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Antifungal effects of volatile compounds produced by *Tetrapisispora* sp. strain 111A-NL1 as a new biocontrol agent on the strawberry grey mold disease

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ABSTRACT

An antagonistic yeast strain was isolated from the strawberry fruit cv. Paros and its antifungal properties against *Botrytis cinerea* causal agent of strawberry grey mold disease were evaluated under *in vitro* and *in vivo* experiments. The isolate was tentatively *identified* as *Tetrapisispora* sp. strain 111A-NL1 *based* on *phenotypic* characteristics and sequence analysis of *D1/D2 domains* of the *26S rRNA* gene. Volatile organic compounds (VOCs) produced by the 111A-NL1 strain inhibited the mycelial growth of fungal pathogen (75.19%) and conidial germination (63.34%); however, inhibition percentage of mycelial growth of pathogen by dual culture test was less (19.49%). Also, the strain produced pectinase, siderophore, chitinase, IAA, as well as gibberellin, and could solubilize phosphate. Additionally, the disease severity of strawberry grey mold was decreased by employing living cells and volatile metabolites methods by 47.61% and 74.05%, respectively, in comparison with untreated control seven days after inoculation. Therefore, its mode of action might consist of antibiosis and VOCs production by yeast strain 111A-NL1 against *B. cinerea*. The VOCs released by strain 111A-NL1 were analyzed, and thirty-three chemical compounds were determined by gas chromatography-mass spectroscopy (GC-MS). Out of them, Decane (12.79%), Squalene (9.60%), Undecane (7.98%), Benzene, 1,2,3-trimethyl- (7.67%), Nonane, 2,6-dimethyl- (5.69%), Benzene, 1-ethyl-3-methyl- (5.55%), Mesitylene (4.17%), and Phenylethyl Alcohol (3.33%) were the major components. In addition, the selected strain reduced natural decay incidence and weight loss of fruit, and preserved quality parameters of strawberry fruit including firmness, soluble solids content, and titratable acidity. This research averred, for the first time, that the creation of VOCs by *Tetrapisispora* sp. strain 111A-NL1 could play an essential role as a biofumigant in the antifungal activity against strawberry grey mold.

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Introduction

Pre-and post-harvest decay caused by pathogenic infections leads typically to heavy losses in fruit and vegetable production chains throughout the globe and might reach up to 30% of total production (1). The widely known fungal pathogens influencing strawberry fruits are *Botrytis cinerea*, *Colletotrichum* spp., *Mucor* spp., *Rhizopus stolonifer*, and *Penicillium* spp. (2). Among them, the most critical pre-and post-harvest disease of strawberries worldwide is grey mold disease, which is caused by *B. cinerea*. It is a major necrotrophic and economically important phytopathogen (3). Grey mold disease leads to remarkable yield and quality losses in strawberries in field production and postharvest storage worldwide (4). It induces leaf blight, blossom blight, and fruit decay in strawberry. Out of all of these, fruits rot is the most significant and is usually responsible for severe pre- and postharvest losses in the strawberry production industry (5,6). Employing chemical fungicides against fungal pathogens is the fundamental management approach, but challenges of enhancing fungicide resistance, human health issues, and recent legal constraints have created reservations about their application (7). As a result, antagonistic microorganisms as an alternative to chemical fungicides have gained importance in recent years.

Biological control and using antagonistic microorganisms have been widely researched recently and considered as favorable alternatives for chemical fungicides to manage fruit pathogens (8). Among biocontrol agents, yeasts have numerous characteristics that make them entirely appropriate as antagonistic agents, because they are tolerant to most

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agrochemicals, have simple nutritional requirements, survive in a wide range of environmental conditions, proliferate as well as colonize fruit surfaces, and also they do not produce anthropogenic compounds (9). Antagonistic yeasts are competing for space and nutrients with other microorganisms on fruit surfaces as biocontrol agents (10). Using yeast species for biological control of the post-harvest disease has been reported for a wide range of vegetables and fruits (11). Several yeasts have been evaluated and reported as effective management of postharvest disease on citrus (12), papaya (13), strawberry (14,15), grape (16), tomato (17), mango (1), and apples (18). Moreover, the application of yeast as biocontrol agents on fruit postharvest disease did not impair the postharvest qualities of fruits and enhanced gene expression and enzyme activity (19,20). Different mechanisms, including competition for nutrients and space, the production of soluble or volatile inhibitory metabolites, parasitism, and the induction of host resistance, have been demonstrated in the biocontrol of postharvest pathogens using microbial antagonists (21). The production of antifungal volatile organic compounds (VOCs) by postharvest biocontrol agents as modes of action has received increased attention (22,23). The use of VOCs produced by biological control agents is an essential strategy acting as an alternative approach for the management of postharvest disease (18). VOCs released by yeasts identified as *Candida sake* strain 41E against *Penicillium expansum* in red Delicious apples (18), *Candida intermedia* against strawberry fruit rot (14), and *Saccharomyces cerevisiae* against citrus black spot (24) have been reported.

