarik et al., 2022). However, this relationship is still considered rough due to the interference of other characters that potentially influence the correlation value (Anshori et al., 2022; Khan et al., 2022). Correction of the role of other characters can be conducted with path analysis. This analysis can separate the independent influence of a character on other characters in influencing a certain main character. This independent influence is expressed as a direct effect (Saleh et al., 2020; Khan et al., 2022; Tilahun et al., 2022). However, the implementation of path analysis with multiple character values is less effective; hence, it requires prior reduction with correlation analysis (Fadhilah et al., 2022). With this context, a combination of correlation and path analysis can be the solution to distinguishing the selection criteria. Several studies have also reported this effectiveness on chili (Tilahun et al., 2022; Amas et al., 2023; Lestari et al., 2023), rice (Alsabah et al., 2019; Saleh et al., 2020), and tomatoes (Fadhilah et al., 2022).

Based on these two analyses, characters, namely, canopy width and fruit weight, can be effectively applied as selection criteria along with the yield. The canopy width is identical to the vegetative properties of fruit chili (Virga et al., 2020; Gupta et al., 2022; Naves et al., 2022). The similarity can also be attributed to the significant positive correlation between canopy width characters and other vegetative characters. Reports of a strong correlation between the canopy width character on chili productivity and the characteristics of other production components also support the correlation results (da Silva et al., 2016; Virga et al., 2020; Arain and Sial, 2022; Bedjaoui et al., 2022; Gupta et al., 2022). Fruit weight is a generative character methodically conjugated with chili yield. This relationship has also been widely reported by Sahid et al., 2020; Tripodi et al., 2021; Amas et al., 2023. This character is also interlinked with the character of other generative result components, unlike its disassociation with canopy width. This indicates that the two characters have distinct roles in supporting the yield. The combination of uncorrelated criteria will make the selection more stringent (Acquaah, 2012). Hence, the combination of the three characters makes the selection more comprehensive, especially in the

TABLE 5 Transgressive Segregation Index on the F3 population of multiple-cross cayenne pepper.

