ASSESSMENT OF YEAST EFFICACY IN CONTROLLING *Botrytis cinerea* YU2403, THE CAUSE OF POST-HARVEST DECAY IN STRAWBERRIES

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SUMMARY

The yeast strains isolated from pickled cabbage brine were used to study their antagonistic ability against *Botrytis cinerea* YU2403, the causative agent of strawberry rot, through co-cultivation under in-vitro conditions. Six yeast strains with over 65% antagonistic effectiveness were selected for further safety evaluation on blood agar and strawberry fruit. Three yeast strains, including DT195-2, PCT02, and SMP902, which did not cause hemolysis or spoilage, were chosen to assess further their effectiveness in controlling *B. cinerea* YU2403 on strawberries. The results showed that two yeast strains, *Kazachstania* sp. YU2401 (PCT02) and *Kazachstania* sp. YU2402 (DT195-2) have the potential to be developed into biocontrol agents to prevent post-harvest strawberry rot, providing a new approach for preserving agricultural products.

Keywords: Strawberries, *Botrytis cinerea, yeast*, post-harvest, *Kazachstania* sp.

INTRODUCTION

Strawberries, delicious and nutritious yet delicate berries, are a prime target for mold infections, including *Botrytis cinerea*. Physical damage during cultivation and post-harvest handling creates opportunities for fungal invasion, leading to fruit rot. Numerous studies have highlighted the severe post-harvest damage caused by *B. cinerea* on strawberries (Abbey *et al*., 2019; Feliziani, Romanazzi, 2016). In gardens, *B. cinerea* spores easily spread from infected to healthy fruits and can be transmitted to other gardens through wind and daily irrigation. Harvesting, transportation, and storage are critical stages where factors such as high humidity, low temperatures, and bruising can create open wounds, further promoting fungal growth.

Chemical treatments have been the preferred method for many years to control and mitigate fungal infections. However, the overuse of synthetic chemical fungicides has detrimental effects on human health and the environment and contributes to the increased resistance of many fungal strains. Therefore, seeking alternative methods using biological agents has become essential (Zhang *et al*., 2020; Damalas, Koutroubas, 2018). In recent years, antagonistic microorganisms, including bacteria, fungi, and yeast, have shown high efficacy in preventing various post-harvest diseases in fruits (Chen *et al*., 2018). Yeasts, in particular, offer several advantages over bacteria, such as lower nutritional requirements, the ability to colonize dry and inaccessible surfaces for extended periods, and rapid growth rates (Parafati *et al*., 2016). Yeasts have been utilized in traditional fermentation products for thousands of years and are now widely used in the food industry. In recent decades, research on the biological control potential of yeasts has received considerable attention and is increasingly being applied.

This study aims to identify potential yeast strains for controlling *Botrytis cinerea* YU2403, which causes post-harvest rot in strawberries. The results are a crucial first step in developing effective and sustainable biological control measures, which will help protect crops, maintain strawberry quality during post-harvest storage, and promote sustainable agricultural economic development.

MATERIAL AND METHODS

Material

The *Botrytis cinerea* YU2403 strain (NCBI Identifier: PP952080), which causes strawberry fruit rot, is stored at the Microbiology Application Laboratory, Practice Experiment Center, Yersin University of Dalat.

The samples of fermented pickle juice were purchased in Dalat City, Lam Dong, Vietnam.

Methods

Isolation and Screening of Yeast Strains with Antagonistic Ability Against B. cinerea YU2403 in-vitro

1 mL of fermented pickle juice was diluted with 9 mL saline solution (0,9% NaCl, Merck). The suspension was then diluted to a concentration of 10^{-3}, and 0,1 mL of the diluted solution was spread onto PDA agar medium containing antibiotics (5 mg/L chloramphenicol) and incubated at 25°C for 3 days. The resulting colonies were purified, and their morphology was recorded before being preserved in glycerol at -20 °C for further experiments.

The antagonistic ability of yeast strains against *B. cinerea* YU2403 was tested using a co-cultivation method based on the studies by Cloutier *et al.,* 2019; Sharma *et al.*, 2019. A 7 mm diameter fungal disc was placed at the center of a petri dish containing 15 mL of PDA agar medium. Yeast colonies were streaked symmetrically at a distance of 2,5 cm from the fungal disc at the center. PDA medium plates containing only the fungal disc were used as control treatments. These experiments were incubated at a temperature of 25±2°C, and results were observed and recorded after 5 days. The percentage of fungal inhibition (GI) was calculated using the following formula:

$$GI=(C-T)/(C-C_1)*100$$

C is the diameter of *B. cinerea* YU2403 in the control treatment (mm), C1 is the initial diameter of the fungal disc (7 mm), and T is the diameter of the fungus in the antagonistic treatment.

