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ORIGINAL ARTICLE

The protective effect of trehalose and monosodium glutamate on yeast viability and antagonistic properties during freeze-drying

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Abstract

In the present eco-conscious era, consumers opt for food choices reflecting ethical and environmental concerns, which increases the demand for organic products. Biocontrol is a viable plant protection method in organic farming. Freeze-drying is a long-term preservation technique for microbial agents, ensuring their genetic stability and viability. To reduce freeze-drying-induced damage to their cells, cryoprotective agents like trehalose and monosodium glutamate are used. This study evaluated the impact of the addition of these substances during the freeze-drying process on chosen yeast isolates' viability, their ability to survive on tomato leaves and maintain antagonistic properties against *Botrytis cinerea* Pers. Yeast isolates 114/73 (*Wickerhamomyces anomalus* E.C. Hansen) and 117/10 (*Naganishia albidosimilis* Vishniac & Kurtzman) were tested on tomato plants under greenhouse conditions before and after the freeze-drying process for both the ability to colonize leaves and as a preventive and interventional treatment against *B. cinerea*. Yeast viability post freeze-drying was evaluated *in vitro*. Both trehalose and monosodium glutamate increased yeast viability during the freeze-drying process. Viability was not very high (from 30.33 to 36.17% for 117/10 and from 10.67 to 16.5% for 114/73). Yeast dehydrated after freeze-drying, protected with trehalose and monosodium glutamate, displayed the same colony count on tomato leaves as before freeze-drying. The efficacy of protective treatments depended on the yeast isolate, the protective substance used during freeze-drying, treatment timing (prevention vs. intervention), and interactions of those factors. Cryopreserved isolate 117/10 performed better than 114/73 with the addition of either trehalose or monosodium glutamate, reducing the disease severity index from 88.3% (control) to 18.75–55.33%. Preventive treatments were more efficient than intervention. The leaf colonization ability and biocontrol efficacy of yeast isolates against *B. cinerea* post-freeze-drying offer promising solutions in sustainable agriculture. However, further research, to analyze the interactions between various factors and to optimize strategies may be needed.

Keywords: biocontrol, *Botrytis cinerea*, freeze-dry, tomato, yeast

Introduction

In today's eco-conscious era, consumer choices in food purchases are shaped by both ethical and environmental considerations. There is a growing need for products sourced from organic agricultural systems. This trend aligns with the European Union's Green Deal, which promotes agricultural methods that diverge from chemical-based approaches. Among crop protection methods in organic farming, biocontrol, particularly using microorganisms, has emerged as a viable option (Kour *et al.* 2020). The market for biocontrol agents is growing fast and it is expected to grow from 5 to 15 billion USD by 2029 (Fernandez-San Millan *et al.* 2023; Marrone 2024). Given this, there is a pressing need to study new sustainable agricultural microbial agents.

Microbial agents should be formulated to ensure their stability during storage, facilitate easy

distribution, and preserve their viability, survivability, and antagonistic properties. Over the years multiple methods for preserving cultures of microorganisms have been established, including the use of sterile distilled water, cryopreservation, freeze-drying, subculturing, and sterile mineral oil. Freeze-drying is the most successful and convenient long-term preservation technique that ensures the genetic stability and viability of the microorganisms for 10 years or longer (Castro-Ríos *et al*. 2021). Freeze-drying pauses the metabolism of microorganisms and removes water from frozen cells through the sublimation of ice under intense vacuum conditions. Cell dehydration, essential to prevent intracellular crystallization during freezing, is controlled by the rate of cooling. To prevent cells from suffering side effects of freeze drying such as denaturation of proteins, cryoprotective agents, including carbohydrates (e.g., glucose, sucrose, maltose, fructose, lactose, mannose, or trehalose), prebiotics (e.g., raffinose, stachyose, inulin, galactooligosaccharides or fructooligosaccharide) and salts (e.g., sodium glutamate or sodium ascorbate) (Marcantonini *et al.* 2022) are needed. This method allows for the storage of microorganisms at room temperature.

Trehalose is a disaccharide made up of two glucose units which can protect subcellular components under stressful conditions (Hubalek 2003; Elbein *et al*. 2003). It maintains internal water balance and protein structure and strengthens the cell membrane to protect its integrity against severe environmental changes (Stefanello *et al*. 2018). Yeasts that can withstand freezing generate significant amounts of trehalose during cryopreservation, indicating a direct relationship between the sugar's presence and increased resistance (Yin *et al.* 2017; Sun *et al*. 2020). Protective effects of monosodium glutamate during freeze-drying involve the preservation of protein formations through interactions between the amino groups of the protectant and the carboxyl groups of the microorganism proteins, coupled with the capacity to hold more residual moisture (Carvalho *et al*. 2003; Tanimomo *et al*. 2016). As a drying agent skimmed milk powder forms a porous texture in the freeze-dried item, facilitating easier rehydration. Additionally, the proteins in milk offer a safeguarding layer for the cells (Abadias *et al*. 2001; Sharma *et al*. 2014).