The potential of *Tetrapisispora* sp. 111A-NL1 to control strawberry grey mold disease has not been found as biological control agent yet. We isolated the antagonistic yeast *Tetrapisispora* sp. 111A-NL1 (GenBank accession no. OL675854) from healthy strawberry fruit, and its antagonistic activity against *B. cinerea* was studied *in vitro* and *in vivo*. Also, the effects of this yeast on the postharvest quality of strawberry fruit were investigated.

Materials and methods

Pathogen

A virulent isolate of *B. cinerea* isolated from decayed strawberry fruits showing typical gray mold infection was obtained from the collection of biological control Laboratory at the University of Kurdistan. The isolate was cultured on potato dextrose agar (PDA) medium for seven days at 25 ± 2 ºC. Spores were harvested by flooding the surface of the culture with sterile distilled water, and the concentration of spores was adjusted to 1×10^6 CFU/mL using a hemocytometer.

Fruits

Healthy strawberry fruit (*Fragaria* × *ananassa* Duch. cv. Paros) was chosen for the experiments based on uniform size, shape, maturity, and no physical damage and infection. The selected strawberry fruits were surface disinfected in 70% ethanol (v/v) for 30 s, rinsed with distilled water, and then air-dried under a laminar hood for further study.

Antagonist isolation and culture media

The *Tetrapisispora* sp. strain 111A-NL1 was isolated from the strawberry healthy fruit c.v Paros in Sanandaj, Iran. Briefly, 100 g of strawberry fruit was sliced into small pieces and added to a 250 mL Erlenmeyer flask containing 100 mL sterile distilled water and incubated with shaking (200 rpm) for 60 min at 25 ± 2 ºC. Then, 100 µL of the sample was cultured in nutrient yeast dextrose agar (NYDA) medium (nutrient broth 8 g/L, Yeast extract 5 g/L, dextrose 10 g/L, agar 20 g/L, and distilled water 1000 mL) supplemented with rose-bengal (0.025 g/L) and chloramphenicol (0.2 g/L) in plates (9 cm in diameter) for 72 h at 25 ± 2 ºC. Then, the selected strain was sub-cultured on the NYDA medium and was incubated at 25 ± 2 ºC for 72 h. Single-cell colonies of selected yeast were obtained by streak plate method on NYDA medium. After that, the cells pellets were re-suspended in sterilized distilled water and the yeast cells concentration was adjusted to 1×10^8 cells/ mL.

Characterization and identification of antagonistic yeast

Morphological, biochemical, and physiological characteristics of the strain 111A-NL1 were studied using the procedures described previously (25). The morphology of cells was examined on Yeast Malt *Agar* (*YM Agar*) medium at 25 ± 2 ºC for 1-4 days. The isolate was tested for the fermentation of glucose,

galactose, sucrose, maltose, lactose, raffinose, and trehalose. The ability of the yeast isolate to assimilate carbon and nitrogen sources was also carried out using yeast nitrogen base (YNB) and yeast carbon base (YCB) as the basal medium, respectively. To further identify this isolate, additional assimilation tests and other growth characteristics such as starch formation, gelatin liquefaction, urease activity, and the range of temperature for growth were performed. The glass bead disruption-based method used to extract DNA has been described previously (26). Polymerase chain reaction (PCR) amplification of the D1/D2 domain of the 26S rDNA fragments was amplified using NL1 (5′-GGATATCAATAAGGGGAGGAAAAG-3′) and NL4 (5′-GGTGGGTGTTTCAAGAGGG-3′) primers (27,28). The purified PCR product was sequenced in both directions with the AB13730xl DNA sequencer by Macrogen Company (Seoul, Korea). Then obtained sequences were compared with those of the most closely-related fungal species in the NCBI database employing the Blast program. The phylogenetic tree was created utilizing identical sequences adapted from NCBI with MEGA7 following the Neighbor-Joining method (29). The bootstrap test was conducted 1000 times to verify the reliability of the phylogenetic tree (30). The sequences of the D1/D2 domains of the 26S rRNA gene of strain 111A-NL1 reported in this research were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

