



OPEN ACCESS

EDITED BY

Ravinder Kumar,
Indian Agricultural Research Institute
(ICAR), India

REVIEWED BY

Junior Bernardo Molina Hernandez,
University of Bologna, Italy
Guadalupe Gutiérrez-Soto,
Universidad Autonoma de Nuevo, Mexico

*CORRESPONDENCE

Ziran Ye

✉ yezr@zaas.ac.cn

Dedong Kong

✉ kongdd@zaas.ac.cn

RECEIVED 27 May 2024

ACCEPTED 22 July 2024

PUBLISHED 05 August 2024

CITATION

Dai M, Tan X, Chen X, Cai K, Zhong Y, Ye Z and
Kong D (2024) Green control for inhibiting
Rhizopus oryzae growth by stress factors in
forage grass factory.
Front. Microbiol. 15:1437799.
doi: 10.3389/fmicb.2024.1437799

COPYRIGHT

© 2024 Dai, Tan, Chen, Cai, Zhong, Ye and
Kong. This is an open-access article
distributed under the terms of the [Creative
Commons Attribution License \(CC BY\)](#). The
use, distribution or reproduction in other
forums is permitted, provided the original
author(s) and the copyright owner(s) are
credited and that the original publication in
this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Green control for inhibiting *Rhizopus oryzae* growth by stress factors in forage grass factory

Mengdi Dai¹, Xiangfeng Tan¹, Xuting Chen¹, Kangfeng Cai²,
Yuanxiang Zhong¹, Ziran Ye^{1,2*} and Dedong Kong^{1*}

¹Institute of Digital Agriculture, Zhejiang Academy of Agricultural Sciences, Hangzhou, China, ²Key
Laboratory of Digital Dry Land Crops of Zhejiang Province, Hangzhou, China

The forage grass factory could break through the restrictions of land resources, region and climate to achieve efficient production throughout the year by accurate and intelligent management. However, due to its closed environment, mold outbreaks in the forage grass factory were severe, significantly affecting barley production. In this study, 9 contaminated barley tissues were collected and 45 strains were isolated in forage grass factory. After ITS sequencing, 45 strains were all identified as *Rhizopus oryzae*. Through stress factor assays, *R. oryzae* growth was seriously hindered by low concentration of sodium nitrate, high pH value and ozone water treatment. High pH and ozone water affected growth mainly by altering membrane integrity of *R. oryzae*. Sodium nitrate inhibited the growth of *R. oryzae* mainly by affecting the amount of sporulation. Low concentration of sodium nitrate and ozone water did not affect the growth of barley. High concentrations of sodium nitrate (100 mM) and pH values (8–8.5) inhibited barley growth. Among them, ozone water had the most obvious inhibition effect on *R. oryzae*. Large-scale ozone water treatment in the forage grass factory had also played a role in restoring barley production. Taken together, the green technology to control mold disease and maintain the safety of forage through different physicochemical methods was selected, which was of considerable application value in animal husbandry.

KEYWORDS

barley, *Rhizopus oryzae*, forage grass factory, stress factor, green control

1 Introduction

The plant factory mainly realizes precise management through intelligent control of various physical and chemical factors such as temperature, humidity, light, and nutrient supply. The factory system enables the production of vegetables throughout the year and improve the productivity and quality of plants, so as to further solve the problems of shortage or instability of vegetables and food supply, resource efficiency, and environmental impact of agricultural activities (Kozai and Niu, 2016; Kozai, 2018). The forage grass factory adopts the design concept of plant factory. Based on the growth and development characteristics of forage grass, it creates a growth environment suitable for soilless cultivation of forage grass through material exchange and environmental regulation of closed system, so as to achieve the purpose of rapid growth, high quality, high yield, and maximum economic benefit of forage grass.

The factory model are especially suitable for arid, semi-arid, non-cultivated land, and alpine areas. The seedlings produced by the forage grass factory have a short production cycle (6–10 d per crop), high planting density, and high yield. At present, forage grass species such as barley, corn, alfalfa, and ryegrass have been produced on a large scale in forage grass factories, but the latter two mainly produce seedling vegetables for human consumption, and do not involve the forage grass function of herbivores (Lee et al., 2010; Fiutak et al., 2019). Among them, barley forage as a kind of high-quality forage grass, has soft and juicy taste, rich nutrition, good palatability of cattle and sheep, rich in vitamins, amino acids and other nutrients, and also contains crude fiber, which is particularly important for maintaining the health of ruminant livestock, and cannot be replaced by grains and other feeds (Fazaeli et al., 2012). Moreover, its digestibility of up to 90% greatly increases the meat production of cattle and sheep (Devendar et al., 2020; Khaziev et al., 2021). It has been used as the main source in forage grass factories for a long time.

In the production process of barley, its performance and nutritional quality are closely related to environmental factors such as cultivation temperature, moisture, air, light, and nutrients (Al-Karaki and Al-Hashimi, 2011; Fazaelil et al., 2011; Naik et al., 2015). The environment with sufficient moisture and warmer cultivation temperature is more suitable for the growth of barley (Podar, 2013). However, such a closed environment is also prone to the growth of microorganisms, especially mold (Grunert et al., 2020). The water and nutrients of the hydroponic system are suitable for the survival and rapid reproduction of microorganisms, and the close proximity and sharing of nutrient solutions will enable the rapid spread of plant diseases (Kanjanamaneesathian, 2015). Due to the large surface and liquid/air boundary, bacteria, and fungi can grow rapidly, causing plant root disease (Dankwa et al., 2020).

Researchers are increasingly realized the implications of microbial development in dairy feed ingredients and feed. The development of mold and other strains in these rations affects not only the nutritional makeup of the food but also its palatability since they produce heat and some mold species produce toxic substances that impair animal health (Mostrom and Jacobsen, 2020). At present, the common pathogens in hydroponic barley include *Pythium*, *Fusarium*, *Rhizopus* and *Phytophthora* (Cao et al., 2022). The main means of control are to prevent the growth of pathogens by regularly cleaning and disinfecting equipment, controlling water temperature, using some beneficial bacteria or fungi products, and some chemical products (Dankwa et al., 2020). However, these methods can not completely remove the contamination of pathogens, and at the same time, whether the use of some agents has side effects on herbivores has not been confirmed.

Rhizopus oryzae is a well-studied fungus that is frequently used to manufacture some traditional oriental dishes. It is mostly known for being an excellent source of lactic acid (Londoño-Hernández et al., 2017). Additionally, the pathogen is the main cause of mucormycosis, a newly discovered, potentially fatal illness with a growth rate above 50% that is characterized by rapid angioinvasive development (Ma et al., 2009). According to previous reports, *R. oryzae* caused “*Rhizopus* rot” on a variety of plants, including apple, banana, mulberry, sweet potato, and tomato (Kwon et al., 2012;

Wang et al., 2017; Khokhar et al., 2019; Gnanesh et al., 2021). Infection by *R. oryzae* causes the infected site to become soft and decayed. And white mycelium grows around the infected area and had an appearance of cobwebs (Kwon et al., 2015). The biological traits of *R. oryzae* have been the subject of numerous investigations. It can withstand temperatures between 25°C to 45°C, with 38°C being its ideal range (Chen et al., 2021). *R. oryzae* growth is influenced by carbons such ribitol, D-arabitol, and β -cyclodextrin (Wang et al., 2018), as well as pH (Chen et al., 2021).

In our study, we first reported that *R. oryzae* infected the roots of barley in forage grass factories and reduced barley yield. Through a variety of physical and chemical methods, a green control was summarized to inhibit the growth of *R. oryzae* in plant factories without affecting barley development.

