

Antifungal Activity of Endophytic Yeasts against Grape Phytopathogens

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Abstract

In this study, the antifungal activity of endophytic yeast strains isolated from the generative parts of plants in Uzbekistan against five important phytopathogenic fungi—*Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium sp1*, and *Penicillium sp2*, was evaluated using the agar diffusion method. The results showed that strain 01 (particularly against *Penicillium sp1*) and strain 3 (against *Fusarium oxysporum* and *Penicillium sp1*) formed the largest inhibition zones, demonstrating broad-spectrum antifungal potential. *Penicillium sp1* was the most sensitive pathogen, while *Aspergillus niger* proved to be the most resistant. Considering the significant growth in Uzbekistan's fruit and vegetable exports during 2023-2025, these endophytic yeasts are promising as commercial biocontrol agents. They offer a sustainable alternative to chemical fungicides and can help reduce post-harvest losses (up to 30%).

Keywords

Endophytic Yeasts, Antifungal Activity, Phytopathogenic Fungi, Biocontrol, *Metschnikowia*, *Fusarium oxysporum*, *Penicillium*, *Aspergillus*, Uzbekistan Fruit Exports

1. Introduction

Endophytic yeasts, including species of the genus *Metschnikowia*, are rapidly developing worldwide as biocontrol agents against fungal diseases in agriculture. Amid the ecological risks of chemical pesticides and the rise of antimicrobial resistance, natural biocontrol has become a core component of sustainable agriculture and Integrated Pest Management (IPM) strategies from 2023 to 2025 [1].

For instance, *Metschnikowia pulcherrima* and *M. fructicola* have been proven

highly effective against major fruit and grapevine diseases, such as *Botrytis cinerea* and *Erysiphe necator*. Their biocontrol mechanisms are multifaceted, including nutrient competition (particularly for iron), production of enzymes (e.g., chitinases), synthesis of volatile organic compounds (VOCs), and activation of plant immune responses.

In 2025, new models based on VOCs from *M. pulcherrima* were proposed [2]. In 2024, *M. fructicola* and *M. pulcherrima* were registered in the European Union as commercial products (e.g., NOLI) [3]. The “zero residue” (NOLI) approach is evolving from an option to an economic necessity, as stated by NOLI founder Samir Dorbi. Chemical pesticides can no longer serve as the sole foundation for plant protection [4].

Integrated Disease Management (IDM) has become the global standard. The global biopesticides market is projected to grow from approximately USD 4.3 billion in 2020 to USD 8.5 billion by 2025, with a compound annual growth rate (CAGR) of 14.7%.

The main risks associated with the continuous use of pre- and post-harvest fungicides include:

- 1) Pesticide residues exceeding Maximum Residue Limits (MRLs), leading to export restrictions.
- 2) Environmental pollution: soil contamination and pathogen resistance.
- 3) Consumer health risks (food safety): residues linked to carcinogenicity and endocrine disruption.
- 4) Economic losses due to reduced export volumes.

For these reasons, biological methods are gaining critical importance in the post-harvest preservation of fruits and vegetables.

In organic vineyards, *Metschnikowia*-based products have demonstrated 70% - 80% efficacy against downy mildew and gray mold [3]. During storage, they reduce decay caused by *Fusarium solani* (potato), *Penicillium digitatum* (citrus), and *Ascosphaera apis* (honeybees) by up to 80% [5]. In 2025, *Metschnikowia* formulations combined with nanobiotechnology (nanoparticle-enhanced) are undergoing testing, with reported increases in efficacy of up to 2-fold [6].

The global biocontrol market is expected to grow by an additional 15% in 2025 (particularly in the EU and USA). Key trends from 2023-2025 include: *M. pulcherrima* achieving 70% - 80% control of gray mold on grapes [7], EU commercial post-harvest applications showing 60% - 75% efficacy, VOC- and nano-formulations exceeding 80% efficacy [6].

This trend represents a significant opportunity for fruit-producing countries such as Uzbekistan.