This study's objective was to assess the impact of adding trehalose and monosodium glutamate in conjunction with skimmed milk powder during the freeze-drying of selected yeast isolates. The viability of these freeze-dried yeast isolates was examined. Subsequently, survival tests were performed on tomato leaves before and after freeze-drying. Additionally, the antagonistic behavior of yeast isolates against *Botrytis cinerea* was evaluated both before and after the freeze--drying process.

Materials and Methods

Microorganisms

The yeasts were isolated from leaves of Virginia mallow (*Sida hermaphrodita* (L.) Rusby) plants collected in Winna Góra, Poland (52°12'21" 17°26'49"E). Two hundred and seventy individual isolates were obtained and subjected to an *in vitro* dual bioassay test to assess their antagonistic properties. Based on the results of this test, 19 isolates, exhibiting promising antagonistic activity, were selected for further analysis and identified biochemically with API 20 C AUX tests (bioMérioux, France). The process of selecting yeast isolates and assessing their antagonistic abilities was described in detail by Remlein-Starosta *et al*. (2016). Effective strains were further assessed in this study.

A *Botrytis cinerea* strain used in the biocontrol test was isolated from the surface of tomato leaves, identified according to its morphological characteristics and Koch's postulates were confirmed. For conidial production, *B. cinerea* was cultured on Potato Dextrose Agar (PDA) (pH 6.5) at 20–25°C until the mycelium appeared, and later at 15°C to induce sporulation. Yeasts were grown on potato dextrose (pH 6.5) at 22°C (PDA, Sigma Chemical Co, St. Louis, MO, USA) and then stored at 4°C.

Yeast freeze-drying procedure using cryoprotectants

Cryoprotectants were evaluated on isolates chosen in the experiments described below. A primary cryoprotective formulation was prepared, consisting of 10% skimmed milk powder (Millipore-Merck, Darmstadt, Germany) in sterile distilled water. This baseline cryoprotectant solution was used on its own (used as a control) and supplemented with 10% trehalose and 10% monosodium glutamate. Protecting solutions were autoclaved at 115°C for 15 min.

Yeast cells were harvested from the PDA surface using 2 ml of 0.9% NaCl and a sterile cell scraper. The resultant cellular mass was adjusted to 2×10^7 CFU · ml^{-1} using a Thoma hemacytometer, transferred to Eppendorf tubes and centrifuged at 3000 rpm for 3 min. NaCl was dispensed and the vials were filled with each cryoprotective medium. For each combination of yeast and protectant, 10 sterile vials were prepared with 1 ml of suspension. They were cooled to −40°C for 2 h, then to −12°C for 6 h, and finally to −20°C for 12 h. The vials were then placed in a lyophilizer chamber at 1 Pa and −45°C and left for 24 h.

Freeze-dried yeasts were stored at room temperature for 6 months. Then they were rehydrated by adding 1 ml of 0.9% NaCl. After 30 min the suspension was poured into Petri dishes containing solidified PDA and incubated at 40°C.

Survivability tests of yeast isolates on tomato leaves before undergoing greenhouse conditions

To choose isolates able to colonize leaf surfaces for the freeze-drying procedure and further experiments, a pot experiment was conducted using tomato (Koralik cultivar) as the model plant. Nineteen yeast isolates, chosen for their strong antagonistic abilities against plant pathogens, were used in the experiment. Suspensions of 3-day-old yeast cultures were prepared at a concentration of 2×10^7 CFU · ml^{-1} using a Thoma hemacytometer and a McFarland scale. The surfaces of tomato plant leaves at the seven-leaf stage on the main stem (BBCH scale: 17) were uniformly treated with 5 ml of the respective yeast suspension using a hand-held spray bottle.

On the 2nd, 4th, 6th, 8th, and 10th days of the experiment, discs measuring 1 cm^2 were cut with a sterile cork borer from a random location on the leaf surface. The discs were placed in Eppendorf tubes, with 2 ml of sterile distilled water added, followed by centrifugation at 3000 rpm for 2 min. From the base of the tubes, 0.1 ml of the suspension was extracted and poured onto Petri dishes containing 10 ml of PDA medium maintained at 45°C. The suspension was then mixed with the medium and allowed to solidify. Post solidification, the Petri dishes were incubated at 23°C for 48 h, and the number of colony-forming units (CFUs) per 1 cm^2 of leaf surface was calculated. A control group of tomato leaves was treated with a yeast-free water solution. The experiment was conducted in five replications. Based on the results the best performing yeast isolates were selected for further experiments.

Viability of yeast cells after freeze-drying, *in vitro* test

Viability was determined for each sample after the freeze-drying process. Each sample was diluted 10 fold and spread in three duplicates onto Petri dishes containing PDA. The Petri dishes were incubated at 23°C for 48 h. Visible colonies were directly counted, and the number of CFU, was obtained per milliliter of the original solutions by applying the corresponding dilution factor (Nf). The initial cell concentration (N0) of each suspension was 2×10^7 CFU · ml^{-1}.