2 Materials and methods

2.1 The growth condition of plant materials

The barley cultivars (*Hordeum vulgare* L.) were grown in an artificial climate chamber and forage grass factory. In artificial climate chamber, barley were grown in culture bottles. And the climate chamber was composed of three microclimate chambers and a management system that controlled the temperature, relative humidity, and LED illumination. Its dimensions were 1,130 mm (L), 795 mm (W), and 1,920 mm (H). The air temperature was set as $25 \pm 2^\circ\text{C}$ with a relative humidity of 85%. The forage grass factory is a 20 inch [6,000 mm (L)*2,400 mm (W)*2,450 mm(H)] container placed in Infite Industrial Park, Hangzhou City, China. The factory was equipped with an environmental control system to control temperature, humidity and carbon dioxide; liquid circulation system to achieve 6–8 times a day spray; automatic control equipment to adjust temperature and humidity, carbon dioxide concentration, and liquid pH value in real time. At the same time, the factory was equipped with two sets of three-layer planting racks [4,000 mm (L)*460 (W) mm*2,190 mm (H)] and 24 plant growth lights, which could achieve multiple rounds of barleys. The air temperature was set as $25 \pm 2^\circ\text{C}$ with a relative humidity of 88%, carbon dioxide concentration of 480 ± 10 ppm in the forage grass factory.

2.2 Isolation and cultivation of strains

The infected barley plants in the forage grass factory were randomly selected and isolated by conventional tissue isolation method (Lozada, 1995). Briefly, the roots of infected barley plants were cut, and tissues of 5 mm \times 5 mm were cut at the junction of diseased and healthy, immersed in 75% alcohol solution for disinfection for 10 s, then put into 3% sodium hypochlorite solution for disinfection for 45 s, washed with sterilized water for 3 times, and cultured on potato dextrose agar (PDA) medium under dark conditions at 25°C. The mycelia on the edge of the colony were selected and transferred to a new PDA plate for purification, and then placed on the PDA slope at 4°C for store.

2.3 Total DNA extraction and identification

Genomic DNA was extracted according to the DNA extraction kit (Tiangen, Shanghai). The internal transcribed spacer (ITS) genes was amplified for identification (Zhu et al., 2022). Primers were listed in [Supplementary Table S1](#). PCR amplification was conducted as described by the method of Zhang et al. (2011). PCR products were sequenced using ABI3730 (Tsingke, Beijing), and the sequencing results were compared with the BLAST sequence. Mega version X was used for the construction of phylogenetic trees.

2.4 Stress assay on *R. oryzae*

The identified fungi *R. oryzae* strain was cultured on potato dextrose agar (PDA, Difco, Detroit, MI, USA) at 25°C in the dark. Fresh hyphae plug was picked up from the edge of *R. oryzae* cultured for 2 d with a diameter of 5 mm stopperborer, and inoculated on the 7 cm medium with different conditions. Under dark conditions, the strain was cultured at 25°C for 1 day, and the colony diameter was measured. The stress medium is as follows (Table 1):

For different carbon sources stress assay, using the basic medium MM (1% glucose) as blank control, the contents of glucose in MM medium were replaced with maltose, fructose, sucrose, mannose, and lactose, respectively.

For different pH stress assay, the pH value of MM medium were adjusted by hydrochloric acid and sodium hydroxide respectively.

For stress assays with different concentrations of nitrate, the concentrations of sodium nitrate in MM medium were replaced with 0.1 mM, 1 mM, 10 mM, and 100 mM concentrations.

For stress assays with different concentrations of ozone water stress, add 5 mL ozone water (the initial concentration is 10 ppm) to 20 mL MM medium (2 ppm), 2.5 mL ozone water to 22.5 mL MM medium (1 ppm), and 500 μ L ozone water to 25 mL medium (0.5 ppm), and pour it into the culture dishes.

For salt stress assays, 0.2 M sodium chloride, 0.3 M sodium chloride, 0.2 M potassium chloride, and 0.3 M potassium chloride were added to PDA medium, respectively.

For stress assays of different phosphorus, 5 mM lecithin, 50 mM lecithin, 5 mM potassium dihydrogen phosphate, and 50 mM potassium dihydrogen phosphate

were added to MM medium, respectively.

2.5 Phenotypic characterization of *R. oryzae* under stress

The strain was cultured on 7 cm culture dish containing 17.5 mL MM medium and stress factors (carbon sources stress, pH stress, different concentrations of nitrate stress, different concentrations of ozone water stress, salt stress, and different phosphorus stress) at 25°C in dark for 1 day. Fifty photos were taken for each treatments and subsequent statistical analysis was performed.

2.5.1 Effect on sporification

To measure sporulation and sporangium, 3 ml sterile water were added into the MM medium. The aerial hypha were scraped on the medium with a spreader, and 15 μ l fluid was absorbed to observe the sporangium production under a light microscope. The remaining fluid was filtered with three layers of filter paper, and the filtrate was centrifuged and 100 μ l sterile water was added to precipitate. The sporulation and sporangium were counted with a hemocytometer under light microscope.

2.5.2 Effect on mycelium reduction

To measure mycelium weight, different stress factors were added to MM liquid medium, and conventional MM medium was used as the control. Five fungal plugs (5 mm) were picked up by a stopperborer and inoculated in the liquid medium, The medium with fungal plugs were shaken at 25°C and 150 rpm/min for 5 d. The mycelia was filtered with three layers of sterile filter paper, and the filtered mycelia was dried with absorbent paper. The mycelium were weighed the fresh weight.

2.5.3 Effect on the integrity of the cell membrane

To measure the integrity of fungal mycelia membrane, mycelia cultured in MM liquid medium was rinsed 3 times with PBS buffer. 0.5 g mycelia was weighed and placed in a 50 mL centrifuge tube, and different stress factors were added respectively. After 2 h treatment, the solution was centrifuged at 5,000 rpm/min, and the solution without mycelia at the same concentration was used as a negative control. The absorption value of supernatant at 260 nm was detected.

2.6 Barley stress assay and co-culture

In artificial climate chamber, barley seeds were ground to remove capsules, then surface-sterilized in 75% ethanol for 5 mins and in 1.0% sodium hypochlorite solution (5% active chlorine) for 10 mins. The seeds' surface was then rinsed with sterile water. Ten sterilized seeds were planted in the 1/2 MS solid medium in a 9 cm culture dish. After 3–4 d, 8 seedlings were transferred

TABLE 1 Different stress assay on *R. oryzae*.

Stress factor	Different treatment
Carbon sources	Glucose, maltose, fructose, sucrose, mannose, and lactose
pH stress	pH = 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5
Nitrate stress	0.1 mM, 1 mM, 10 mM, and 100 mM
Ozone water stress	2 ppm, 1 ppm, 0.5 ppm
Salt stress	0.2 M sodium chloride, 0.3 M sodium chloride, 0.2 M potassium chloride, and 0.3 M potassium chloride
Phosphorus stress	5 mM lecithin, 50 mM lecithin, 5 mM potassium dihydrogen phosphate, and 50 mM potassium dihydrogen phosphate

into a sealed tissue culture bottle (the bottom diameter is 95 mm, the height is 185 mm, the capacity is 1,000 ml), which contained 1/2 MS solid medium. For stress assay, the 1/2 MS medium was supplemented stress factors as described above. For co-culture, each culture bottle was planted barley seedlings with three fungal plugs of *R. oryzae*. Each treatment had three tissue culture bottles, and each experiment was repeated three times. In forage grass factory, the barley seeds were rinsed in water to remove dented grains, and then soaked in sodium hypochlorite solution for 2 h. Then 50 g seeds were evenly scattered in the plastic square dish with holes in the bottom [135 mm (L)*83 mm (W)*53 mm (H)] and placed in the container for planting. After 4 d, the stress solution was prepared, and equal amounts were poured into the barley seedling tray for stress treatment. The stress treatment was carried out once a day until harvest.

2.7 Phenotype measurement on barley

After 7 d planting, phenotype measurement was conducted at a fixed time point. Five barley seedlings were randomly selected for each tissue culture bottle to measure the plant height. The plant height was the linear distance between the highest point of the canopy leaf and the medium. Since there is only 8 barleys in the tissue culture bottle and the weight is relatively light, the fresh weight is mainly determined in forage grass factory. The above-ground part of barley seedlings was cut and the fresh weight was measured.

2.8 Determination of physiological indexes

Plant leaves that were fully developed and in good health were chosen at random from each treatment for analysis. Three repeats of the study were conducted for every physiological index. The crude fat content of plants was determined by Soxhlet extraction method. The total starch content of plants was measured by acid hydrolysis method-DNS. The content of crude protein was determined by using sulfuric acid-catalyst digestion and Kjeldahl nitrogen determination method.