Uzbekistan ranks among the world's leading fruit producers, with an annual production of approximately 5 million tons (including apples, dates, peaches, pomegranates, and others) and steadily increasing export volumes.

The present study evaluates the antifungal activity of endophytic yeasts isolated from grapes against major phytopathogenic fungi. Based on these findings, future

research continues to focus on extending the shelf life of fruits through biological means.

2. Research Methods

Isolation and Identification of Endophytic Yeasts.

2.1. Sample Collection, Isolation, and Identification of Endophytic Yeasts

Endophytic yeasts were isolated in the Laboratory of Biochemistry and Biotechnology of Physiologically Active Compounds at the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan. A total of 50 endophytic yeast isolates were obtained using the enrichment technique from the generative and storage organs of 18 different plant species collected from trees and shrubs growing in Tashkent city and the surrounding Tashkent region.

Flower samples were collected in March and April from trees in the Yunusobod district of Tashkent several hours prior to the experiments. Fruit samples were gathered in May from various regions of Uzbekistan, including Victoria strawberry (*Fragaria vesca*) from Hasanboy (Tashkent region), Korolevskiy variety strawberry from Qibray district (Tashkent region), apricots from Surkhandarya region, white and black mulberry from Yangiyo'l district (Tashkent region), and sweet cherry from Namangan region. In addition, apples stored from the previous season were included in the study. All plant materials were identified based on morphological characteristics.

Samples were processed under aseptic conditions. Surface sterilization was carried out using 70% ethanol (3 - 5 minutes), followed by 3% sodium hypochlorite, and then rinsed three times with sterile distilled water. The final rinse water was plated onto agar medium to verify sterilization efficiency; no microbial growth was observed, confirming the effectiveness of the surface sterilization procedure.

Sterilized materials were cut into approximately 5 mm pieces. Plant pieces were transferred into flasks containing liquid Sabouraud medium supplemented with cefotaxime (200 mg/L) to inhibit bacterial growth during the initial enrichment phase. Cefotaxime was not used in subsequent cultivation steps. Cultures were incubated at 28 °C with shaking at 140 rpm for 3 - 5 days.

After visible changes (turbidity and gas production) were observed, the enriched cultures were streaked onto antibiotic-free Sabouraud dextrose agar (SDA) plates to obtain pure cultures. Yeast-like colonies were examined microscopically and repeatedly subcultured on SDA to ensure purity.

The obtained isolates were preliminarily identified according to the methods described by Abdel-Hafez *et al.* (2015) and Kurtzman *et al.* (2000). Yeast cultures were centrifuged at 6000 rpm for 15 minutes to separate the culture supernatant, which was collected for subsequent analyses.

Identification of Yeast Isolates

Initial identification of yeast isolates was performed based on morphological and

cultural characteristics. Colony shape, color, size, and texture on Sabouraud dextrose agar after 3 - 5 days of growth, as well as the microscopic morphology of the cells, were examined.

For more precise identification, isolates were subcultured twice under the original isolation conditions. MALDI-TOF MS analysis was performed on third-generation pure cultures using a 4800 Plus MALDI TOF/TOF™ Analyzer (AB SCIEX, Framingham, MA, USA) as previously described (Welham *et al.*, 2020).

Briefly: A small amount of fresh biomass from 48 - 72 h single colonies grown on Sabouraud medium was suspended in 70% ethanol, vortexed, and centrifuged. The pellet was subjected to protein extraction using formic acid/acetonitrile according to the manufacturer's recommendations. One microliter of the extract was spotted onto a polished steel target plate, air-dried, and overlaid with 1 µL of α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 mg/mL in 50% acetonitrile containing 0.1% trifluoroacetic acid).

Mass spectra were acquired in positive linear mode. Spectra were converted to .txt files using Data Explorer 4.0 software (AB SCIEX) and then imported into BioNumerics 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Spectral profiles were compared using the Pearson product-moment correlation coefficient, and a dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) clustering algorithm. Homogeneous clusters consisting of isolates with visually identical or nearly identical mass spectra were delineated and identified at the genus level and, where possible, at the species level.