Rank	Lines	Real value			z-value			Ratio TS			Index
		CW	FW	Yield	CW	FW	Yield	CW	FW	Yield	
1	G3-2-7-3	49.04	1.75	209.67	−0.24	102.25	36.12	−0.07	4.96	8.89	31.00
2	G3-1-5-11	48.92	1.15	204.71	−0.51	−23.76	22.16	−0.14	−1.15	5.45	15.29
3	G2-6-9-10	72.97	1.92	241.32	11.90	13.92	11.81	3.25	0.67	2.91	10.81
4	G2-6-5-10	75.63	1.85	219.58	3.84	29.24	8.58	1.05	1.42	2.11	8.06
5	G7-12-3-13	52.97	1.87	224.76	0.41	22.68	9.12	0.11	1.10	2.24	7.75
6	G6-5-10-8	46.46	1.28	199.66	−0.64	−0.77	9.23	−0.18	−0.04	2.27	6.70
7	G1-12-9-8	55.43	1.70	208.18	2.63	13.65	7.79	0.72	0.66	1.92	6.67
8	G4-7-2-8	93.17	1.73	224.22	4.91	2.44	6.62	1.34	0.12	1.63	5.61
9	G9-5-4-7	50.99	1.12	212.86	0.16	−44.80	10.05	0.04	−2.17	2.47	5.53
10	G1-9-2-10	62.59	1.19	215.08	2.76	−8.69	7.51	0.76	−0.42	1.85	5.52
11	G1-7-1-7	56.96	1.19	213.47	3.05	−23.58	7.84	0.83	−1.14	1.93	5.17
12	G2-6-10-10	49.04	1.33	203.26	−0.22	−0.50	6.23	−0.06	−0.02	1.53	4.55
13	G5-12–1-8	62.14	1.67	204.53	4.12	3.08	5.17	1.13	0.15	1.27	4.46
14	G10-5-8-7	68.97	1.55	208.40	3.63	0.80	5.38	0.99	0.04	1.32	4.46
15	G7-12-2-8	76.72	1.55	223.81	3.91	0.75	5.30	1.07	0.04	1.30	4.43
16	G5-5-8-7	51.39	1.13	214.24	0.18	−13.70	6.73	0.05	−0.66	1.66	4.41
17	G10-9-6-11	69.71	1.32	196.59	6.08	−0.50	4.59	1.66	−0.02	1.13	4.13
18	G7-7.5–7	60.97	1.46	215.41	1.72	0.30	4.86	0.47	0.01	1.20	3.82
19	G9-6-1-8	61.19	1.16	206.86	6.44	−12.37	4.74	1.76	−0.60	1.17	3.78
20	G4-5-2-12	57.15	1.41	199.77	1.61	0.03	4.77	0.44	0.00	1.17	3.73
21	G1-7-8-13	42.41	1.65	213.28	−3.56	2.03	5.01	−0.97	0.10	1.23	3.34
22	G6-8-7-8	58.97	1.97	238.75	2.59	5.29	3.73	0.71	0.26	0.92	3.30
23	G7-3-8-8	49.63	1.58	206.50	−0.44	1.33	4.07	−0.12	0.06	1.00	3.00
24	G2-11–5-8	60.99	1.14	197.89	2.85	−15.81	4.33	0.78	−0.77	1.07	2.89
25	G7-12-5-10	56.62	1.27	203.95	1.85	−1.86	3.45	0.51	−0.09	0.85	2.70
26	G5-7-4-11	42.60	1.83	205.50	−1.31	13.66	2.54	−0.36	0.66	0.63	2.30
27	G2-1-10-9	69.13	1.79	208.55	7.65	2.76	1.21	2.09	0.13	0.30	1.97
28	G5-7-1-6	46.43	1.36	198.11	−1.06	−0.22	2.70	−0.29	−0.01	0.66	1.85
29	G9-5-1-5	66.38	1.31	195.85	5.95	−0.41	1.47	1.63	−0.02	0.36	1.82
30	G7-12–10-7	51.68	1.36	197.72	0.33	−0.23	2.28	0.09	−0.01	0.56	1.72
31	G8-3-9-14	52.25	1.76	209.40	0.30	3.47	1.97	0.08	0.17	0.48	1.64
32	G10-9-1-12	72.86	1.43	195.88	3.73	0.19	1.52	1.02	0.01	0.37	1.60
33	G4-11–1-13	51.68	1.80	210.92	0.16	3.84	1.64	0.04	0.19	0.40	1.39
34	G4-7-4-11	37.64	1.86	220.09	−1.72	6.52	1.79	−0.47	0.32	0.44	1.38
35	G1-9-5-11	61.47	1.70	200.86	3.41	2.77	1.03	0.93	0.13	0.25	1.31
36	G9-1-7-13	51.25	1.77	205.47	0.12	3.93	1.52	0.03	0.19	0.37	1.30
37	G10-7-1-1	51.65	1.78	227.93	0.19	2.08	1.56	0.05	0.10	0.38	1.26
38	G1-7-10-7	23.97	1.23	193.17	−5.38	−17.33	3.56	−1.47	−0.84	0.88	1.21
39	G1.12–3-7	41.12	1.06	199.30	−1.19	−16.49	2.73	−0.33	−0.80	0.67	1.16
40	G10-7-5-11	68.71	1.67	194.48	3.46	2.83	0.82	0.95	0.14	0.20	1.16
41	G9-12-9-11	47.23	1.01	184.64	−0.74	−14.25	2.42	−0.20	−0.69	0.59	1.08
42	Dewata F1	40.00	1.12	232.82	−3.66	−34.87	4.06	−1.00	−1.69	1.00	1.05
105	Bara	38.65	1.11	173.38	−5.71	−46.36	−0.69	−1.56	−2.25	−0.17	−3.21
112	Ungara	24.00	1.76	95.46	−12.99	8.04	−9.65	−3.55	0.39	−2.38	−8.42
113	Katokkon	28.63	2.47	78.74	−5.19	20.63	−13.30	−1.42	1.00	−3.27	−9.59

CW, canopy width; FW, fruit weight; TS, transgressive segregation.