Viability was calculated with the equation:

$$\text{Viability } [\%] = (\text{Nf}/\text{N0}) \times 100.$$

The results ranged from 0 (no viable cells) to 100 (all cells viable).

Survivability tests on rehydrated yeast isolates on tomato leaves after freeze-drying under greenhouse conditions

The ability of rehydrated yeast isolates to colonize plant surfaces was tested. A greenhouse experiment was conducted using the methodology corresponding to the methods of the previously described yeast survivability tests. The Koralik cultivar was used as a model plant (BBCH scale 17). The water suspensions of viable cells of two chosen rehydrated yeast (3-day-old yeast cultures) isolates (114/73 (*Wickerhamomyces anomalus* E.C. Hansen) and 117/10 (*Naganishia albidosimilis* Vishniac & Kurtzman) were used at a concentration of 2×10^7 CFU · ml^{-1} to uniformly treat tomato plant leaves.

Every other day, starting on the 2nd day of the experiment, discs (1 cm^2) were cut from a random location of the leaf surface and centrifuged in Eppendorf tubes with 2 ml of sterile distilled water added at 3000 rpm for 2 min. Then, 0.1 ml of the suspension was extracted and poured onto Petri dishes, mixed with 10 ml of the PDA medium and solidified. They were then were incubated at 23°C for 48 h, and the number of CFUs per 1 cm^2 of leaf surface was calculated. The control group of tomato leaves was treated with a yeast-free water solution. The experiment was conducted in five replications.

Determination of antagonistic activity of chosen yeast isolates against *Botrytis cinerea* on tomatoes before and after freeze-drying under greenhouse conditions

To evaluate the yeast isolate's ability to mitigate symptoms on tomato leaves caused by *B. cinerea* two experiments with both preventive and interventional treatments were performed. In the first experiment the antagonistic activity of two yeast isolates, 117/10 and 114/73 which were able to maintain a high population on tomato leaves was evaluated against gray mold. Tomato plants of the Koralik cultivar were individually sown in pots. Two-week-old plants were inoculated with 7-day-old *B. cinerea* mycelium. A water suspension of pathogen spores with a density of 3×10^5 · ml^{-1} was sprayed onto the plants using a handheld spray bottle, applying 1 ml per plant.

Two types of protective treatments, i.e., preventive and interventional, were performed using a water suspension of selected antagonist isolates with an optical density of one according to the McFarland scale (a concentration of 2×10^7 CFU · ml^{-1}). The preventive treatment was applied 3 days before pathogen inoculation, while the interventional treatment was applied 5 days post pathogen inoculation, upon the appearance of the first symptoms. Disease incidence assessment

was conducted 7 days after the interventional treatment when the tomato plants were at the 16–17 BBCH growth stage. The evaluation was based on a nine-point scale indicating the percentage of leaf infection, where 0 represented no infection and 8 indicated 100% leaf infection (Sobolewski and Robak 2004). Then a disease severity index (DSI) was calculated:

$$\text{DSI [\%]} = \frac{\text{sum (class frequency} \times \text{score of rating class)}}{\text{total number of plants} \times \text{maximal disease index}} \times 100.$$

The plants were kept under natural light conditions at an average temperature of 18°C. Control plants were inoculated and treated with distilled water. Each treatment was replicated three times on 10 tomato plants.

A second experiment was conducted in a greenhouse using viable cells of two rehydrated yeast isolates, with the experimental procedure the same as the methodology described above.

Statistical analysis of the results

Analysis of variance (ANOVA) was performed on all collected data. The Shapiro-Wilk test was conducted initially to verify the normality of data distribution. The equality of variances across groups was confirmed using Levene's test. ANOVA with the Tukey HSD post hoc test was used to identify significant differences between the groups ($p < 0.05$). The dispersion of the data set was assessed by calculating the standard deviation.

Results

Survivability tests of yeast isolates on tomato leaves before undergoing greenhouse conditions

Before freeze-drying 19 individual isolates displayed varied survivability on the surface of tomato leaves. Isolates 102/5, 102/45, and 114/20 did not survive on the leaf surface for 48 h. After 2 days, colonies of isolates 113/69, 114/24, and 115/61 were no longer being isolated from the leaf surface. During the subsequent 6 days, the quantity of yeast isolated from the leaf surface systematically decreased, and 10 days post-treatment, only colonies of isolates 114/73, 117/10, and 117/60 could still be found on the leaf surface. On the 10th day of the experiment 0.5, 1.0, and 0.3, respectively, colony-forming units were isolated from 1 cm^2 (Fig. 1). Isolates 114/73 and 117/10 had the highest number of isolated colonies throughout the experiment. The largest drop in colony numbers for isolate 114/73 occurred after 6 days (from 14.5 to 3.8 CFU · ml^{-2}), and for isolate 117/10 after 4 days