A total of 50 endophytic yeast isolates were obtained during the isolation procedure. From these, 25 representative strains were selected for antifungal activity screening based on the following criteria: 1) clear yeast-like morphology and stable growth after repeated subculturing; 2) distinct MALDI-TOF MS spectral profiles to avoid redundancy among closely related isolates; 3) representation of the most frequently occurring genera (*Metschnikowia*, *Meyerozyma*, *Hanseniaspora*, and *Pichia*); 4) preliminary qualitative antifungal activity observed during pilot screening experiments.

MALDI-TOF MS identification confidence Species-level identification by MALDI-TOF MS was accepted when spectral similarity scores were ≥ 2.0 , indicating high-confidence identification, while scores between 1.7 and 1.99 were considered reliable for genus-level assignment. Isolates with scores below 1.7 were excluded from further analysis. Only isolates meeting these confidence thresholds were included in the antifungal assays.

2.2. Evaluation of Antifungal Activity of Endophytic Yeasts

Endophytic yeast isolates obtained from fruits were inoculated into 100 mL of liquid Sabouraud medium and incubated on a rotary shaker at 180 rpm and 28°C for 5 days. After incubation, cultures were centrifuged at 6000 rpm for 15 minutes, and the supernatant was used for antifungal activity testing. After centrifugation, the culture supernatants were passed through sterile 0.22 µm membrane filters to

remove residual yeast cells before antifungal assays. Antifungal activity was evaluated using the agar well diffusion method as described by Coda *et al.* (2008). Phytopathogenic fungi used as test organisms were cultivated on potato dextrose agar (PDA) at 28°C for 7 days. Fungal spore suspensions were prepared and adjusted to 0.5 McFarland standard ($\approx 1 \times 10^7$ CFU/mL). Aliquots (0.1 mL) of each fungal suspension were evenly spread on PDA plates and allowed to absorb for 30 minutes. Sterile cork borers were used to create 10 mm diameter wells in the agar, and 100 μ L of yeast culture supernatant was added to each well. Positive control wells were filled with clotrimazole (1 mg/mL), negative control wells contained sterile Sabouraud medium without yeast extract, and plates inoculated only with the pathogen served as pathogen controls. All plates were incubated at 28°C for 7 days. After incubation, the diameter of the inhibition zone around each well was measured using a standard ruler. Each experiment was performed in triplicate (biological replicates) on different Petri dishes, and the average inhibition zone diameter was calculated.

To minimize non-specific effects, all yeast culture supernatants were standardized prior to antifungal testing. Yeast cultures were incubated under identical conditions (28°C, 180 rpm, 5 days) with the same initial inoculum density (approximately 1×10^6 cells/mL). After centrifugation and filtration, the pH of the supernatants was measured and confirmed to fall within a narrow range (pH 5.6 - 6.0).

Sterile, uninoculated Sabouraud medium processed under identical conditions served as a negative control to rule out medium-derived or pH-related inhibition effects.

Test Phytopathogenic Fungi

The phytopathogenic fungi used in this study (*Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus flavus*, and *Penicillium* spp.) were obtained from the culture collection of the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan. All fungal strains were previously isolated from infected fruits and grapevine tissues and maintained on potato dextrose agar (PDA) at 4°C.

Differentiation of *Penicillium* sp1 and *Penicillium* sp2

Penicillium sp1 and *Penicillium* sp2 were distinguished based on consistent differences in colony morphology (color, texture, and growth rate on PDA), microscopic characteristics of conidiophores and conidia, and sporulation patterns. Although molecular identification was not performed, the stable phenotypic differences observed over repeated subcultures justified their separation as two distinct operational taxonomic units for antifungal screening purposes.