2.9 Statistical analysis

One-way ANOVA and independent-samples *t*-test were used to statistically analyze the data using SPSS (version 16.0, IBM, Chicago, IL, USA) software. The values demonstrated the mean \pm standard error.

3 Results

3.1 Discovery and identification of mold in forage grass factory

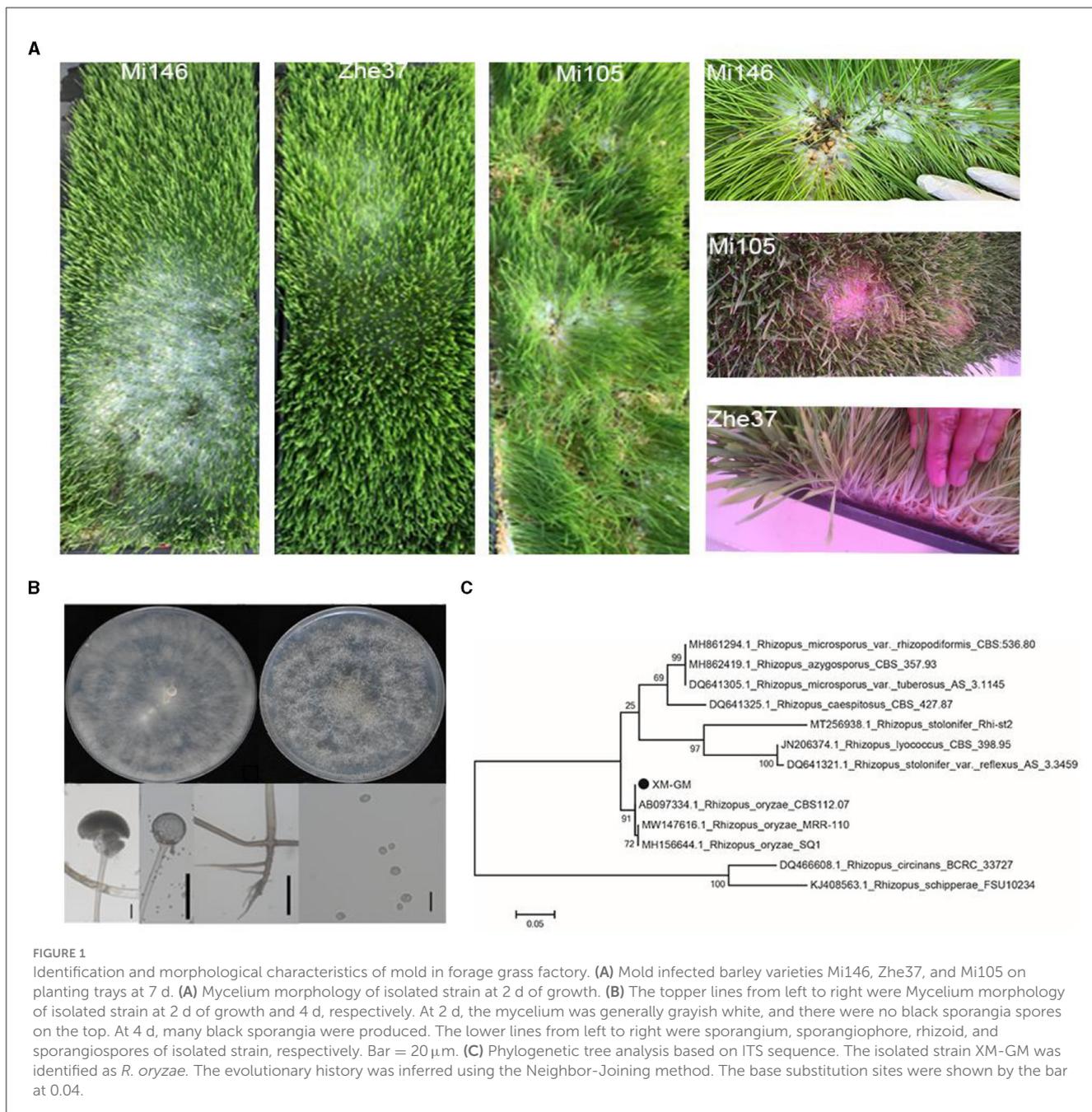
We planted 9 different varieties of barley seedlings in the forage grass factory. After growing under the same environmental

control condition for 7 d, we had a phenotypic observation and found that three barley varieties Mi146, Mi105, and Zhe37 had serious mold outbreaks. There is a large amount of white mycelium in the roots of barley, and the mycelium has black spots on the top. In severe cases, the white mycelium extends to the barley leaves (Figure 1A). We randomly selected ninety contaminated barley tissues to isolate the contaminated fungi and obtained 45 isolated strains. The morphological characteristics of the contaminated fungi were observed on the PDA medium. The results showed that the morphological characteristics of the 45 strains were the same species. The colony was initially white, then many black sporangia were produced. The sporangium was spherical or sub-spherical, with a diameter of $72.37 \pm 4.21 \mu\text{m}$ ($n = 50$). The rhizoid was dark brown and $4.37 \pm 1.05 \mu\text{m}$ wide ($n = 50$). The sporangiospores were non-septate, globose or sub-globose, and about $4.35 \pm 0.74 \mu\text{m}$ in diameter ($n = 50$) (Figure 1B). Five typical strains were selected for ITS sequence determination. By phylogenetic tree analysis, the isolated strain XM-GM and *Rhizopus oryzae* clustered into a clade, and the clade support was 91% (Figure 1C). In summary, the morphological characteristics of the barley seedling contaminated were observed, ITS sequence was determined, and phylogenetic tree analysis of ITS sequence was performed. The results showed that the contaminated fungi of barley seedling was *Rhizopus oryzae* of *Rhizopus*.

3.2 Impact of stress factors on *Rhizopus oryzae*

To observe the growth of *R. oryzae* under different stresses, we conducted plate experiments on the response of *R. oryzae* to stress factors, and found out the stress factors affecting the growth of *R. oryzae*. The results showed that the growth of *R. oryzae* was inhibited under alkaline condition (pH = 7–8.5) (Figure 2A). Mycelium weight and colony diameter decreased significantly when pH = 7–8.5 compared with acidic environment (Figures 2B, C). Under different nitrogen source conditions, it was found that *R. oryzae* was more able to utilize organic nitrogen than inorganic nitrogen source (Figure 2D). The colony diameter and fungal biomass of *R. oryzae* grew under 0.1 mM sodium nitrate stress were significantly lower than other nitrogen conditions (Figures 2E, F). Under different concentrations of ozone water, the growth of *R. oryzae* was significantly inhibited (Figure 2G). And the inhibition effect gradually decreased with the decrease of concentrations (Figures 2H, I). Under different carbon sources, *R. oryzae* could grow normally (Supplementary Figures S1A, B). Different concentration of salt stress factors and phosphorus culture conditions could promote the growth of *R. oryzae* (Supplementary Figures S1C–F).