In vitro inhibitory effects assay

Dual culture assay

Inhibition of *B. cinerea* by the strain 111A-NL1 was conducted by investigating growth rate inhibition by employing a dual culture technique on an NYDA medium. For this purpose, the mycelial disc plug (5 mm in diameter) from a 5-day-old culture of *B. cinerea* was placed at one centimeter from the edge of plates (9 cm diameter) and then selected antagonistic yeast strain was on the opposite side of the plate. Also, the plates inoculated with the *B. cinerea* alone were utilized as a control. All plates were incubated at 25 ± 2 ºC in 12 h light/12 h dark for 5 days. The diameter of the *B. cinerea* colony was assessed, and inhibition percentage (I) was measured employing the following formula: $I (\%) = [(d_c - d_t)/d_c] \times 100$, in which d_c is the radial growth of the pathogen in control, d_t is radial growth of the pathogen in treatment.

Volatile compounds antifungal test on mycelial growth

The two-sealed-base plate's method was applied, and the selected yeast strain was cultured onto the NYDA medium, and a mycelial disc (5 mm in diameter) from a 5-day-old culture of *B. cinerea* was inoculated on the PDA medium plate. Following that, the plate of the *B. cinerea* was placed on top of a yeast strain plate and then sealed tightly with parafilm (17). The inhibition percentage of the mycelial growth of pathogen for the selected yeast strain was calculated based on the formula as mentioned earlier (Section 2.5.1).

Volatile compounds antifungal test on conidial germination

For this experiment, 10 µL of spore suspension (1×10^6 spores/mL) of *B. cinerea* were pipetted and cultured on water agar medium in plates (90 mm diameter). Then 100 µL of yeast cells (1×10^8 cells/ mL) of selected yeast strain were cultured on NYDA medium in separate plates. Then, the plate, including conidial suspension of *B. cinerea,* was placed upside the selected yeast culture plates, and two dishes were sealed tightly with parafilm. Three replications were utilized for each treatment. Plates containing sterile water (without yeast strain) were used as a control. All plates were incubated at 25 ± 2 ºC. The conidial germination was calculated after 18 h with an optical microscope (Olympus BX51, japans; 60 × magnification using micrometer), and 100 conidia were considered for each replication (14,31). The inhibition percentage of conidial germination of *B. cinerea* (I) was calculated based on the following formula: $I (\%) = [(N_c - N_t)/N_c] \times 100$, in which N_c is the number of germinated conidia in control and, N_t is the number of germinated conidia in treatment.

Secondary metabolites and enzymes

Production of secondary metabolites and enzymes by selected yeast strain were also evaluated. Briefly, production of hydrogen cyanide (32), proteases enzymes secretion (33), pectin (34), chitinase (35), siderophore (36), indole-3-acetic acid (37), gibberellin (38) and phosphate solubilization (39) were estimated.

In vivo antifungal activity assay

The *in vivo* assay was conducted based on the method explained previously with minor modification (14). For this work, the impacts of selected yeast were examined on strawberry fruit decay development by two methods employing living cells and volatile metabolites of the 111A-NL1 strain.

In both methods, seven days after inoculation, strawberry fruits showing symptoms of soft rot and gray mold were recorded. For this work, each strawberry fruit was considered to have a conical shape. Then, the total surface area was recorded by measuring the lateral and base area. After that, the disease severity (DS) was measured by AutoCAD software based on the method of Alijani et al. (2019) using the following formula (31):

$DS = A\ /H \times 2\pi r$

Where, (A) is the infected area, (H) and (r) are the height and the base radius of the fruit, respectively.

Volatile compounds extraction and GC-MS analysis

Fifty microliters of 48-h-old liquid culture of selected yeast strain (1×10^8 cells/ mL) were inoculated in vials containing 5 mL of NYDB medium and used for volatile compounds extraction. The vials were sealed with parafilm and placed on a rotary shaker (120 rpm) at 25 ± 2 ºC for 48 h. Vials without yeast inoculation were used as a control. Then 5 mL ethyl acetate was added to each vial (v/v) and then placed on a magnet for 60 min. Finally, samples were centrifuged at 6000 rpm for 15 min to extract all trapped volatile compounds in treatment and control (40). Three replications were used for each treatment. The chemical analysis of VOCs produced by yeast strain 111A-NL1 was carried out by Agilent 7890A gas chromatography (GC) coupled with Agilent 5977B mass spectrometry (MS) (Agilent Technologies, USA), using an HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The analytical conditions were described according to Amini et al. (2016) (41).