Safety Assessment of Potential Yeast Strains

The yeast strains that could resist *B. cinerea* YU2403 were selected to evaluate their safety *in vitro* and on strawberries. The hemolysis test was performed by spot-inoculating the yeast strains on Columbia agar supplemented with 5% sheep blood. After 48 hours of incubation at 25±2°C, the hemolysis zone around the colonies was observed and recorded. The safety test on strawberries was conducted according to Chen's description, with minor modifications. A 30 µL suspension containing yeast spores (10^6 spores/mL) was directly injected into physically wounded strawberries, which were then incubated at 25±2°C for 5 days. Observations on the strawberries, including the color around the wound and any fermentation odor, were recorded and evaluated.

Evaluation of the effectiveness of control B. cinerea* YU2403 *on strawberries

Strawberries that showed no signs of disease or physical damage were sterilized using 2% NaClO, and then rinsed three times with distilled water. The strawberries were sterilized with 70% ethanol under sterile conditions, rinsed twice with distilled water to remove the alcohol, and air-dried on a sterilized towel. Before the experiment, all strawberries were wounded with a 2 mm diameter and 2 mm deep incision. A 30 µL spore suspension of *B. cinerea* YU2403 (10^5 spores/mL) was injected into the wound, allowed to dry at room temperature for 30 minutes, followed by an injection of 30 µL yeast spore suspension (10^6 spores/mL) and allowed to dry at room temperature for 30 minutes. Distilled water was used as a control in place of the yeast. The strawberries were stored individually at 25±2°C with 90-95% relative humidity for 5 days. The Disease Index (DI) and Control Efficacy (CE) were calculated according to Chen's description. The rot index of strawberries caused by *B. cinerea* YU2403 was calculated as follows: 0 = no lesions; 1 = a few scattered lesions covering < 2% of the fruit surface; 2 = extensive lesions covering > 2% but < 5% of the fruit surface; 3 = extensive lesions covering > 5% but < 25% of the fruit surface; and 4 = extensive lesions covering > 25% of the fruit surface.

$$DI = (\sum_{i=0}^{4} n_i \times i)/(4 \times \sum_{i=0}^{4} n_i) \times 100\%$$

Where i is the disease severity (0–4) and n_i is the number of strawberries with a severity of i. The DR was calculated using the following formula: CE (%) = 100 × (DI-B_c − DI-test)/DI-B_c. Where B_c is *B. cinerea* YU2403.

***Identification of antagonistic yeast strains against Botrytis cinerea* YU2403**

Yeast colonies were extracted using the ABT DNA extraction kit according to the standard procedure. The total DNA was then amplified for the ITS gene using the primer pair ITS-1 (5'- TCCGTAGGTGAACCTGCGG- 3'), ITS-4 (5'TCCTCCGCTTA TTGATATGC- 3'). The thermal cycling conditions for the PCR reaction were as follows: 95ºC for 3 minutes, then 35 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 1 minute. Finally, the reaction was held at 72ºC for 5 minutes, and the samples were maintained at 4ºC. The PCR products were sequenced using the Sanger method at DNA SEQUENCING Co., Ltd.. The sequencing results were processed using BioEdit 7.2 software. These sequences were then blasted into the NCBI database to identify similar known sequences. Mega 7.0 software was used to construct the phylogenetic tree, with a bootstrap analysis of 1000 iterations to ensure the tree's reliability. This tree helps understand the evolutionary relationships and confirms the identification of the yeast strains.

Data analysis

The experiments were repeated three times to determine the mean values and standard deviations (SD). This repetition ensures the reliability and accuracy of the data.

RESULT AND DISCUSS

The results of yeast isolation and screening for antagonistic activity against *B. cinerea* YU2403

From 32 samples of pickled mustard juice, 24 yeast strains were isolated. These strains were distinguished from bacteria based on their oval cell morphology and budding capability. The 24 strains were screened for their ability to antagonize the fungus *B. cinerea* YU2403. The antagonistic results are shown in Figure 1, where most tested strains demonstrated the ability to inhibit *B. cinerea* YU2403. These results can be categorized into three levels of effectiveness: six strains exhibited strong antagonistic effects above 65% (with strain DT195-2 being the most effective), thirteen strains showed moderate antagonistic effects ranging from 50,23% to 62,1%, and five strains had low antagonistic effects below 50% (with strain DK03 being the least effective). The inhibition capability of these yeast strains against *B. cinerea* YU2403 is superior compared to the study by Chen *et al.* (2019), which isolated yeast strains with antagonistic effects on *B. cinerea* JYC2142 ranging from 18,7% to 36,5%. The yeast strains antagonize *B. cinerea* through mechanisms such as the production of cell wall-degrading enzymes, iron competition, nutrient competition, and space competition. The study by Parafati *et al.* (2016) demonstrated that certain yeast strains isolated from food products could inhibit the growth of *B. cinerea*. Therefore, the strains with strong antagonistic effects were selected for further experiments.