2.3. Statistical Analysis

All experiments were performed in triplicate. Results are expressed as mean \pm standard deviation (SD). Statistical analysis was carried out using one-way ANOVA, and differences were considered significant at $p < 0.05$. Statistically significant differences ($p < 0.05$) were observed among yeast strains within the same pathogen group, particularly for *Penicillium* sp1 and *Fusarium oxysporum*, where strains

M. guilliermondii 01 and *M. pulcherrima* 3 showed significantly higher inhibition zones compared to other isolates.

3. Results and Discussion

3.1. Isolation and Identification of Endophytic Yeasts

Endophytic yeasts were isolated from various plant parts [8]. In this study, endophytic yeasts were obtained from different parts of local plants growing in Uzbekistan, a process consistent with existing data on the global distribution and diversity of endophytic yeasts. According to the review article by Ling *et al.* (2020), endophytic yeasts live in symbiotic relationships within plant tissues (including roots, stems, leaves, fruits, and seeds), and their diversity is influenced by environmental factors (temperature, humidity, precipitation), plant species, and tissue characteristics [8]. The main genera of endophytic yeasts (*Rhodotorula*, *Pichia*, *Candida*, and *Debaryomyces*) are most frequently found in fruits and leaves, with their abundance and species composition affected by seasonal variations (higher occurrence in spring and autumn) [9]. As emphasized in that article, the diversity of endophytic yeasts depends on the internal microenvironment of the plant (pH, nutrient availability) as well as external factors (negative impact of chemical fungicides) [10]. Similar observations were made in our study: the number and quality of isolated strains appear to be related to the sampling season (March-May and September-October) and geographic locations (Tashkent city and surrounding regions).

During the research process, a total of 50 endophytic yeast isolates were obtained from the generative (flowers, fruits) and storage organs of 18 different plant species. More specifically, flower samples were collected in March-April from trees in the Yunusobod district of Tashkent city. Fruit samples were obtained in May from various regions of Uzbekistan: Victoria variety strawberry (*Fragaria vesca*) from Hasanboy (Tashkent region), Korolevskiy variety strawberry from Qibray district (Tashkent region), apricots from Surkhandarya region, white and black mulberry from Yangiyo'l district (Tashkent region), and sweet cherry from Namangan region. In addition, apples (*Malus domestica*) stored from the previous season were included in the study. All plant materials were identified based on morphological characteristics.

These results align with the data presented by Ling *et al.* (2020), who reported a high abundance of endophytic yeasts in the leaves, bark, and internal fruit tissues of fruit crops such as citrus (*Citrus reticulata*), apple (*Malus domestica*), and pear (*Pyrus communis*) [11]. According to their findings, ascomycetous yeasts (*Pichia*, *Hanseniaspora*) predominate in internal leaf and fruit tissues, while basidiomycetous yeasts (*Rhodotorula*, *Cryptococcus*) are more common on fruit surfaces. A similar pattern was observed in our isolates: *Pichia* and *Metschnikowia* genera were more frequently isolated from the internal parts of fruits and flowers.

The isolates identified through MALDI-TOF MS and morphological analysis belonged to six genera, with *Pichia* (19 strains), *Hanseniaspora*, and *Metschni-*

kowia (a combined 10 strains) being the most prevalent. Overall, these isolation and identification results provide a strong foundation for the potential application of endophytic yeasts as biocontrol agents against post-harvest diseases in fruit production in Uzbekistan.

3.2. Evaluation of Antifungal Activity of Endophytic Yeasts

The antifungal activity of endophytic yeasts was assessed using their culture supernatants, with grapevine pathogens serving as test cultures (Table 1). In this study, the antifungal activity of 25 yeast strains against five pathogenic fungi—*Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium sp1*, and *Penicillium sp2*, was evaluated by the agar diffusion method. Activity was measured as the diameter of the inhibition zone (mm), with no activity indicated by a dash (“—”) symbol, which was interpreted as zero inhibition.

Table 1. Antifungal activity of endophytic yeast culture fluid.