TABLE 6 Transgressive segregation of F3 cayenne pepper with potential anthocyanins.

Rank	Lines	Index	Anthocyanin (mg L^{-1})	Category
1	G3-2-7-3	31.00	34.09	Transgressive segregant and high anthocyanin
2	G3-1-5-11	15.29	0.00	Transgressive segregant
3	G2-6-9-10	10.81	64.01	Transgressive segregant and high anthocyanin
4	G2-6-5-10	8.06	16.54	Transgressive segregant and moderate anthocyanin
5	G7-12-3-13	7.75	3.01	Transgressive segregant
6	G6-5-10-8	6.70	0.00	Transgressive segregant
7	G1-12-9-8	6.67	22.59	Transgressive segregant and moderate anthocyanin
8	G4-7-2-8	5.61	7.14	Transgressive segregant
13	G5-12–1-8	4.46	12.53	segregant transgressive and moderate anthocyanin
14	G10-5-8-7	4.46	12.03	segregant transgressive and moderate anthocyanin
15	G7-12-2-8	4.43	0.00	Transgressive segregant
18	G7-7-5-7	3.82	0.00	Transgressive segregant
20	G4-5-2-12	3.73	3.87	Transgressive segregant
42	Dewata F1	1.05	25.07	check variety

TABLE 7 Validation of transgressive segregation index and anthocyanin content in the F4 population.

Genotype	Means			Variance			*z*-value			F4 TSI	AC (mg L^{-1})
	CW	FW	Yield	CW	FW	Yield	CW	FW	Yield		
G1-9-2-10	54.01	1.43	123.41	72.00	0.064	18.09	−0.61	0.98	0.09	0.85	58.00
G10-5-8-7	39.53	1.59	156.5	91.93	0.189	297.68	−5.86	0.34	0.52	−0.84	35.00
G10-9-6-11	51.08	1.22	134.52	50.12	0.039	186.22	−2.19	1.94	0.24	1.42	63.00
G4-5-2-12	60.78	1.5	159.91	238.17	0.120	50.28	1.21	0.59	1.39	5.25	0.00
G5-12–1-8	48.67	2.04	149.96	77.80	0.080	164.17	−2.72	−0.51	0.57	0.01	8.00
G5-5-8-7	50.79	1.64	123.5	62.81	0.072	37.60	−2.08	0.42	0.07	−0.39	7.00
G1-7-1-7	48.40	1.37	120.01	38.79	0.084	113.13	−4.00	0.99	−0.05	−1.11	11.00
G2-6-9-10	55.73	1.10	124.42	9.35	0.100	70.06	0.28	1.45	0.08	1.64	0.00
G3-2-7-3	56.08	1.30	164.69	39.45	0.052	20.46	0.33	1.44	2.46	8.80	27.50
G9-5-4-7	49.92	1.45	134.14	9.00	0.030	15.44	−6.53	1.35	0.81	0.60	63.50
Bara	56.79	1.25	144.24	21.23	0.031	122.13	1.00	2.07	0.52	3.85	0.00
Dewata	57.14	1.46	159.53	69.28	0.054	95.66	0.70	1.00	1.00	4.20	56.00
Ungara	44.19	1.29	80.01	20.06	0.030	19.93	−8.87	1.96	−2.45	−9.70	24.00
μ populations	55.48	1.82	121.96								

CW, canopy width; FW, fruit weight; TS, transgressive segregation; TSI, transgressive segregation index; AC, anthocyanin content; μ, means.

formation of the selection index (Anshori et al., 2021, 2022). However, establishing a selection index also requires consideration of the genetic role of the three characters (Reddy and Jabeen, 2016; Farid et al., 2022; Amas et al., 2023). This is crucial in predicting the response of the next generation to selected genotypes. One of the effective genetic parameters in F3 selection is the narrow-sense heritability.