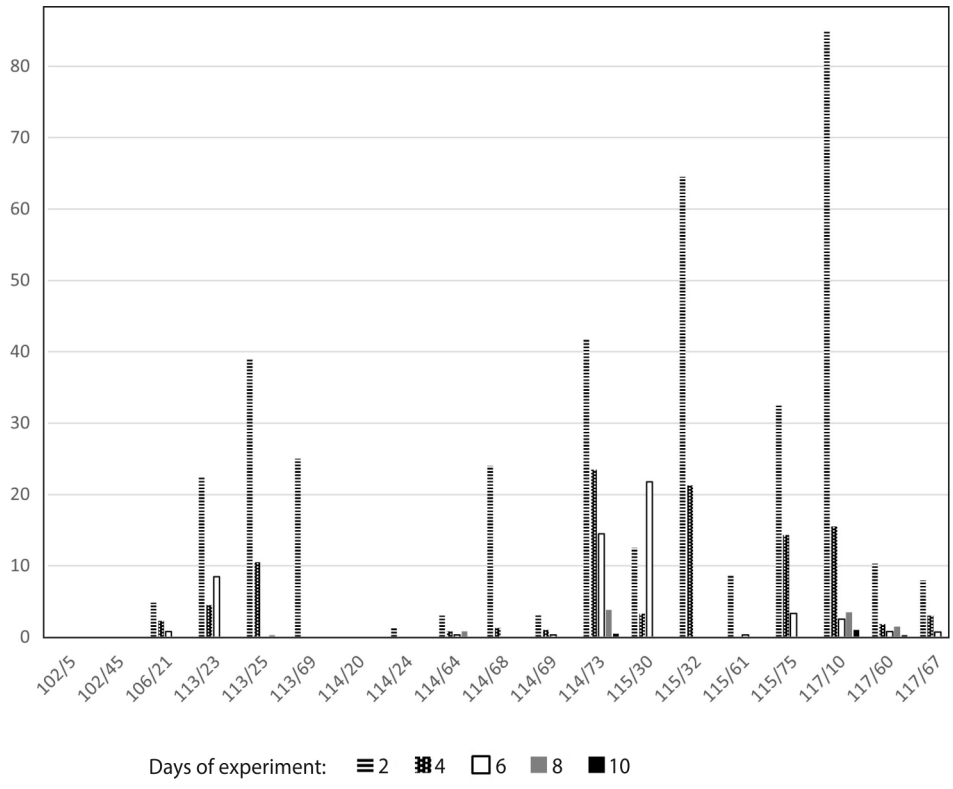

Fig. 1. The average number of yeast colonies in the tomato phyllosphere [CFU · cm^{-2}] in the subsequent days of the experiment

(from 15.5 to 2.5 CFU · ml^{-2}). No yeast isolates were obtained from the control plants. Isolates 114/73 (*W. anomalus*) and 117/10 (*N. albidosimilis*) were chosen for further experiments.

Viability of yeast cells after freeze-drying, *in vitro* test

For isolate 114/73, the addition of monosodium glutamate (MG) resulted in the highest viability (16.5%), followed by trehalose with 10.67%, and skimmed milk (SM) on its own with 6.97%. While the viability was significantly higher with MG than SM, no significant difference was found between MG and trehalose treatments and neither treatment reached viability over 20%.

For isolate 117/10, the addition of both trehalose and monosodium glutamate resulted in significantly higher viability than using SM on its own, with MG giving the highest viability (36.17%), followed closely by trehalose (30.33%), and then the control (10%).

Both trehalose and monosodium glutamate demonstrated greater protective effects than skimmed milk for preserving yeast viability post-lyophilization, with monosodium glutamate giving the best results for both isolates (Fig. 2).

Survivability tests on rehydrated yeast isolates on tomato leaves after freeze-drying under greenhouse conditions

The impact of various protectants, on the preservation of yeast colonies in the tomato phyllosphere following freeze-drying was investigated. The control group represented the number of yeast colonies before freeze-drying, serving as a reference for comparison. Isolates protected with trehalose and MG treatments displayed high average colony counts and were statistically the same as the control before freeze-drying, suggesting their potential efficacy in preserving yeast's ability to colonize the tomato leaf surface during freeze-drying. Isolates protected with SM on its own consistently showed the lowest average number of yeast colonies, indicating its limited protective efficacy (Fig 3).

Determination of antagonistic activity of chosen yeast isolates against *Botrytis cinerea* on tomatoes before and after freeze-drying under greenhouse conditions

This study evaluated the efficacy of yeast water suspensions as protective treatments against *B. cinerea* infestation in tomato leaves. Before freeze-drying the preventive treatment with isolate 117/10 reduced leaf infestation, achieving a DSI of 11.25, significantly lower than the control group's 84.17. On the other hand, the intervention treatment with isolate 114/73 showed no substantial reduction in infestation compared to the control, with a similar DSI of 75.42. This implies the potential use of preventive yeast suspensions, specifically isolate 117/10, as an effective biological control against *B. cinerea* in tomato plants (Table 1).