Effect of yeast strain on quality parameters of strawberry fruits

Similar healthy fruits (without disinfection) were dipped in a yeast cell suspension of 111A-NL1 strain (1×10^8 yeast cells/ mL) for five min and air-dried under a laminar flow hood for 30 min. The treated fruits in sterile distilled water were used as the control. All fruits were then placed into sterile poly-ethylene boxes and maintained at 25 ± 2 ºC, humidity (75%) under 12 h of light and 12 h of darkness for four days. Three replicates (boxes) per treatment containing 10 fruits were examined. The quality parameters of strawberry fruit, including weight loss (WL), firmness, solid soluble content (SSC), titratable acidity (TA), pH, and decay incidence was measured at t = 0 (1 h after treatment), 2, and 4 days according to the previous methods (42,43).

Statistical analysis

All experiments were designed in a completely randomized design (CDR) and repeated twice. The data analysis was carried out using the analysis of variance (ANOVA) via SAS software (version 8.2; SAS Institute, Cary, NC, USA, 2013). The data were illustrated as mean values ± standard deviation (SD). LSD test ($P \leq 0.05$) was employed to calculate the statistical significance of various treatments.

Results and discussion

Identification of yeast isolate 111A-NL1

Initial characterization and identification of the selected isolate 111A-NL1 were carried out based on morphological and biochemical characteristics. The cells are ovoid to the ellipsoid (2–4 × 3.5–7 µm) and exist singly or in pairs after being placed in a YM medium for 1-2 days at 25 °C. Budding is multilateral. Ascospore and pseudohyphae formation occurred on YM agar media after 4-9 days of incubation at 25 ºC (Fig. 1). Glucose and galactose are fermented, but not sucrose, maltose, lactose, raffinose, and trehalose. In carbon assimilation tests, growth is present on glucose and galactose while growth is absent on arabinose, inulin, sucrose, raffinose, trehalose, maltose, cellobiose, glycerol, ribose, mannitol, ribitol, succinate, citrate, ethanol, and methanol. In nitrogen-assimilation tests, no growth is observed in nitrate, nitrite, ethylamine, glucosamine, and vitamin-free medium. Other Biochemical traits suggested that the isolate was negative for starch formation, urease activity, and gelatin liquefaction. The yeast isolate could grow at temperatures 19 °C, 25 °C, 30 °C, 37 °C, and not at 40 °C. Based on morphological and biochemical characteristics, the isolate was tentatively

identified as *Tetrapisispora* sp. strain 111A-NL1. For further confirmation, PCR amplification using the universal primers NL1 and NL4 and sequencing of the *D1/D2 domain* of the *26S rRNA* gene were completed. Based on the BLASTn results, the most identical sequences were reported to be sequences of strains of the genus *Tetrapisispora*. Furthermore, strain 111A-NL1 demonstrated a 98% identity with *Tetrapisispora fleetii* (*GenBank accession no.* AY645662). The *phylogenetic tree revealed strain* 111A-NL1 *among* the type *strains* of its closest related species in the genus *Tetrapisispora* (Fig. 2). In addition, the strain 111A-NL1 was able to solubilize phosphate and produced pectinase, siderophore, chitinases, and IAA (1352.50 µg/L), gibberellin (274.39 µg/L), and negative for HCN, protease and cellulase production.

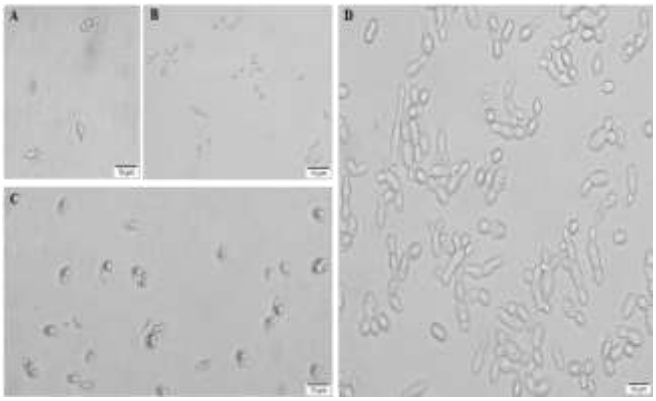

Figure 1. *Tetrapisispora* sp. 111A-NL1. (A, B) Budding cells on YM agar medium after 2 days. (C, D) Ascospore and pseudohyphae after 4-9 days on YM agar medium. Incubation was at 25 ºC for all cultures. Scale bar = 10 µm.