Yeast strains	<i>Fusarium oxysporum</i>	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Penicillium sp1</i>	<i>Penicillium sp2</i>
	Inhibition zone (mm)				
<i>control</i>	25 ± 2.1	25 ± 1.8	21 ± 1.6	25 ± 1.9	22 ± 1.8
<i>M. pulcherrima 021</i>	16 ± 1.5	13 ± 1.6	25 ± 2.1	27 ± 1.6	25 ± 1.5
<i>M. guilliermondii 022a</i>	17 ± 1.4	15 ± 2.1	22 ± 1.8	25 ± 1.6	26 ± 1.2
<i>M. guilliermondii 023a</i>	18 ± 1.8	13 ± 1.5	25 ± 1.2	22 ± 1.6	23 ± 1.2
<i>M. guilliermondii 025a</i>	13 ± 1.8	14 ± 2.1	23 ± 2.1	27 ± 1.5	22 ± 1.4
<i>M. pulcherrima 026</i>	13 ± 1.4	-	27 ± 1.8	26 ± 1.6	25 ± 1.4
<i>M. guilliermondii 027a</i>	13 ± 1.6	11 ± 1.2	25 ± 1.2	25 ± 1.2	23 ± 1.4
<i>M. guilliermondii 028a</i>	13 ± 1.4	12 ± 2.1	23 ± 1.4	23 ± 1.4	27 ± 1.4
<i>M. guilliermondii 17</i>	19 ± 1.6	17 ± 2.1	-	29 ± 1.4	24 ± 1.4
<i>M. guilliermondii 18k</i>	15 ± 1.4	16 ± 1.4	25 ± 1.8	25 ± 1.4	23 ± 1.4
<i>H. uvarum 52a</i>	18 ± 1.6	12 ± 1.8	13 ± 1.2	28 ± 1.4	-
<i>H. uvarum 101a</i>	18 ±	12 ±	17 ± 2.1	22 ± 2.1	25 ± 1.4
<i>M. carpopyila 103</i>	18 ± 1.8	12 ± 1.8	22 ±	28 ± 1.8	28 ± 1.2
<i>M. guilliermondii 01</i>	15 ± 1.8	21 ± 1.6	28 ± 2.1	40 ± 1.8	27 ± 1.4
<i>M. guilliermondii 02a</i>	18 ± 1.2	-	23 ± 1.4	23 ± 1.6	13 ± 1.4
<i>M. guilliermondii 03a</i>	18 ± 1.4	23 ± 2.1	25 ± 2.1	25 ± 1.4	13 ± 1.2
<i>M. pulcherrima 67</i>	18 ± 1.4	26 ± 1.6	25 ± 1.4	25 ± 1.4	21 ± 1.2
<i>M. pulcherrima 1</i>	-	18 ±	13 ± 1.2	27 ± 1.4	18 ± 1.4
<i>M. pulcherrima 2</i>	17 ± 1.4	13 ± 1.6	-	25 ± 1.4	23 ± 1.4
<i>M. pulcherrima 3</i>	30 ± 1.2	23 ± 1.6	22 ± 1.8	30 ± 1.4	25 ± 1.4
<i>M. pulcherrima 5</i>	17 ± 1.8	16 ± 1.6	-	25 ± 2.1	-
<i>M. pulcherrima 6</i>	15 ± 1.4	18 ± 1.8	15 ± 1.8	24 ± 1.4	21 ± 1.4

Continued

<i>H. uvarum</i> 14	17 ± 1.2	24 ± 2.1	-	28 ± 2.1	28 ± 1.2
<i>P. kudriavzevii</i> 66	17 ± 1.4	18 ± 1.4	15 ± 1.8	27 ± 1.6	16 ± 1.6
<i>M. pulcherrima</i> 67b	15 ± 1.6	13 ± 1.8	10 ± 2.1	23 ± 1.6	13 ± 1.4
<i>H. uvarum</i> 108a	20 ± 1.8	19 ± 2.1	-	13 ± 2.1	13 ± 1.6

The strongest strains were *M. guilliermondii* 01 (particularly showing maximum activity against *Penicillium sp1*) and *M. pulcherrima* 3 (demonstrating maximum activity against both *Fusarium oxysporum* and *Penicillium sp1*), while the weakest strain, 108, exhibited minimal activity against *Aspergillus* and *Penicillium* species. These results confirm the pathogen-specific selective antifungal potential of the strains (Figure 1).