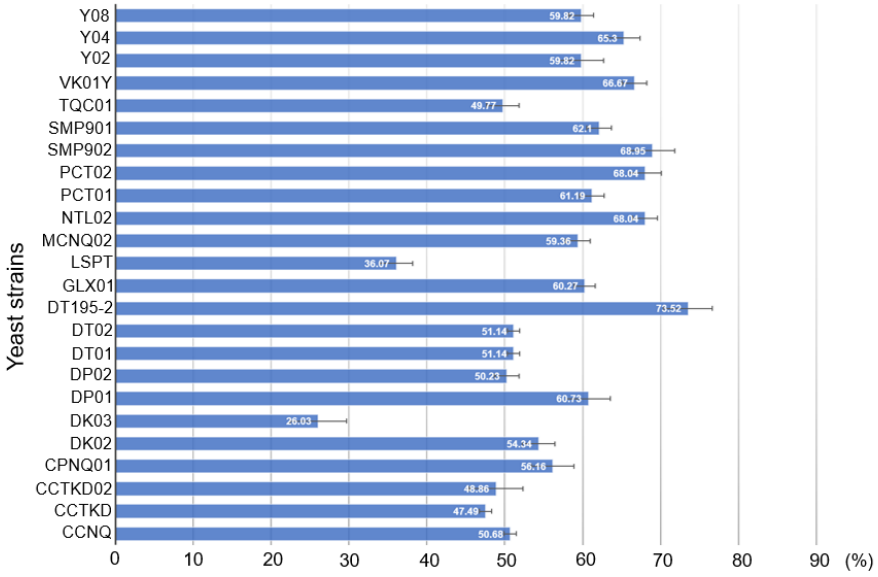

Figure 1. The ability of yeast strains to control *Botrytis cinerea* YU2403

Safety characteristics of yeast strains

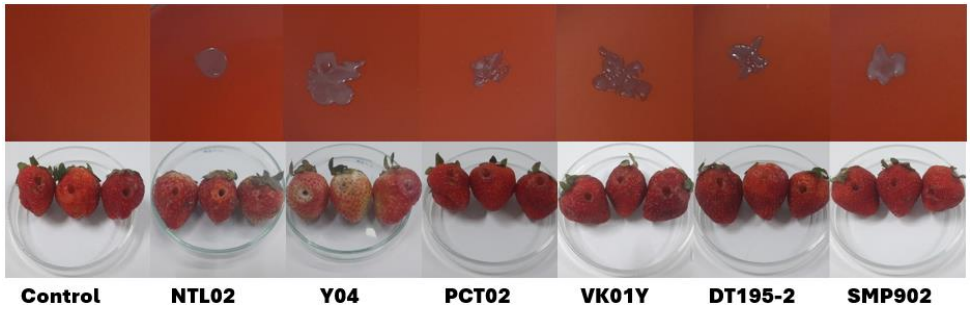

Figure 2. Safety test results on blood agar and strawberries

Six yeast strains VK01Y, NTL02, Y04, PCT02, SMP902, and DT195-2, which can resist *B. cinerea* under in-vitro conditions, were further used to evaluate safety efficacy on blood agar and strawberries. The safety test results on blood agar, as shown in Figure 2, indicated that all six tested yeast strains did not create a resolution zone around the colony (hemolysis γ). For the safety test on strawberries, after five days of observation, strawberries tested with the strains Y04, NTL02, and VK01Y showed small lesions, with some mycelium appearing around the wounds, exuding fluid, and a slight fermentation odor. The remaining three strains, PCT02, DT195-2, and SMP902, showed no surface lesions or fruit flesh fermentation. The wound areas treated with these yeast strains were dry, not mushy, and did not exude fluid, similar to the control.