To observe whether green biocontrol fungi could inhibit the growth of *R. oryzae*, the experiment was conducted between *R. oryzae* and *Piriformospora indica* and *Bacillus subtilis*. The diameter of *R. oryzae* was not significantly different from that of the control, indicating that green biocontrol fungi could not directly inhibit the growth of *R. oryzae* and did

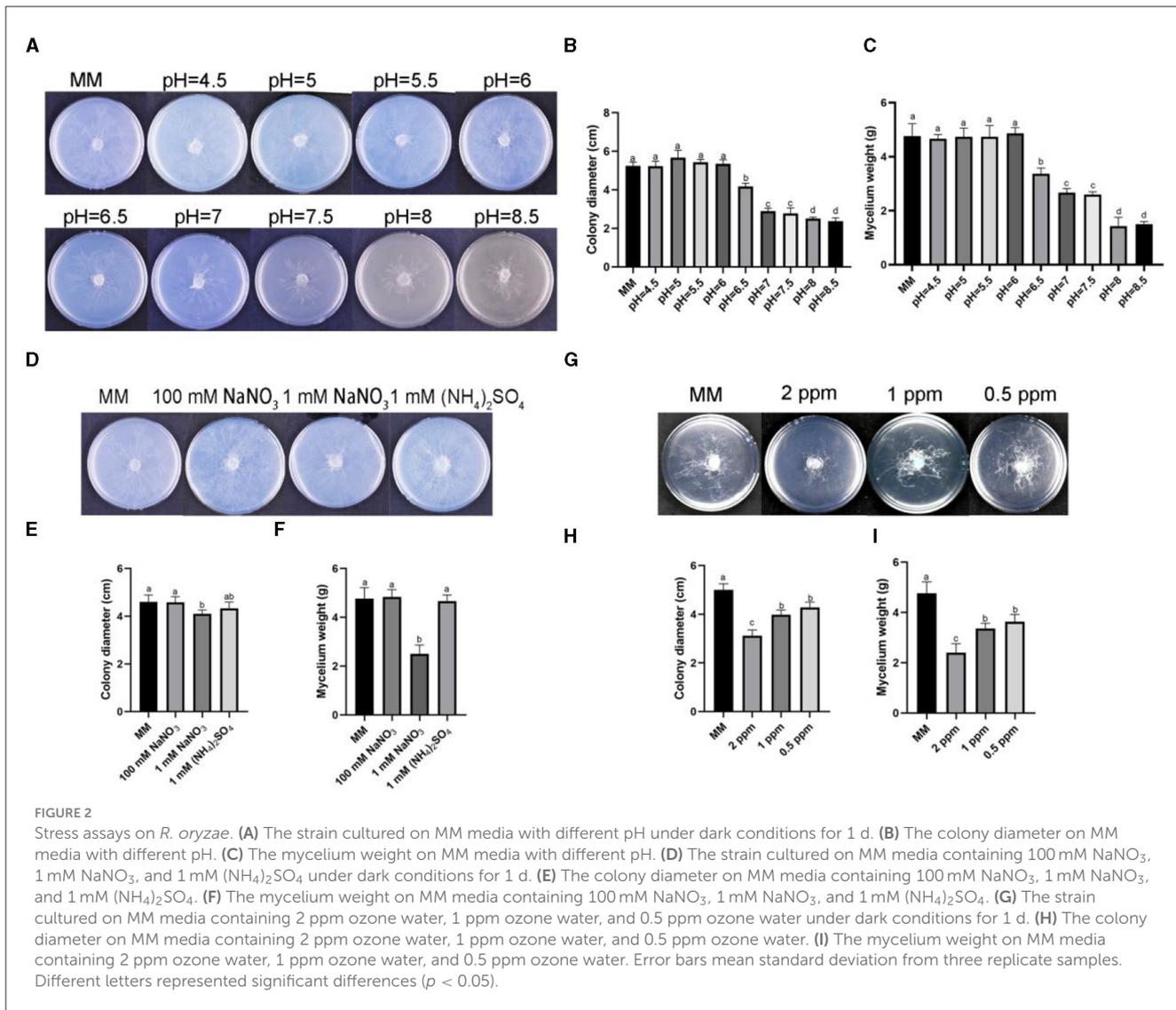


not secrete any antagonistic substances against *R. oryzae* (Supplementary Figure S2).

3.3 Mechanism of stress factors on *R. oryzae*

To clarify the mechanism of different stress conditions affecting the growth of *R. oryzae*, we observed the aerial hyphae and sporangium of *R. oryzae* growing on stress medium. The results showed that the aerial hyphae of *R. oryzae* growing under ozonated water stress was thinner than that of the control (Figure 3A). It

was found that ozone water could inhibit sporospore production. In 2 ppm ozone water treatment, its sporulation was only 1/3 of the control (Figure 3B). With the decrease of concentrations, the spore production also increased. Through observation of sporangium, it was found that *R. oryzae* grown on MM medium produced a large number of sporangium, while the number of sporangium of *R. oryzae* was significantly reduced under ozone water treatment, and the sporangium inhibition rate reached 77.38% under 2 ppm ozone water treatment (Figure 3C). When the concentrations decreased, the sporangium inhibition rate decreased, indicating that ozone water could significantly inhibit the production of sporangium in *R. oryzae*. By measuring the integrity of mycelium membrane, it was found that the absorption



value of the solution was increased by the treatment of ozone water, indicating the leakage of nucleic acid. However, the absorption value of ozone water without adding mycelium did not change with the change of concentrations, suggesting that this phenomenon was not caused by the presence of ozone water in the solution, but by the change of membrane integrity of *R. oryzae*. The results showed that ozone water could destroy the membrane structure of *R. oryzae* and cause the change of membrane integrity (Figure 3D).

Besides, we also observed the growth condition of *R. oryzae* under different pH and different concentrations of sodium nitrate stress (Figures 3E, J). The results showed that different concentrations of nitrate also affected the sporulation and sporangium generation of *R. oryzae*, and with the increase of nitrate concentration, sporangium production gradually increased, and sporangium inhibition rate gradually decreased (Figures 3F, G). However, the increase of nitrate concentration did not affect the change of absorption value in *R. oryzae* solution, indicating that the change of nitrate concentration did not affect the integrity of mycelia (Figure 3H). In addition, pH affected sporulation,

sporangium generation and mycelium integrity of *R. oryzae*, thus inhibiting the growth of *R. oryzae*. And this inhibition gradually increased with the increase of pH value (Figures 3I, K, L).

3.4 Effects of stress factors on the barley-*R. oryzae* interaction relationship

In order to test whether these three stress factors could inhibit the growth of *R. oryzae* without affecting the growth of barley, we conducted co-culture assay between barley and *R. oryzae* under the above stress conditions (Figures 4A, D, E). The results showed that, consistent with the results of the previous barley tissue culture assays (Supplementary Figure S3), except for the conditions of 100 mM sodium nitrate, pH = 8 and pH = 8.5, the barley growing under other conditions could grow normally compared with the control, indicating that the influence of *R. oryzae* on the growth of barley was less than that under stress conditions (Figures 4B, F, H). In different concentrations of ozone water treatment, 2