This study investigated the efficacy of yeast isolates 117/10 and 114/73 in preventing *B. cinerea* symptoms on tomato leaves following their cryopreservation with protective treatments. When cryopreserved with SM on its own, both yeast isolates resulted in higher DSIs when used either as prevention (85.83 and 87.92% for 117/10 and 114/73, respectively) or intervention (86.67 and 88.33%). In contrast, cryopreservation with trehalose or MG led to lower DSIs. Isolate 117/10, protected with Tre or MG and used as prevention, resulted in DSIs of 18.75%, whereas when used as an intervention, the DSIs were 53.33 and 29.58%, respectively. For isolate

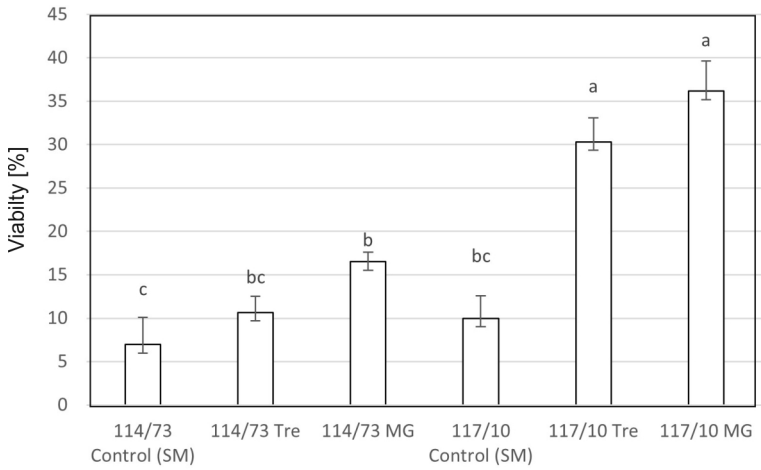

Fig. 2. Effect of different cryoprotectants (Control – Skimmed Milk, Tre – Trehalose, MG – monosodium glutamate) on the viability of the isolates 114/73 and 117/10 after lyophilization. Measurements with the same letter do not show significant differences ($p < 0.05$)

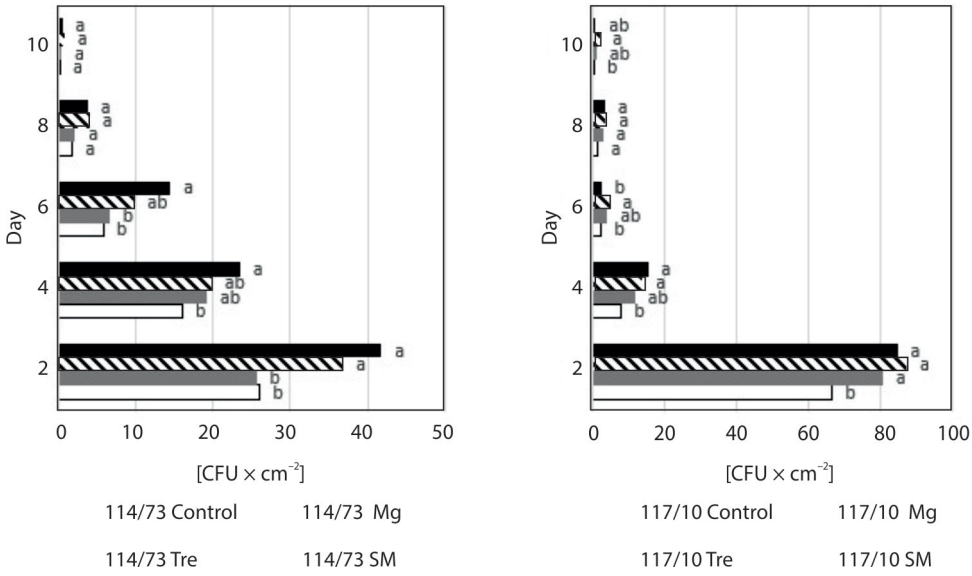

Fig. 3. Effect of different cryoprotectants (Control – pre-dry-freezing, Tre – Trehalose, MG – monosodium glutamate, SM – skimmed milk) on the ability to maintain colonies on the tomato leaf surface of isolates 114/73 and 117/10 after lyophilization. Measurements with the same letter do not show significant differences ($p < 0.05$)

Table 1. The effect of different protective treatments with yeast isolates on *Brassica cinerea* Disease Severity Index [%] on tomato leaves before freeze-drying

Control	Protective treatment			
	117/10 prevention	117/10 intervention	114/73 prevention	114/73 intervention
84.17 ± 1.56 a	11.25 ± 3.68 d	39.17 ± 2.12 c	48.75 ± 3.55 b	75.42 ± 1.56 a

a, b, c, d – homogenous groups at $p \leq 0.05$

114/73, the respective DSIs were 82.08 and 49.17% (prevention), and 87.5 and 74.58% (intervention). The most effective approach was the preventive application of the 117/10 isolate, after using either trehalose or MG as a cryoprotectant, achieving the minimum DSI of 18.75%. This demonstrated the significant impact of protective treatments during cryopreservation on the performance of yeast isolates against *B. cinerea* (Table 2).