Narrow-sense heritability is a direct approach that entails a comparison of selection responses and selection differentials (Acquaah, 2012; Lstibůrek et al., 2018). This concept is considered effective in evaluating the stability of the selection response of a character from two different generations with distinct environmental influences (Acquaah, 2012; Lstibůrek et al., 2018; Farid et al., 2022). The effectiveness of this concept was also reported by Farid et al. (2022) on tomato F3 populations. Following this narrow-sense heritability, the yielding principle is a character that is considered relatively stable in comparison with the other two characters. This demonstrates that the heritability value of the yield is high, exceeding 50%, and is still in the rational range of 0–100 (Acquaah, 2012). Contrastingly, the other two characters are outside the optimal range of heritability. However, the fruit weight character has a high heritability in comparison to the canopy width, thereby prioritizing the fruit weight character in comparison with the canopy width character. These results entail good consideration when constructing a selection index, especially when assessing the transgressive segregation of cayenne pepper in a multiple-cross F3 population.

Forming a transgressive segregated selection index has an auxiliary approach to the selection index in general. This selection index is approximated by the value of the transgressive segregation ratio (Koide et al., 2019). This ratio refers to the concept of transgressive segregation itself, where the assessment is based on the

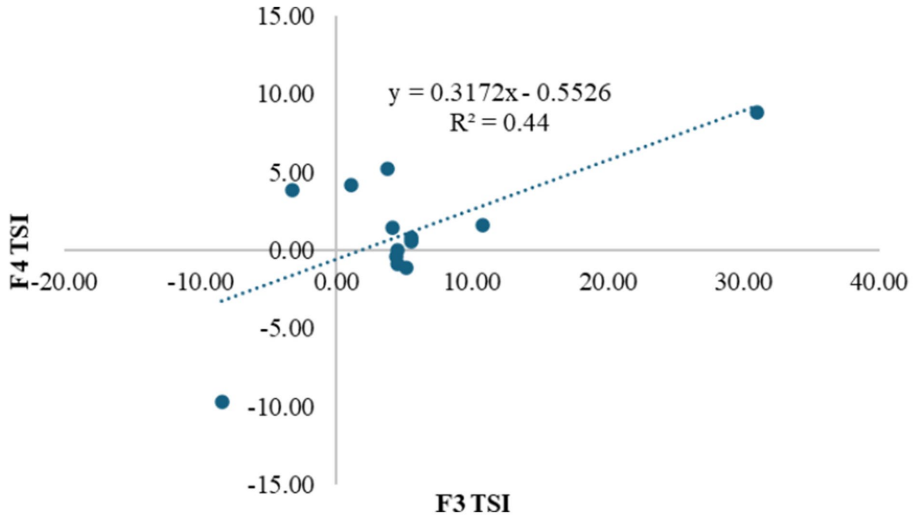

FIGURE 4
Interaction of F3 transgressive segregation index to its validation in the F4 population.

best mean and lowest variance of a strain against the best comparison parents (Alimi et al., 2013; de los Reyes, 2019; Koide et al., 2019; Pabuayon et al., 2021). However, the assessment of more than one character requires standardized comparisons. So, the overall value of the selection criteria is converted to a z-value or selection intensity utilizing the standard deviation of each of these genotypes (Acquaah, 2012; Syukur et al., 2015; Shwetha et al., 2022). This allows the z-value to distinguish the characteristics of variance and the median values of the lines to parents and to integrate the potential of the lines for each selection criterion (Feng et al., 2020; Chung and Liao, 2022; Farid et al., 2022; Shwetha et al., 2022). Therefore, the z-value ratio approach between lines and parents is innovative in the selection concept, especially for the accumulative transgressive segregation potential.