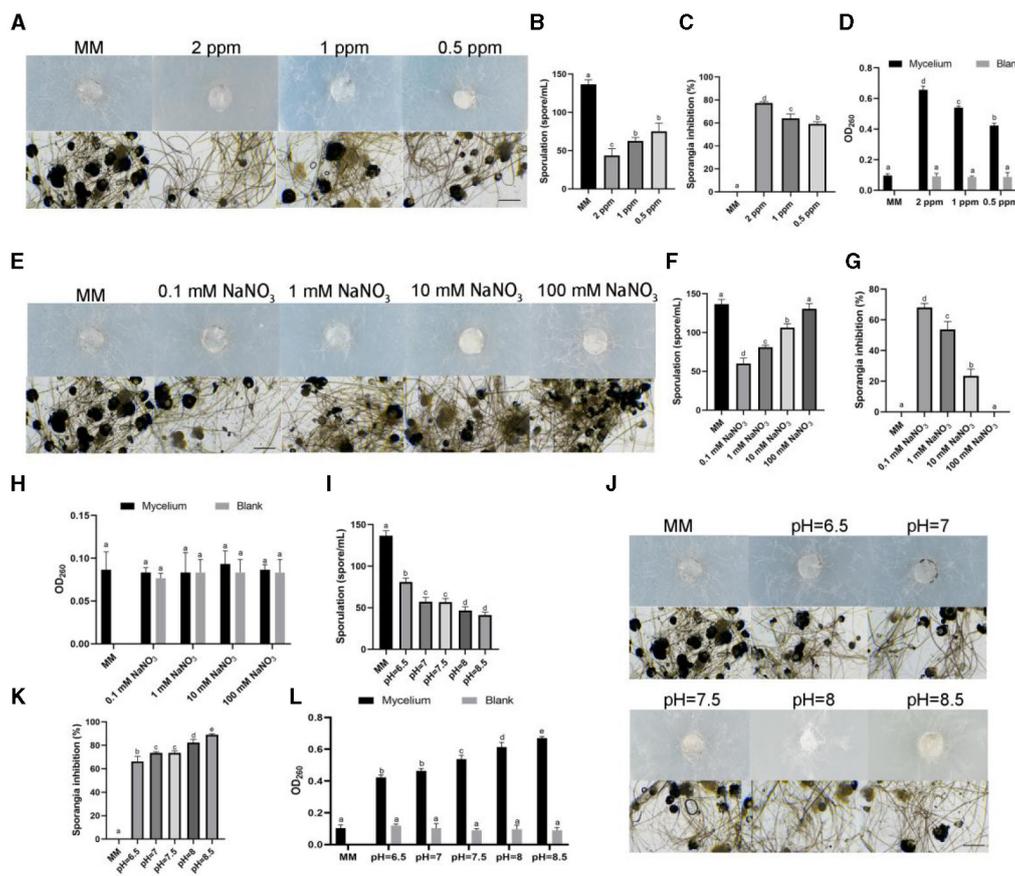


FIGURE 3 Effects of different stress factors on sporangium, sporulation and membrane integrity of *R. oryzae*. (A) The aerial hyphae and sporangium morphology of *R. oryzae* growing under 2 ppm ozone water, 1 ppm ozone water, and 0.5 ppm ozone water. Bar = 10 μm. (B) The sporulation of *R. oryzae* growing under 2 ppm ozone water, 1 ppm ozone water, and 0.5 ppm ozone water. (C) The sporangium inhibition of *R. oryzae* growing under 2 ppm ozone water, 1 ppm ozone water, and 0.5 ppm ozone water. (D) The membrane integrity of *R. oryzae* growing under 2 ppm ozone water, 1 ppm ozone water, and 0.5 ppm ozone water. (E) The aerial hyphae and sporangium morphology of *R. oryzae* growing under 0.1 mM NaNO₃, 1 mM NaNO₃, 10 mM NaNO₃, and 100 mM NaNO₃. Bar = 10 μm. (F) The sporulation of *R. oryzae* growing under 0.1 mM NaNO₃, 1 mM NaNO₃, 10 mM NaNO₃, and 100 mM NaNO₃. (G) The sporangium inhibition of *R. oryzae* growing under 0.1 mM NaNO₃, 1 mM NaNO₃, 10 mM NaNO₃, and 100 mM NaNO₃. (H) The membrane integrity of *R. oryzae* growing under 0.1 mM NaNO₃, 1 mM NaNO₃, 10 mM NaNO₃, and 100 mM NaNO₃. (I) The aerial hyphae and sporangium morphology of *R. oryzae* growing under different pH. Bar = 10 μm. (J) The sporulation of *R. oryzae* growing under different pH. (K) The sporangium inhibition of *R. oryzae* growing under different pH. (L) The membrane integrity of *R. oryzae* growing under different pH. Error bars mean standard deviation from three replicate samples. Different letters represented significant differences ($p < 0.05$).

ppm inhibition of *R. oryzae* was the best, and the colony diameter was 1.8 cm, which was much smaller than that of the control (4 cm) (Figure 4C). In different concentrations of sodium nitrate treatment, 0.1 mM sodium nitrate (2.55 cm) and 1 mM sodium nitrate (2.475 cm) could achieve better inhibition effect on *R. oryzae* (Figure 4G). Under different pH treatments, the inhibition effect on *R. oryzae* was obvious when pH = 8 and 8.5, but the growth of barley was poor (Figure 4I). In summary, considering the inhibition effect and operability, we thought that 2 ppm ozone water was more suitable for controlling *R. oryzae* in forage grass factories.

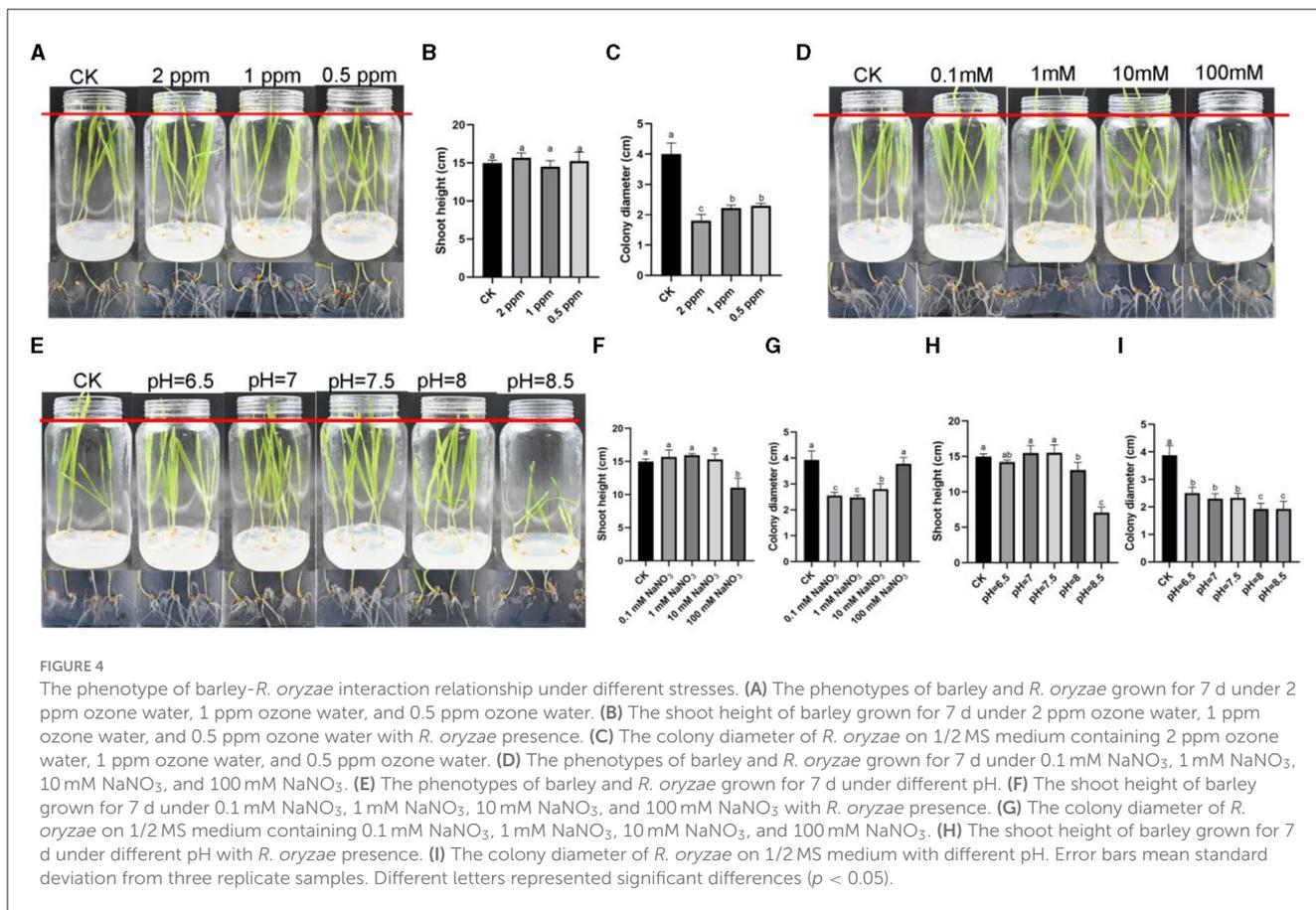
3.5 Application of ozone water in forage grass factory

To verify whether ozone water could effectively inhibit the outbreak of *R. oryzae* in forage grass factory, we planted three barley varieties including Mi105, Zhe37, and Mi146 which were

susceptible to *R. oryzae*. It could be seen from the results that the mold in the root of barley was controlled to a certain extent by spraying 2 ppm ozone water daily (Figure 5A), and the above ground of fresh weight was also increased compared with the control. The fresh weight of barley variety Mi105 was 11.39 g, which increased by 45% compared with untreated (Figure 5B). Barley quality indexes including crude fat content, crude protein content, and total starch content were determined. It was found that the barley quality was not affected by 2 ppm ozone water treatment, and there was no significant difference compared with the control (Figures 5C–E).