Effectiveness of controlling *B. cinerea* YU2403 on strawberries

Table 1. Effectiveness of yeast in protecting strawberries from *B. cinerea* YU2403 infection

Treatment	(%) DI	(%) CE
Control	100±0	0
DT195-2	0	100±0
SMP902	45,42±6,17	54,58±6,17
PCT02	0	100±0

After 5 days, all strawberries in the control treatment showed damage with a disease index of 100%. In contrast, the disease indices for the treatments with strains DT195-2, SMP902, and PCT02 were 0%, 45,42%, and 0%, respectively. This corresponds to Control Efficacy indices of 100%, 54,58%, and 100%, as shown in Table 1. This indicates that the two strains DT195-2 and PCT02, effectively protected strawberries against *B. cinerea* YU2403. In the control treatment, 100% of the strawberries were completely covered by fungal hyphae. In the treatment with strain SMP902, some strawberries showed hyphae around the wounds, which gradually spread. Meanwhile, when treated with DT195-2 and PCT02, strawberries showed no disease or fungal growth, as shown in Figure 3.

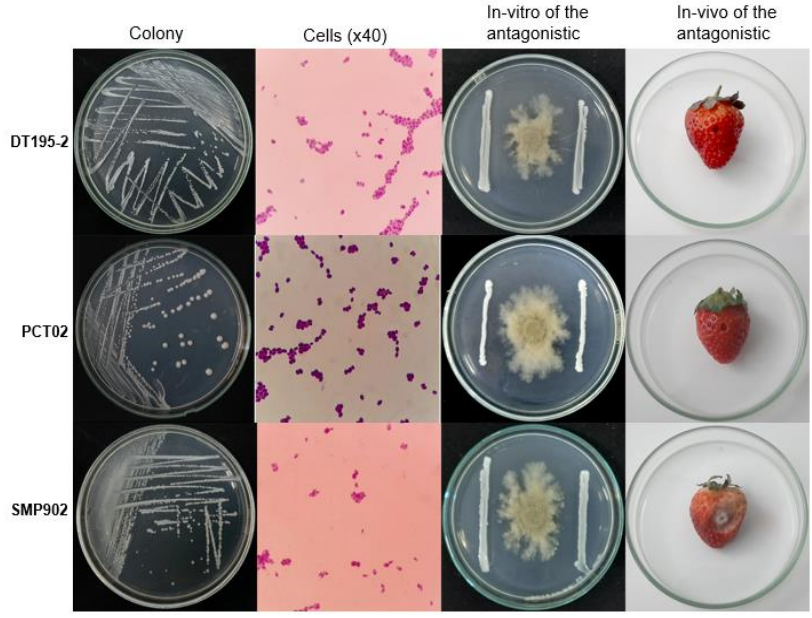

Figure 3. Effectiveness of yeast strains in controlling *B.cinerea* YU2403

The study by Ximena demonstrated that the decay rate of grapes caused by *B. cinerea* significantly decreased when treated with yeast strains. This is attributed to the yeast quickly colonizing the wound's surface, competing for nutrients and space, preventing the spores of *B. cinerea* from germinating. *W. anomalus* strains have shown the ability to reduce the growth of *B. cinerea* in in-vivo experiments (Maluleke *et al*., 2022). The identified volatile compounds were grouped into various chemical families, such as higher alcohols, aldehydes, esters, organosulfur compounds, monoterpenes, ketones, and aromatic hydrocarbons. Notably, phenylethyl alcohol, isoamyl alcohol, n-butanol, and 2,5-dimethylpyrazine, which appear to originate from the yeast, have been demonstrated to effectively inhibit the germination of spores and the growth of hyphae of *B. cinerea*, Aspergillus, and Penicillium species.

Molecular identification of yeast strains

Based on the identification results from the NCBI gene bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the two strains DT195-2 and PCT02 are similar to *Kazachstania* sp. Additionally, the phylogenetic tree analysis results show that the strains DT195-2 and PCT02 are in the same branch as *Kazachstania* sp. B24 15, *Kazachstania* sp. B24 3, *Kazachstania* sp. B2 TP1 4, and *Kazachstania* sp. IMB BR1 (Figure 4). Therefore, the two strains DT195-2 and PCT02 are two strains of *Kazachstania* sp. YU2402 (NCBI gene bank identification code: PP952090) and *Kazachstania* sp. YU2401 (NCBI gene bank identification code: PP957694). Further in-depth studies are needed to identify the species of these two bacterial strains.