Table 2. Comparison of *Brassica cinerea* Disease Severity Index [%] on tomato leaves following cryopreservation of yeast isolates using various protective treatments: skimmed milk (SM), trehalose (Tre), and monosodium glutamate (MG)

Control	Protective treatment					
	117/10 SM prevention	117/10 SM intervention	117/10 Tre prevention	117/10 Tre intervention	117/10 MG prevention	117/10 MG intervention
88,33 ± 1.59a	85.83 ± 3.84 a	86.67 ± 3.28 a	18.75 ± 2.04 e	53.33 ± 0.59 c	18.75 ± 1.02 e	29.58 ± 0.59 d
	Protective treatment					
	114/73 SM prevention	114/73 SM intervention	114/73 Tre prevention	114/73 Tre intervention	114/73 MG prevention	114/73 MG intervention
	87.92 ± 0.59 a	88.33 ± 2.57 a	82.08 ± 3.86 a	87.50 ± 1.02 a	49.17 ± 0.59 c	74.58 ± 0.59 b

a, b, c, d, e – homogenous groups at $p \leq 0.05$

Discussion

Freeze-drying is a low-temperature dehydration process that is considered a gentle drying method. The process is divided into three distinct steps, each of which poses the risk of potential cellular damage: freezing, primary drying, and secondary drying. The solutes are frozen and a vacuum is applied to remove frozen water by sublimation with its onset below the triple point of water (primary drying). Subsequently, unfrozen water is removed during secondary drying at elevated shelf temperatures. The initial product can be retrieved by adding the amount of removed water, which is called rehydration (Wolker *et al*. 2002). It is an important dehydration technique for yeast and other cells, leading to a stable product. It may cause an initial drop in viability, but the integration of protective agents during this process offers enhanced preservation, with the degree of protection depending upon the specific compound incorporated (Tang *et al*. 2020; Rockinger *et al.* 2021). Such additives not only augment the longevity and viability of the yeast but also play a pivotal role in determining its functional efficacy post-dehydration (Bond 2007; Wolkers and Oldenhof 2015). The type of protectant depends on microorganisms, but some protectants, such as skimmed milk, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose, and polymers (for example, dextran and polyethene glycol) can be used with many species (Morgan *et al*. 2006).

In this study, the influence of two cryoprotectants, i.e., MG and trehalose (with the addition of skimmed milk powder) during the freeze-drying process was investigated. Specifically, the impact on viability, the ability to colonize the tomato phyllosphere and antagonistic properties against *B. cinerea* of two chosen yeast isolates were assessed.

Monosodium glutamate and trehalose significantly enhanced the viability of chosen yeast isolates after freeze-drying, with MG yielding the best results for both tested isolates, as shown in Figure 2. However, the viability did not exceed 40%, so a study with additional substances and combinations might be needed. Based on literature, it can be concluded, that different yeasts react divergently to the addition of different protectants. Various cryoprotectants, including MG and trehalose (as used in this study), and a combination of those protectants, have been described which enhance the viability of yeast strains to similar levels or higher. For example, the cell viability of yeast protected with trehalose (40.5%) while freeze-drying surpassed that with maltodextrin (34.4%) (Chu-Ky *et al*. 2013). Trehalose and xylo-oligosaccharides had the best protective effects on the viability of the probiotic yeast *Saccharomyces boulardii* Seguela, Bastide & Massot (Shu *et al*. 2018). Skimmed milk combined with trehalose proved optimal for *Yarrowia lipolytica* (Wick., Kurtzman & Herman) Van der Walt & Arx PII6a, *Candida kefyr* (*Kluyveromyces marxianus* E.C. Hansen) PII1b, and *Candida sphaerica* (*Kluyveromyces lactis* Stell.-Dekk.) FII7a viability after freeze-drying, while *Candida famata* (*Debaryomyces hansenii* Zopf) MI1a survival rate was the highest with the addition of skimmed milk with MG (Polomska *et al*. 2012). Post freeze-drying, the cell viability of these yeast strains ranged from 74 to 80%, which is much higher than the viability achieved in this study. This viability remained relatively consistent over a year of storage, with *C. famata* being the exception. The initial growth patterns of the yeast were largely retained throughout 6 months of storage. Chen *et al*. (2017) found that the optimal composition of cryoprotectants for *S. boulardii* consisted of lactose, trehalose and MG, which significantly enhanced its freeze-drying survival rate. In a study by Bae *et al*. (2022) the protective effect of turanose combined with skimmed milk maintained the viability of *S. cerevisiae* at 36.8% after 60 days of storage compared to 11.6% for the control. Among established protectants for yeast isolates, other than trehalose and MG, sucrose, maltodextrin and chitosan are used. In the study by Stefanello *et al.* (2019) the addition of 10% sucrose allowed 25% viability until 90 days of storage of the yeast *W. anomalus* IAL 4533. In another study (Silva *et al.* 2021) sucrose (100 $g \cdot l^{-1}$) allowed an 89.63% survival rate of *Candida* spp. on Yeast Extract Glucose Chloramphenicol (YGC) agar. The addition of maltodextrin was found to increase yeast cell viability to over 55% (from about 30% for control) after 56 days of storage by improving their cell integrity (Li *et al.* 2023a) and inhibiting ice-recrystallization (Li *et al*. 2023b). It was demonstrated that chitosan, along with glucose and trehalose, had varying effects on the viability of *Escherichia coli* and *S. cerevisiae* during lyophilization, with trehalose showing better performance for *E. coli* and all treatments being satisfactory for *S. cerevisiae* throughout the 60 days evaluated (Freitas *et al.* 2020). In a different study the addition of chitosan in combination with other protectants such as skim milk powder and lactose significantly improved the stability and survival rate of *S. cerevisiae* after freeze-drying, leading to enhanced fermentation performance without significant physicochemical changes (Park *et al.* 2023). Novel protectants can also improve the viability of yeast during freeze-drying by enhancing the survival rate of the yeast cells. The use of turanose showed a substantial improvement in the survival rate of *S. cerevisiae* during freeze-drying compared to trehalose (Bae *et al.* 2022). A compound protectant containing glycerin, skimmed milk powder, β-cyclodextrin, and sucrose was optimized to enhance the viability and fermentation performance of bread yeast during freeze-drying (Han *et al.* 2021).