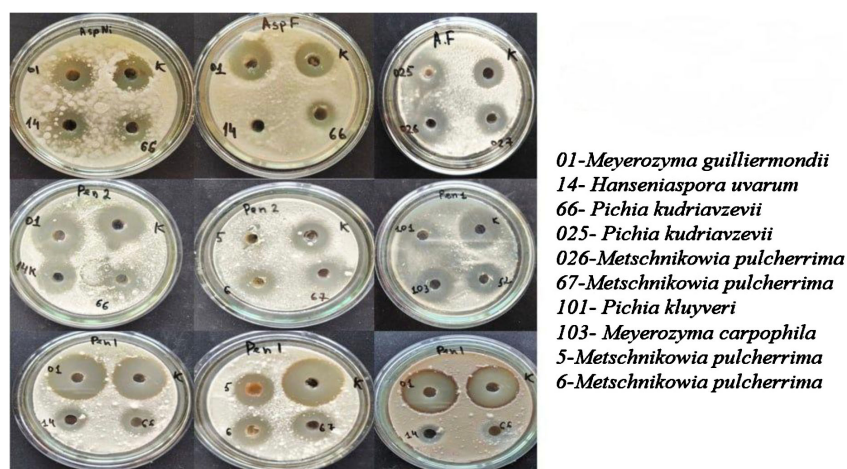


Figure 1. Antifungal activity of endophytic yeasts (mm).

It can be stated as a discussion that fungi such as *Fusarium oxysporum*, *Alternaria alternata* cause 20% - 40% post-harvest losses of the harvested produce (Figure 2). Chemical pesticides cause a decrease in export volumes, and fruit exports are difficult due to chemical pesticide residues [12] [13]. *Metschnikowia* (e.g. *M. pulcherrima*) as a biofungicide solves the MRL (Maximum Residue Limit) problem, as it leaves no residue and complies with EU/US standards (in the case of the NOLI product). For countries with high fruit exports like Uzbekistan (Table 1) (apples, peaches—5+ million tons/year) in cases where the EU (European Union) market is limited due to MRLs (20% - 40% loss), endophytes such as *M. pulcherrima* 3 are an ideal alternative, since the residue is 0 mg/kg [14]. Postharvest fruit rot in Uzbekistan reaches 30%, with *Fusarium* and *Alternaria* being the main causes, and endophytic yeasts are proving their potential as a solution [15].

The obtained results, taking into account the dynamics of Uzbekistan's fruit and vegetable exports, highlight the practical significance of the antifungal activity of endophytic yeasts. According to Figure 3, both export volume and value are steadily increasing from 2023 to 2025. In particular, significant growth is observed

for apples (2023: ~200 thousand tons, 2025: ~400 thousand tons) and grapes (2023: ~100 thousand tons, 2025: ~150 thousand tons), with corresponding increases in export value in million USD (for example, for apples: 2023: ~150 million USD, 2025: ~300 million USD).