The formation of the selection index is also inextricable from the weighting system for each character (Chung and Liao, 2022). The weighting system can be carried out subjectively, objectively, and semi-objectively (Alsabah et al., 2019; Chung and Liao, 2022; Fadhilah et al., 2022; Farid et al., 2022). This is following the objectives and consideration of various factors in the selection (Chung and Liao, 2022; Farid et al., 2022). Established on the considerations in this study, the weighting system can be carried out semi-objectively. This is derived from considerations that can be objectively and subjectively together. The direct influence of the path analysis can be the basis for weighting the selection criteria. The principal selection criteria are assessed with a weight of 1, while the supporting selection criteria are given a weight according to their direct influence. Sabouri et al. (2008) and Fadhilah et al. (2022) have also reported this hypothesis. However, the weighting cannot elucidate the role of genetics in the index. Hence, the consideration of genetic factors, such as narrow-sense heritability, is imperative for harmonization (Acquaah, 2012; Lstibůrek et al., 2018; Farid et al., 2022). However, the narrow-sense heritability in this study is still considered to be overestimated so that heritability assessment can be done subjectively (Farid et al., 2022). The yield, as the predominant character constituting high heritability, is rationally given more priority than fruit weight and canopy width, so the weight is multiplied by 3. The fruit weight character is also a character with high heritability. However, this character has a heritability value above its rational value; hence, the weight of this character is multiplied by 2. Meanwhile, the canopy width character with low heritability is not multiplied.

The selection results established on the transgressive segregation selection index demonstrated that 41 lines were better than the index value of the best parents. This is the basis for determining lines that can be continued in the F4 generation. The concept of comparison with the best parents has also been reported by Suwarno Lubis et al. (2009), Fadhilah et al. (2022), Anshori et al. (2022), and Farid et al. (2022). However, determining the reality of transgressive segregation is predicated on two criteria. First, these lines have an index value above 6. This is achieved by the minimum optimization for each transgressive segregation ratio, which is 1, so the minimum TSI from transgressive segregation accumulation is 6. The second criterion is that these lines have an index value below 6. Still, the transgressive segregation ratio is above 1 for the yielding character and positive for fruit weight and canopy width criteria. This criterion tolerates the process, so the transgressive selection does not become too stringent. In addition, this criterion considers the minimum potential of a line transgressive to the main character by considering the stability of adaptation to other selection criteria. Based on these two criteria, G3.2.7, G3.1.5, G2.6.9, G2.6.5, G7.12.3, G6.5.10, G1.12.9, G4.7.2, G10.5.8, G5.12.1, G7.12.2, G7.7.5, and G4.5.2 are recommended as F3 transgressive segregated lines in cayenne pepper.

Anthocyanin levels identified the 13 transgressive segregated lines. Several lines have anthocyanin responses, categorized as high or moderate. This category also refers to the best comparison principle owned by Dewata F1. This concept was also introduced by Arnnok et al. (2012) On *Capsicum annum* L. Accounting for this, lines G3.2.7, G2.6.9, G2.6.5, G1.12.9, G10.5.8, and G5.12.1 are recommended as lines with high productivity potential and superior anthocyanins. The six lines require validation for the stability of the anthocyanin content in the next generation or at the preliminary yield test stage. The stability of the yield and anthocyanins will be a desirable novelty to be released into functional cayenne pepper varieties with high anthocyanins.

The validation results showed an excellent TSI value between F3 and F4. This was indicated by the percentage of genotypes with consistent TSI between F3 and F4. In addition to that, inconsistent genotypes have small negative values in their F4. This inconsistency

is also caused by the influence of different agroclimates on the cultivation of the F3 population and its validation (F4). According to Widowati et al. (2023), differences in climatological parameters significantly influence the response of genotypes. This difference was also reported by Sayekti et al. (2021) and Sahmat et al. (2024), which show drastic differences in response between chili genotypes to differences in environmental and climatological factors. This indicates that the TSI value can still be used as a good reference in assessing the potential for transgressive segregants, especially for segregants with high TSI values in the F3 population. It was reflected in the genotypes G3-2-7-3, G2.6.9–10, G5-12–1-8, and G4.5.2–12, which consistently have positive TSI values in their F4. Apart from that, genotype G3-2-7-3 also has good anthocyanin potential, along with G10.5.8–5. These two genotypes also have consistent anthocyanin content in F3 and F4. However, this concept still needs to be developed by linking the concepts of other approaches so that a selection formulation with a higher determination value is formed. In addition, the anthocyanin content also needs to be analyzed precisely, such as metabolomics, proteomics, transcriptomics, and genomics. Nevertheless, based on the overall validation results, the TSI approach can still be used as a reference in assessing the potential of transgressive segregants.