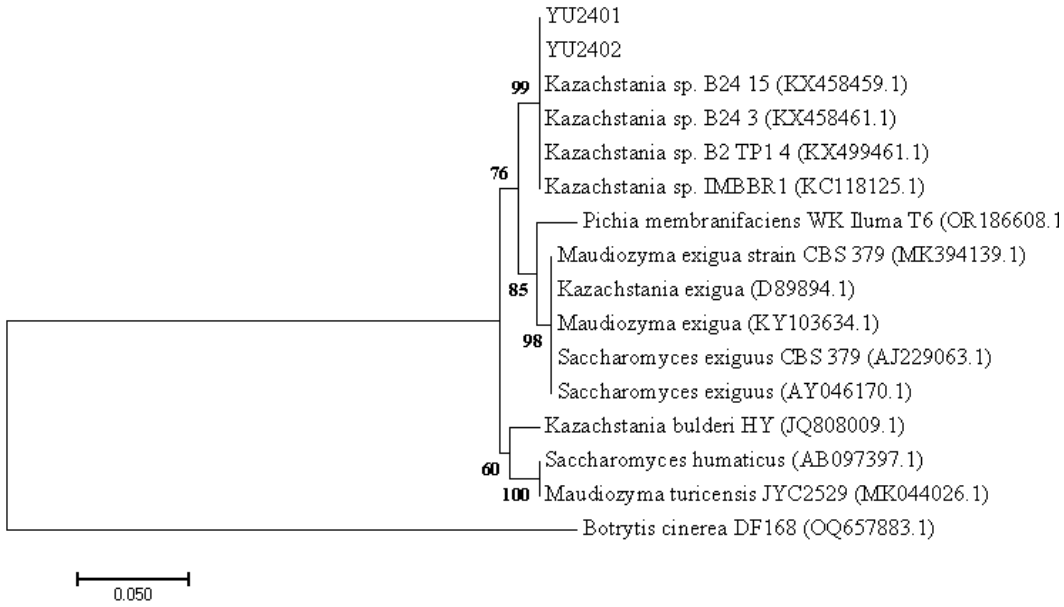

Figure 4. Phylogenetic tree of the strains *Kazachstania* sp. YU2401 and *Kazachstania* sp. YU2402

CONCLUSION

From the samples of fermented mustard greens, 24 yeast strains were isolated. Among these, six strains (VK01Y, NTL02, Y04, YU2401, SMP902, YU2402) demonstrated significant antagonistic activity against *B. cinerea* YU2403, achieving inhibition rates of over 65%. Notably, three strains (DT195-2, SMP902, PCT02) exhibited strong antifungal properties and showed no hemolysis or rot symptoms on strawberries. This indicates their potential safety for application in post-harvest disease control. Among them, two strains, PCT02 and DT195-2, identified as *Kazachstania* sp. YU2401 and *Kazachstania* sp. YU2402 were evaluated as promising candidates for controlling *B. cinerea* YU2403-induced rot in strawberries. However, further advanced biological techniques are needed to accurately classify and identify these two yeast strains, as well as to provide deeper insights into their mechanisms of action and ensure their effectiveness and safety in practical applications.

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ĐÁNH GIÁ HIỆU QUẢ CỦA NẤM MEN TRONG KIỂM SOÁT *Botrytis cinerea* YU2403, NGUYÊN NHÂN GÂY THỐI QUẢ SAU THU HOẠCH Ở DÂU TÂY

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TÓM TẮT

Các chủng nấm men được phân lập từ dịch dưa cải muối được sử dụng để nghiên cứu khả năng đối kháng nấm *Botrytis cinerea* YU2403, tác nhân gây bệnh thối quả dâu tây, thông qua phương pháp đồng nuôi cấy trong điều kiện *in-vitro*. Sáu chủng nấm men có hiệu quả đối kháng trên 65% đã được chọn để tiếp tục đánh giá an toàn trên môi trường thạch máu và thử nghiệm an toàn trên quả dâu tây. Ba chủng nấm men gồm DT195-2, PCT02 và SMP902 không gây tan máu và không gây ra bất kỳ các hiện tượng thối hỏng đã được lựa chọn để tiếp tục đánh giá hiệu quả kiểm soát *B. cinerea* YU2403 trên quả dâu tây. Kết quả cho thấy hai chủng nấm men *Kazachstania* sp. YU2401 (PCT02) và *Kazachstania* sp. YU2402 (DT195-2) có khả năng ứng dụng phát triển thành chế phẩm sinh học ngăn ngừa thối quả dâu tây sau thu hoạch do nấm *B. cinerea* gây ra, mở ra triển vọng mới cho việc bảo quản nông sản.

Từ khóa: Dâu tây, *Botrytis cinerea*, nấm men, sau thu hoạch, *Kazachstania* sp.

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