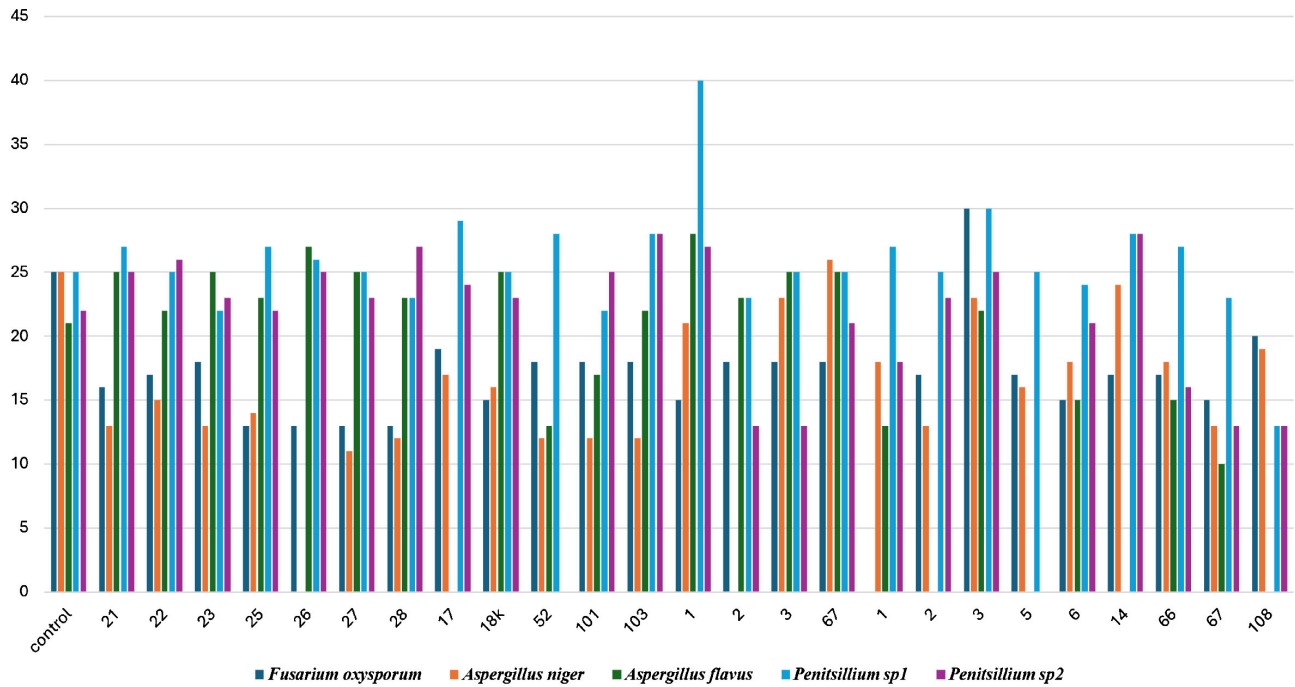


Figure 2. Antifungal activity of endophytic yeasts (mm).