Comparable results were obtained for microorganisms other than yeast. For example, *Lactobacillus plantarum* (Orla-Jensen) S32 and *Pediococcus acidilactici* (Lindner) S56 obtained optimum viability (94.01 and 91.30%, respectively) with trehalose (Assohoun 2022). The optimal formulation for protectants used while freeze-drying *Lentilactobacillus hilgardii* (Douglas and Cruess) Q19 freeze-dried cytoprotectants was skimmed milk powder, yeast extract powder, and MG with a survival rate of 87.85 (Wang *et al*. 2023). The addition of MG helped to achieve a freeze-dried *Lactobacillus acidophilus* (Johnson) CRL2074 survival rate of 96% after 30 days of storage (Aristimuño Ficoseco *et al*. 2023). In the same study, *Limosilactobacillus fermentum* (Beijerinck) CRL2085 and *Limosilactobacillus mucosae* (Roos) CRL2069 reached maximal viability when suspended in fructose, sucrose, trehalose, MG and Whey Protein Concentrate (WPC) mixture and WPC trehalose mixture. Monosodium glutamate and the WPC with trehalose were among the best-suspending media to preserve the bacteria's high viability. In another study, the most effective protectants for freeze-drying were identified as glycerol, MG and skim milk. When *Streptococcus thermophilus* (Orla-Jensen) STX2 was freeze-dried using this optimal formulation, it achieved a peak cell viability of 93.58% (Lu *et al*. 2017).

For the implementation of a biological plant protection agent in agronomic settings, it must follow specific criteria. For example, the agent should demonstrate rapid and efficient colonization capabilities on the plant surface, thereby ensuring a consistent suppression of pathogens in a competitive and challenging habitat (Iftikhar *et al*. 2020; Grabka *et al*. 2022; Nigam and Mukerji 2023). Our research findings indicate that both yeast isolates, i.e., 117/10 and 114/73, exhibited environmental stability before being freeze-fried (Fig. 1). In maintaining the isolates' ability to survive on the leaf surface post-freeze-drying, both MG and trehalose treatments seemed to effectively preserve it. The average yeast colony counts for these protectant-treated isolates were statistically similar to those of the control group before freeze-drying, suggesting that these protectants successfully retained the yeast isolates' colonizing ability through freeze-drying. Studies on the dynamics of yeast colonies on the surface of plant leaves before and after lyophilization have received limited attention. Nevertheless, some research has been dedicated to understanding how yeast colonies behave on different plant surfaces, including fruit during storage processes. For example, in the study by Navarta *et al.* (2020) yeast *Cryptococcus laurentii* (Kuff.) cryoprotected with 10% skimmed nonfat milk, 0.5% yeast extract, and 1% glucose after reaching its maximum level between 10 and 15 days remained on apples for 40 days. In another study on freeze-dried yeast on apples, trehalose significantly protected and increased the population of *Cryptococcus laurentii* (Kuff.) Skinner and *Rhodotorula glutinis* (Fresen.) Harrison in apple wounds, mainly during the first 48 hours after inoculation (Li *et al.* 2008). However, in those experiments no comparison was made with pre-freeze-drying yeast populations. Our study seems to present a new approach to the problem.