4 Discussion

Mold contamination causes huge economic losses to food industry (Dantigny et al., 2005). These losses are difficult to assess because they fluctuate with location, season, and type of spoiled



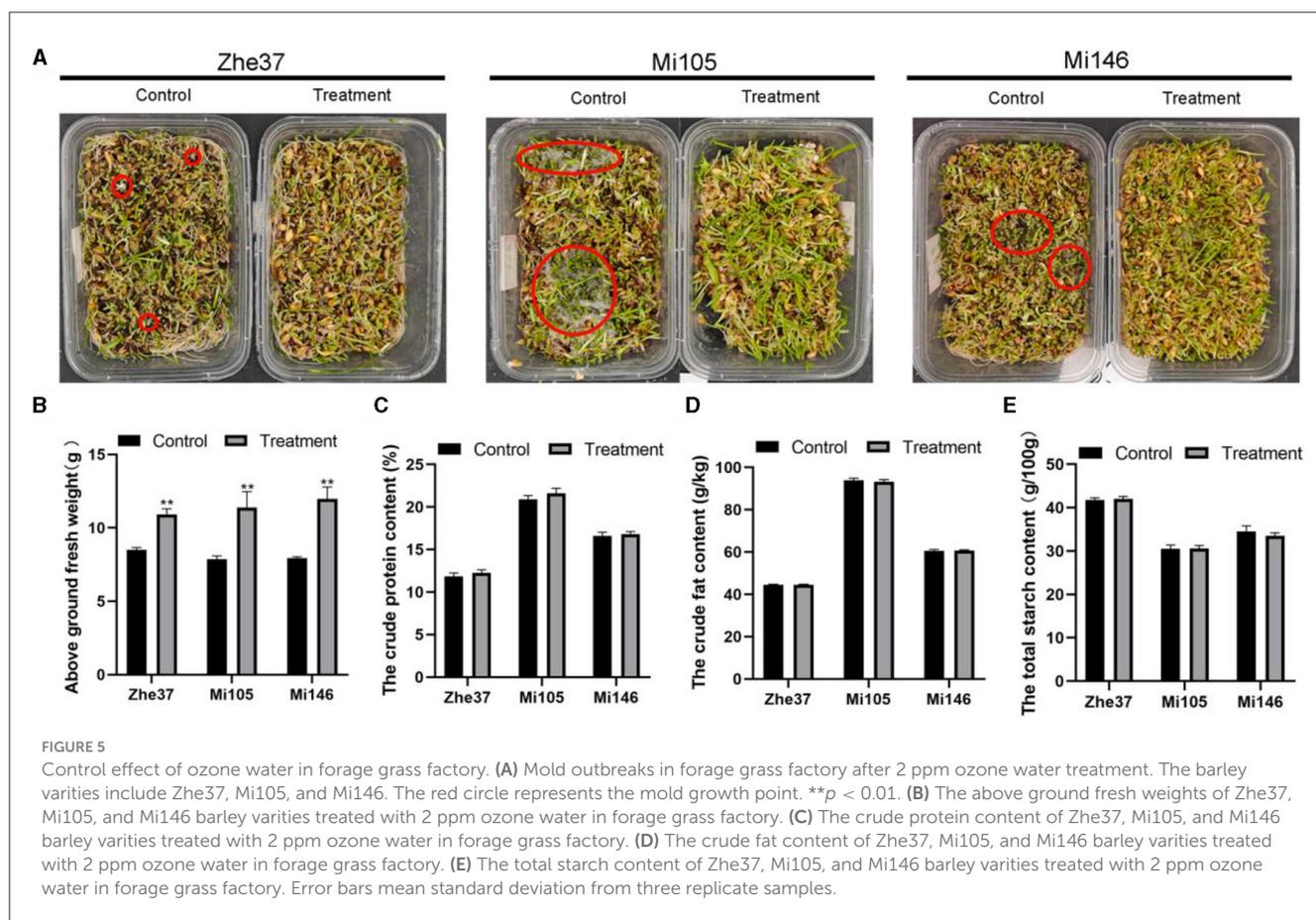
product. 131 notifications about the presence of mold in various animal and vegetable food products as well as dietary supplements were approved between 2018 and 2022 in the European Union (RASFF).¹ And the highest incidence of mold in these products was in cereal and bakery products. In our study, we also found mold outbreak in forage grass factory. And the contamination caused the decrease of barley yield (Figure 1A). By ITS sequencing, we identified *R. oryzae* as a main contaminator in the forage grass factory (Figures 1B, C). The interaction between *R. oryzae* and barley didn't report in other study. The main reason for this interaction might be due to the special environment in plant factory. Different from the natural environment outside, the humidity inside the plant factory is high, the temperature changes are small, the air circulation is slow, and it is easier to form an acidic environment. Such environmental conditions promoted *R. oryzae* to become the dominant colony, interact with barley, and affect the yield. Therefore, plant factory as a special habitat environment, its internal microbial dynamic changes, microbial-plant interaction forms, deserves our further study.

Nitrogen, as an essential element for biological growth and development, is supplemented in plant factories mainly by nutrient solution (Vidal et al., 2020). Different organisms have different

preferences for different concentrations and forms of nitrogen (Marschner, 1995; Chen et al., 2004). In recent study, it was found that around 25 nitrogen sources (amino acids) significantly inhibited the growth of *R. oryzae* (Li et al., 2023). In this study, it was found that *R. oryzae* preferred nitrates for growth (Figure 2D), indicating that *R. oryzae* might have the ability of denitrification. As a pathway for protein synthesis, nitrogen also closely related to the growth of mycelia (Morschhäuser, 2011). From the results, we also found that when nitrate nitrogen was reduced, the sporulation and biomass of *R. oryzae* were reduced (Figure 3E). Thus, by controlling the concentration of nitrate, we could effectively inhibit the growth of *R. oryzae* and control the outbreak of *R. oryzae* from the perspective of nitrogen source utilization. Moreover, it is important for plants to maintain appropriate nitrate concentrations. From our results, we found that when the concentration of sodium nitrate in the environment reached 100 mM, barley growth was hindered. When sodium nitrate concentration decreased, barley grew normally. In the barley-*R. oryzae* symbiosis system, the barley grew normally under the condition of inhibiting *R. oryzae* growth only by maintaining the appropriate sodium nitrate concentration (0.1 mM and 1 mM).

Besides, pH is also a limiting factor for fungal-plant interaction. The pH value suitable for mold growth varies with different species. The optimal pH of common *Penicillium*, *Aspergillus*, *Streptomyces* and other strains is about pH 5–6. The low pH preference of some molds that cause fruit decay is likely an adaptation to the growth of acidic host fruits (Buijs et al., 2021). In the forage grass factory,

¹ European Community's Rapid Alert System for Food and Feed (RASFF). Available online at: <https://webgate.ec.europa.eu/rasff-window/screen/search> (accessed 17 January 2024).



a large number of barley seeds were laid on the planting tray. The too-dense seeds planting made the barley undergo anaerobic respiration and generate acetic acid, resulting in a decrease in environmental pH. Lower pH was suitable for the growth of molds such as *R. oryzae* (Figures 1A, 2A). *R. oryzae* is mainly used in fermentation industry and is suitable for growing in acidic environment (Sebastian et al., 2021). From the result, it was also inferred that when the pH value exceeded 6.5, the growth of *R. oryzae* was significantly limited (Figure 2A), which might be related to the alkaline condition disrupting membrane integrity (Figure 3L). Similarly, barley prefers to grow in slightly acidic and neutral environment (pH value between 6–7.8). So even though alkaline conditions can inhibit the outbreak of *R. oryzae*, they will also inhibit the growth and development of barley. When the pH value of the environment was 7–7.5, the expansion of *R. oryzae* could be better inhibited than control without affecting the growth of barley (Figure 4E). Therefore, the outbreak of *R. oryzae* could be inhibited by adjusting the pH value of nutritional solution properly during the subsequent cultivation of barley in forage grass factory.