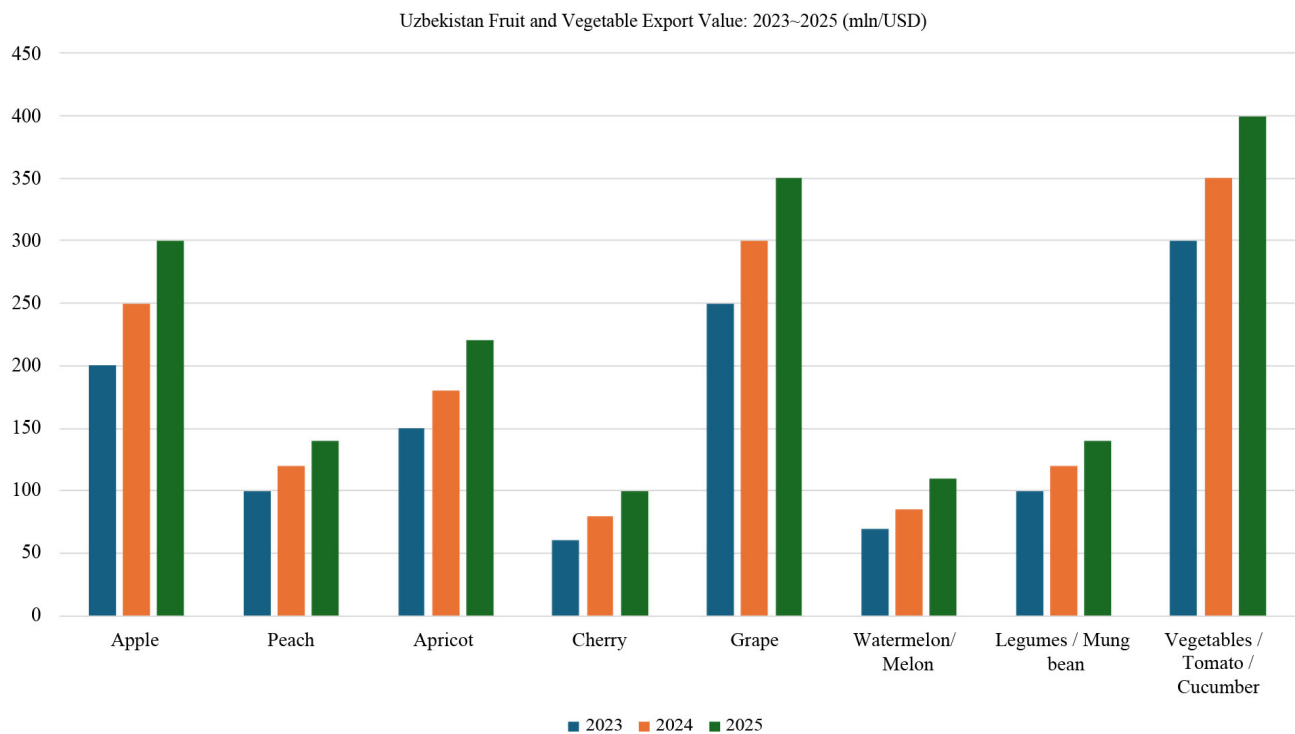


Figure 3. Uzbekistan’s fruit and vegetable export volume value (in million USD) (2023-2025).

However, this growth is at risk due to postharvest losses (up to 30%) caused by phytopathogens such as *Fusarium oxysporum* and *Penicillium* species, which can reduce export volumes by 20% - 40% (due to Maximum Residue Level (MRL) restrictions in the EU market). The high activity of endophytic yeast strains such as *M. guilliermondii* 01 and *M. pulcherrima* 3 (especially against *Penicillium* sp1 and *Fusarium oxysporum*) as promising biocontrol yeasts, particularly *Metschnikowia pulcherrima* and *Meyerozyma guilliermondii*, since they leave no residues and comply with export standards [3] [14].

These findings align with global trends (the biopesticides market is projected to reach approximately 8 - 10 billion USD by 2025 according to various forecasts), offering Uzbekistan opportunities to extend fruit shelf life and increase exports. Nevertheless, future *in vivo* experiments combined with field trials are necessary.

4. Limitations of the Study

This study evaluated antifungal activity using cell-free culture supernatants under *in vitro* conditions, which does not fully reflect the complex interactions occurring *in vivo* on fruit surfaces or in field environments. Whole-cell mechanisms such as nutrient competition, colonization ability, and induced host resistance were not assessed. Therefore, the observed inhibitory effects should be interpreted as preliminary evidence of antifungal potential. Claims regarding commercial biocontrol application remain conditional and require validation through *in vivo* fruit assays and field trials.

5. Conclusion

The study results confirm the high antifungal activity of endophytic yeasts (in particular, strains *M. guilliermondii* 01 and *M. pulcherrima* 3) against important phytopathogens such as *Fusarium oxysporum* and *Penicillium* species. These strains have potential as a sustainable alternative to chemical fungicides for reducing post-harvest decay and protecting Uzbekistan's growing fruit and vegetable exports (in the period 2023-2025). It is recommended to continue research on *in vivo* testing of selected strains and the development of commercial formulations (nano- and VOC-based). This approach will contribute to the implementation of Integrated Pest Management (IPM) and the "zero residue" strategy in Uzbekistan's agriculture.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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