5 Conclusion

This study demonstrates the effectiveness of developing a semi-objective-based selection index as an innovative methodology in segregated transgressive F3 chili pepper selection. Fruit weight and canopy width are recommended for selection criteria, along with the yield. The transgressive selection index formed in this study was 3 yield +0.88 fruit weight + 0.46 canopy width. This index is considered adequate based on its validation in predicting transgressive segregants. The recommended lines as transgressive segregants with high productivity were G3-2-7-3, G2.6.9–10, G5-12–1-8, and G4.5.2–12. Meanwhile, line G3-2-7-3 was recommended as a line with high productivity potential and superior anthocyanins. However, this concept still needs to be developed by linking the concepts of other approaches so that a selection formulation with a higher determination value is formed. In addition, the anthocyanin content also needs to be analyzed precisely, like the omics concept. Nevertheless, these segregant lines can still be recommended to proceed to the yield testing stage or be crossed to form hybrid lines with high yield potential and anthocyanin content.

5.1 Resource identification initiative

The project uses STAR 2.0.1 from IRRI, Rstudio with the corrplot package, and the Excel Office 2016 version. For lines resources from multiple cross-generations.

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 in the article/supplementary material.

Author contributions

MA: Writing – original draft, Visualization, Validation, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. YM: Writing – review & editing, Supervision, Resources, Conceptualization. ND: Writing – original draft, Conceptualization. NW: Writing – original draft, Investigation, Conceptualization. AA: Writing – original draft, Investigation, Conceptualization. AM: Writing – original draft, Conceptualization. FF: Writing – original draft, Investigation. MF: Writing – review & editing, Supervision, Resources. AD: Writing – review & editing, Supervision, Data curation. AN: Writing – original draft, Investigation.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia through the scheme of Penelitian Fundamental Kolaboratif UNHAS (UNHAS Fundamental Research Collaborative) with grant Number: 00323/UN4.22/PT.01.03/2023.

Acknowledgments

The authors are grateful to the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia through the UNHAS Fundamental Research Collaborative scheme. Furthermore, we thank publication management center of Hasanuddin University for providing facilities, such as training workshops and Enago proofreading for this article.

Conflict of interest

The authors declare that the research was conducted without 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/fsufs.2024.1288579/full#supplementary-material

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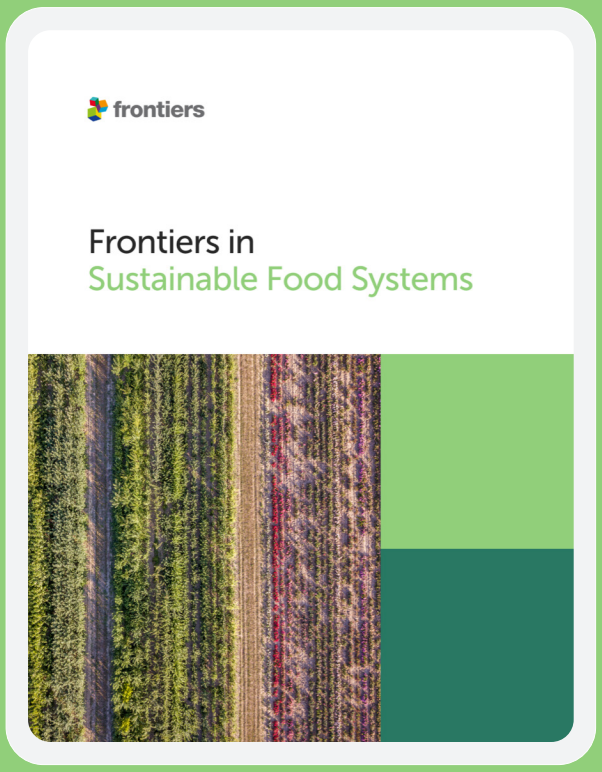

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