Ozone has a potent microbicidal action against bacteria, fungi, parasites, and viruses when they are present in low-ozone-demand media. As a safe antibacterial agent, the FDA authorized ozone for the food industry about 20 years ago (Asokapandian et al., 2018). Ozone can be used as an aqueous or gaseous substance. The benefits of ozone over other chemical oxidants are as follows: (a) it can be produced on-site; (b) it can be administered in both

gaseous and aqueous forms; (c) it doesn't leave any residue after contact; (d) it can be produced in large quantities; and (e) it doesn't have any hazardous disposal requirements (Pandiselvam et al., 2019; Torres et al., 2019). Ozone has the ability to deactivate a variety of microorganisms, including viruses and protozoa (Brié et al., 2018), pathogenic bacteria like *Salmonella typhimurium*, *Escherichia coli*, and *Listeria monocytogenes* (Alwi and Ali, 2014; Song et al., 2015), and fungi (Allen et al., 2003; Watanabe et al., 2010). The ozone's inactivation strategies against bacteria include ozone penetrating cells, targeting components of cell membranes, inactivating enzymes, and breaking down gDNA and total RNAs genetic materials. These activities finally lead to cell lysis and the leaking of cellular contents. In our study, we produced ozone water to control *R. oryzae*, and found that it had the best inhibitory effect on *R. oryzae* without affecting barley growth (Figures 4A–C). And the main reason was by changing the integrity of its cell membrane, resulting in the leakage of cellular substances (Lee and Kim, 2022) (Figure 3D). This treatment does not affect the normal growth of the barley due to its ability to self-decomposition, rapid action, and strong oxidative properties to produce oxygen (Sarron et al., 2021). By using ozone water in the forage grass factory, it was found that it suppressed the outbreak of *R. oryzae* and restored barley production (Figure 5). In addition, unlike the other two influencing factors that require changing the composition of the nutrient solution, ozone water can enter the grass factory through additional addition, which is more operational. Therefore, ozone water is more suitable for the

application of ozone (whether in liquid or gas form). Ozone will be a promising industrial fungistatic technology and has gained great interest in the food industry.

5 Conclusion

Based on the controllable environment of forage grass factory, this study explored the influence of different stress factors on the growth of mold and barley. The fungi were identified by ITS sequencing and the dominant fungus *R. oryzae* was isolated. By exploring the effect of different stress factors on the prevention and control of mold disease and the safety of barley, the green-technology of the prevention and control of mold disease in forage grass factory was studied. We found out the best means of controlling *R. oryzae*, that is, by ozone water to inhibit the growth, while not affecting the growth of barley. The molecular mechanism of ozone water treatment is to change the membrane integrity of *R. oryzae*. It was also used in the forage grass factory and obtained a significant prevention effect. These results will play an important role in the promotion of industrial forage grass culture technology and the development of animal husbandry.

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

MD: Investigation, Writing – original draft. XT: Methodology, Writing – review & editing. XC: Investigation, Methodology, Writing – original draft. KC: Conceptualization, Data curation,

References

- Al-Karaki, G. N., and Al-Hashimi, M. (2011). Green fodder production and water use efficiency of some forage crops under hydroponic conditions. *Isrn Agron.* 1:188. doi: 10.5402/2012/924672
- Allen, B., Wu, J., and Doan, H. (2003). Inactivation of fungi associated with barley grain by gaseous ozone. *J. Environ. Sci. Health Part B.* 38, 617–630. doi: 10.1081/PFC-120023519
- Alwi, N. A., and Ali, A. (2014). Reduction of *Escherichia coli* O157, *Listeria monocytogenes* and *Salmonella enterica* sv. *Typhimurium* populations on fresh-cut bell pepper using gaseous ozone. *Food Control.* 46, 304–311. doi: 10.1016/j.foodcont.2014.05.037
- Asokapandian, S., Periasamy, S., and Swamy, G. J. (2018). “Ozone for fruit juice preservation,” in *Fruit juices*, eds. G. Rajauria, B. K. Tiwari (Cambridge: Academic Press), 511–527. doi: 10.1016/B978-0-12-802230-6.00025-4
- Brié, A., Boudaud, N., Mssihid, A., Loutreul, J., and Bertrand, I., Gantzer, C. (2018). Inactivation of murine norovirus and hepatitis A virus on fresh raspberries by gaseous ozone treatment. *Food Microbiol.* 70, 1–6. doi: 10.1016/j.fm.2017.08.010
- Buijs, V. A., Zuidgeest, X. C. L., Groenewald, J. Z., Crous, P. W., and de Vries, R. P. (2021). Carbon utilization and growth-inhibition of citrus-colonizing Phyllosticta species. *Fungal Biol.* 125, 815–825. doi: 10.1016/j.funbio.2021.05.003
- Cao, D., Lou, Y., Jiang, X., Zhang, D., and Liu, J. (2022). Fungal diversity in barley under different storage conditions. *Front. Microbiol.* 13:895975. doi: 10.3389/fmicb.2022.895975

Writing – review & editing. YZ: Investigation, Writing – review & editing. ZY: Funding acquisition, Investigation, Writing – original draft. DK: Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Key R&D Program of Zhejiang Province (Grant No. 2023C02048) and Key Laboratory of Digital Upland Crops of Zhejiang Province (Grant No. 2022E10012).

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1437799/full#supplementary-material>

- Chen, B. M., Ma, Z. H., Li, S. X., Wang, G. X., Song, H. X., and Wang, X. N. (2004). Effects of nitrate supply on plant growth, nitrate accumulation, metabolic nitrate concentration and nitrate reductase activity in three leafy vegetables. *Plant Sci.* 167, 635–643. doi: 10.1016/j.plantsci.2004.05.015

- Chen, Q. L., Wang, H. C., Huang, Y., Li, W. H., Cai, L. T., Tan, Q. Q., et al. (2021). Pathogenicity and metabolic phenotypic characteristics of *Rhizopus oryzae* in tobacco under different osmotic pressure and pH environments. *Yancao Keji.* 2022, 16–23. doi: 10.16135/j.issn1002-0861.2021.0081

- Dankwa, A. S., Machado, R. M., and Perry, J. J. (2020). Sources of food contamination in a closed hydroponic system. *Lett. Appl. Microbiol.* 70, 55–62. doi: 10.1111/lam.13243

- Dantigny, P., Guilmar, A., and Bensoussan, M. (2005). Basis of predictive mycology. *Int. J. Food Microbiol.* 100, 187–196. doi: 10.1016/j.ijfoodmicro.2004.10.013

- Devendar, R., Kumari, N. N., Reddy, Y., Rao, K. S., Reddy, K. K., Raju, J., et al. (2020). Growth performance, nutrient utilization and carcass characteristics of sheep fed hydroponic barley fodder. *Anim. Nutr. Feed Technol.* 20, 321–331. doi: 10.5958/0974-181X.2020.00029.3

- Fazaeli, H., Golmohammadi, H., Tabatabayee, S. N., and Asghari-Tabrizi, M. (2012). Productivity and nutritive value of barley green fodder yield in hydroponic system. *World Appl. Sci. J.* 16, 531–539.