The phyllosphere presents a challenging and ever-changing habitat where yeasts and other microorganisms encounter various biotic and abiotic stresses (Koskella 2020; Bashir *et al.* 2022; Perreault and Laforest-Lapointe 2022). These include limited nutrient access, exposure to ultraviolet rays, fluctuations in temperature and humidity, and the presence of harmful substances like mycotoxins and fungicides (Bechtold *et al.* 2021; Liu *et al.* 2022; Gouka *et al.* 2022; Bhandari *et al.* 2023; Lin *et al.* 2023). In yeast, trehalose is responsible for a response to a variety of stress factors, preventing cellular damage by protein stabilization and suppression of denatured proteins. Additionally, acting as a water substitution it preserves the properties of yeast hydrated membrane (Argüelles 2000; Mahmud *et al.* 2010; Asada *et al.* 2022). Its presence can alter cellular processes, nucleotide metabolism, protein modification, and amino acid metabolism (Dhanasekaran *et al.* 2021). It can prevent oxidative stress damage by eliminating the hydroxyl radicals formed by a Fenton reaction (Moon *et al.* 2020; Lin *et al.* 2021) and increase the content of intracellular malondialdehyde (MDA) and adenosine triphosphate (Li *et al.* 2023a). Monosodium glutamate is vital for the maintenance of redox balances, biosynthesis of important metabolites and production of organic substrates in yeast (Mara 2018). Yeast can utilize MS as a source for lipid production (Gong *et al.* 2016) and use it to overcome nutritional stress (Dey and Rangarajan 2021). Therefore, it can be assumed that protectors used during the freeze-drying process can have a positive impact on yeast's ability to survive in the phyllosphere post-re-hydration.

Chosen yeast isolates, i.e., 117/10 and 114/73, demonstrated different antagonistic efficacy against *B. cinerea* symptoms on tomato leaves. Before freeze-drying, isolate 117/10 was effective in both prevention and intervention approaches, while isolate 114/73 showed a significant reduction only in the prevention strategy (Table 2). Post-cryopreservation results varied according to the protective treatments used. With skimmed milk as the sole protectant, neither yeast isolate exhibited an ability to significantly reduce *B. cinerea* symptoms. However, when trehalose was added to skimmed milk, 117/10 retained its antagonistic properties in both prevention and intervention, while 114/73 lost its efficacy. Upon the addition of MG to the cryoprotection, both yeast isolates demonstrated strong antagonistic capabilities against *B. cinerea* across both kinds

of treatment, significantly reducing disease symptoms on tomato leaves. The preventative application of the 117/10 isolate, when protected with either trehalose or MG during freeze-drying, resulted in the lowest DSI, demonstrating these protectants' ability to maintain the biocontrol efficacy of the yeast isolates against *B. cinerea*. As in this study, previous reports have stated that various cryoprotectants can help to maintain yeast antagonistic abilities against plant pathogens. For example, freeze-dried yeast *Sporidiobolus pararoseus* ZMY-1 protected with skimmed milk powder, polyethylene glycol and trehalose retained good biocontrol efficacy against *B. cinerea* in apples (Han *et al*. 2021a). In other studies, increasing trehalose levels in *C. laurentii* improved its ability to control symptoms caused by *B. cinerea* in apples after freeze-drying the yeast (Li and Tian 2020; Navarta *et al*. 2014). Skimmed milk, yeast extract and glucose were successfully used as cryoprotectants for a mixed formulation of two biological control agents, *Kosakonia radicincitans* and *C. laurentii* against the same pathogen in apples (Navarta *et al*. 2020). A formula consisting of skimmed milk powder, polyethene glycol and trehalose was used while freeze-drying the marine yeast *Sporidiobolus pararoseus* (H.C. Olson & B.W. Hammer) ZMY-1 to maintain its ability to reduce disease incidences and lesion diameters in apples caused by *Penicillium expansum* (Link), *B. cinerea* and *Alternaria alternata* (Fr.) Keissl. (Han *et al*. 2021b).

Cryoprotectants are crucial for safeguarding microorganisms during freeze-drying. Since different microorganisms respond differently to freezing, it is essential to choose the right cryoprotectant or a combination of them. This ensures that they remain viable after lyophilization. Therefore, a customized approach, backed by thorough research, is vital to select the best cryoprotectants for each microorganism, optimizing their survival and preserving their ability to reduce detrimental impacts caused by phytopathogens.

In this study it was demonstrated that protectants such as MG and trehalose can play a significant role in the freeze-drying process of yeast isolates, impacting their viability, the ability to colonize leaf surfaces, and their antagonistic activity against *B. cinerea*.

The efficacy of the protective treatment with antagonistic yeast depended upon a few variables. These included the specific yeast isolate being used, the supplementary substance incorporated during the lyophilization process, the timing of the treatment (prevention and intervention), and the interaction among these factors.

The treatment with yeast isolate 117/10 was effective when either trehalose or MG was added for the freeze-drying process. The preventive treatments showed superior efficacy in comparison to the intervention.

On the other hand, for yeast isolate 114/73, the addition of trehalose did not contribute to the preservation of its antagonistic capabilities, while the addition of MG helped to preserve them. When MG was added, prevention treatment with the 114/73 isolate was more effective than intervention.

To summarize, isolate 117/10 was more effective when cryopreserved with either trehalose or MG. The preventive treatments were more efficient than intervention.

These findings emphasize the nuanced role of a range of factors in determining the effectiveness of treatments with antagonistic yeast. Further research is needed to analyze these interactions and optimize treatment strategies. The enhancement of the viability, colonization ability, and biocontrol efficacy of yeast isolates against *B. cinerea* post-freeze-drying offers promising applications in sustainable agriculture.

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