- Fazaileh, H., Golmohammadi, H., Shoayee, A. A., Montajebi, N., and Masharaf, S. H. (2011). Performance of feedlot calves fed hydroponics fodder barley. *J. Agric. Sci. Technol.* 13, 367–375.
- Fiutak, G., Michalczyk, M., Filipczak-Fiutak, M., Fiedor, L., and Surówka, K. (2019). The impact of LED lighting on the yield, morphological structure and some bioactive components in alfalfa (*Medicago sativa* L.) sprouts. *Food Chem.* 285, 53–58. doi: 10.1016/j.foodchem.2019.01.086
- Gnanesh, B. N., Tejaswi, A., Arunakumar, G. S., Supriya, M., Manojkumar, H. B., and Tewary, P. (2021). Molecular phylogeny, identification and pathogenicity of *Rhizopus oryzae* associated with root rot of mulberry in India. *J. Appl. Microbiol.* 131, 360–374. doi: 10.1111/jam.14959
- Grunert, O., Hernandez-Sanabria, E., Buysens, S., De Neve, S., Van Labeke, M. C., Reheul, D., et al. (2020). In-depth observation on the microbial and fungal community structure of four contrasting tomato cultivation systems in soil based and soilless culture systems. *Front. Plant Sci.* 11:520834. doi: 10.3389/fpls.2020.520834
- Kanjanamaneesathian, M. (2015). Biological control of diseases of vegetables grown hydroponically in thailand: Challenge and opportunity. *Recent Pat. Biotechnol.* 9, 214–222. doi: 10.2174/1872208310666160414102201
- Khaziev, D., Gadiev, R., Yusupova, C., Kazanina, M., and Kopylova, S. (2021). Effect of hydroponic green herbage on the productive qualities of parent flock geese. *Vet. World.* 14, 841–846. doi: 10.14202/vetworld.2021.841-846
- Khokhar, I., Wang, J., Jia, Y., and Yan, Y. (2019). First report of rhizopus soft rot on tomato (*Lycopersicon esculentum*) caused by *Rhizopus oryzae* in China. *Plant Dis.* 5, 1041–1041. doi: 10.1094/PDIS-10-18-1848-PDN
- Kozai, T. (2018). “Current status of plant factories with artificial lighting (PFALs) and smart PFALs,” in *Smart plant factory*, ed. T. Kozai (Singapore: Springer Nature Singapore Pte Ltd.), 3–13. doi: 10.1007/978-981-13-1065-2_1
- Kozai, T., and Niu, G. (2016). “Plant factory as a resource-efficient closed plant production system,” in *Plant Factory: an indoor vertical farming system for efficient quality food production*, ed. T. Kozai (Cambridge: Academic Press), 69–90. doi: 10.1016/B978-0-12-801775-3.00004-4
- Kwon, J. H., Kang, D. W., Lee, S. D., and Kim, J. (2015). Outbreak of rhizopus rot caused by *Rhizopus oryzae* on seedlings of grafted cucumber on pumpkin rootstock in South Korea. *J. Phytopathol.* 163, 670–674. doi: 10.1111/jph.12304
- Kwon, J. H., Ryu, J. S., Chi, T. T., Shen, S. S., and Choi, O. (2012). Soft rot of *Rhizopus oryzae* as a postharvest pathogen of banana fruit in Korea. *Mycobiology.* 40, 214–216. doi: 10.5941/MYCO.2012.40.3.214
- Lee, H. S., and Kim, Y. (2022). Myricetin disturbs the cell wall integrity and increases the membrane permeability of *Candida albicans*. *J. Microbiol. Biotechnol.* 32, 37–45. doi: 10.4014/jmb.2110.10014
- Lee, N. Y., Lee, M. J., Kim, Y. K., Park, J. C., Park, H. K., Choi, J. S., et al. (2010). Effect of light emitting diode radiation on antioxidant activity of barley leaf. *J. Korean Soc. Appl. Biol. Chem.* 53, 685–690. doi: 10.3839/jksabc.2010.104
- Li, Z., Shi, C. H., Huang, Y., Wang, H. C., Li, W. H., and Cai, L. T. (2023). Phenotypic analysis and genome sequence of *Rhizopus oryzae* strain Y5, the causal agent of tobacco pole rot. *Front. Microbiol.* 13:1031023. doi: 10.3389/fmicb.2022.1031023
- Londoño-Hernández, L., Ramírez-Toro, C., Ruiz, H. A., Ascacio-Valdés, J. A., Aguilar-Gonzalez, M. A., Rodríguez-Herrera, R., et al. (2017). *Rhizopus oryzae*—ancient microbial resource with importance in modern food industry. *Int. J. Food Microbiol.* 257, 110–127. doi: 10.1016/j.jfoodmicro.2017.06.012
- Lozada, A. F. (1995). Isolation and identification of mycotoxigenic fungi in selected foods and feeds. *Food Addit. Contam.* 12, 509–514. doi: 10.1080/02652039509374337
- Ma, L. J., Ibrahim, A. S., Skory, C., Grabherr, M. G., Burger, G., Butler, M., et al. (2009). Genomic analysis of the basal lineage fungus *Rhizopus oryzae* reveals a whole-genome duplication. *PLoS Genet.* 5:e1000549. doi: 10.1371/journal.pgen.1000549
- Marschner, H. (1995). *Mineral Nutrition of Higher Plants*. London: Academic Press.
- Morschhäuser, J. (2011). Nitrogen regulation of morphogenesis and protease secretion in *Candida albicans*. *Int. J. Med. Microbiol.* 301, 390–394. doi: 10.1016/j.jimm.2011.04.005
- Moström, M. S., and Jacobsen, B. J. (2020). Ruminant mycotoxicosis: an update. *Vet. Clin. North Am. Food Anim. Pract.* 36, 745–774. doi: 10.1016/j.cvfa.2020.08.011
- Naik, P. K., Swain, B. K., and Singh, N. P. (2015). Production and utilization of hydroponics fodder. *Indian J. Anim. Nutr.* 32, 1–9.
- Pandiselvam, R., Subhashini, S., Banuu Priya, E. P., Kothakota, A., Ramesh, S., and Shahir, S. (2019). Ozone based food preservation: a promising green technology for enhanced food safety. *Ozone Sci. Eng.* 41, 17–34. doi: 10.1080/01919512.2018.1490636
- Podar, D. (2013). Plant growth and cultivation. *Methods Mol. Biol.* 953, 23–45. doi: 10.1007/978-1-62703-152-3_2
- Sarron, E., Gadonna-Widehem, P., and Aussenac, T. (2021). Ozone treatments for preserving fresh vegetables quality: a critical review. *Foods.* 10:605. doi: 10.3390/foods10030605
- Sebastian, J., Dominguez, K. V., Brar, S. K., and Rouissi, T. (2021). Fumaric acid production using alternate fermentation mode by immobilized *Rhizopus oryzae*—a greener production strategy. *Chemosphere* 281:130858. doi: 10.1016/j.chemosphere.2021.130858
- Song, W. J., Shin, J. Y., Ryu, S., and Kang, D. H. (2015). Inactivation of *Escherichia coli* O157:H7, *Salmonella Typhimurium* and *Listeria monocytogenes* in apple juice at different pH levels by gaseous ozone treatment. *J. Appl. Microbiol.* 119, 465–474. doi: 10.1111/jam.12861
- Torres, A. M., Palacios, S., Yerkovich, N., Palazzini, J., Battilani, P., Leslie, J., et al. (2019). Fusarium head blight and mycotoxins in wheat: Prevention and control strategies across the food chain. *World Mycotoxin J.* 12, 333–355. doi: 10.3920/WMJ2019.2438
- Vidal, E. A., Alvarez, J. M., Araus, V., Riveras, E., Brooks, M. D., Krouk, G., et al. (2020). Nitrate in 2020: Thirty years from transport to signaling networks. *Plant Cell.* 32, 2094–2119. doi: 10.1105/tpc.19.00748
- Wang, H. C., Zhang, M., Zhang, C. Q., Chen, X. J., Tan, Q. Q., Ma, J., et al. (2018). Pathogen detection, carbon metabolic phenotype analysis of *Rhizopus oryzae* from tobacco and its sensitivity to seven fungicides. *J. Pestic. Sci.* 20, 743–748. doi: 10.16801/j.issn.1008-7303.2018.0095
- Wang, R. Y., Gao, B., Chen, S. L., Ma, J., Li, X. H., and Xue, C. (2017). First report of *Rhizopus oryzae* causing soft rot on storage roots of sweetpotato in China. *Plant Dis.* 6:1039. doi: 10.1094/PDIS-09-16-1272-PDN
- Watanabe, M., Masaki, H., Mori, T., Tsuchiya, T., Konuma, H., Hara-Kudo, Y., et al. (2010). Inactivation effects of uv irradiation and ozone treatment on the yeast and the mold in mineral water. *J. Food Prot.* 73, 1537–1542. doi: 10.4315/0362-028X-73.8.1537
- Zhang, N., Zhao, S., and Shen, Q. (2011). A six-gene phylogeny reveals the evolution of mode of infection in the rice blast fungus and allied species. *Mycologia.* 103, 1267–1276. doi: 10.3852/11-022
- Zhu, J. N., Yu, Y. J., Dai, M. D., Zeng, Y. L., Lu, X. J., Wang, L., et al. (2022). A new species in *Pseudophialophora* from wild rice and beneficial potential. *Front. Microbiol.* 13:845104. doi: 10.3389/fmicb.2022.845104