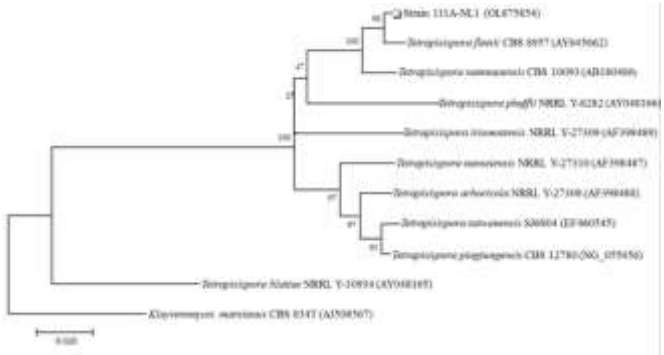

Figure 2. Phylogenetic tree for strain 111A-NL1 and related species constructed by the neighbor-joining method based on the *D1/D2 domain* of the *26S rRNA* gene. The numbers shown at the branch points indicate *percentage bootstrap* values from *1000 datasets.* GeneBank accession numbers are indicated in parentheses. *Kluyveromyces marxianus* was used as an outgroup.

In vitro antagonistic activity assays

The outcomes of antagonistic tests of selected yeast strain demonstrated that *Tetrapisispora* sp. 111A-NL1 had an appropriate antagonistic activity against *B. cinerea*. The strain 111A-NL1decreased mycelia growth of *B. cinerea* under *in vitro* experiment by dual culture with 19.49% inhibition (Table 1). Volatile metabolites of strain 111A-NL1 significantly reduced mycelial growth and conidial germination of *B. cinerea* by 75.19% and 63.34% compared to control, respectively (Table 2).

Table 1. Inhibitory effect of *Tetrapisispora* sp. 111A-NL1 on mycelial growth of *Botrytis cinerea* under *in vitro* tests by dual culture test after 5 days

Treatments	Colony diameter (mm)	Inhibition %
strain 111A-NL1	4.26 ± 0.15 b	19.49
Control	5.3 ± 0.26 a	-
LSD (5%)	0.49	-

Mean followed by different letters within the column represents significant differences according to the LSD test ($p \leq 0.05$). Data are the mean of three replicates with ± standard deviation (SD).

Table 2. Inhibitory effect of volatile organic compounds of *Tetrapisispora* sp. 111A-NL1 on mycelial growth and conidial germination of *Botrytis cinerea* under *in vitro* tests after 5 days

Treatments	Mycelial growth		Conidial germination	
	Colony diameter (mm)	Inhibition %	Germinated conidia	Inhibition %
strain 111A-NL1	2.16 ± 0.12 b	75.19	34.33 ± 1.45 b	63.34
Control	8.73 ± 0.14 a	-	93.00 ± 1.76 a	-
LSD (5%)	0.52	-	6.42	-

Mean followed by different letters within the column represents significant differences according to the LSD test ($p \leq 0.05$). Data are the mean of three replicates with ± standard deviation (SD).

In vivo antagonistic activity assays

The effect of *Tetrapisispora* sp. 111A-NL1 on disease severity of strawberry grey mold disease *in vivo* was investigated employing living cells and volatile metabolites methods seven days after inoculation. Under *in vivo* experiments revealed that strain 111A-NL1 substantially reduced the strawberry fruit decay development and suppressed strawberry grey mold disease more than control. The biocontrol efficacy of strain 111A-NL1 was 47.61% and 74.05% using living cells and volatile compounds, respectively (Table 3 and Fig. 3).

Volatile compounds detection using GC-MS

As for determining VOCs created by *Tetrapisispora* sp strain 111A-NL1, extracted compounds were analyzed with GC-MS. Results of

GC-MS analysis are shown in Table 4. Thirty-three various compounds were identified by GC-MS analysis. Eight compounds with highest area percent were Decane (12.79%), Squalene (9.60%), Undecane (7.98%), Benzene, 1,2,3-trimethyl- (7.67%), Nonane, 2,6-dimethyl- (5.69%), Benzene, 1-ethyl-3-methyl- (5.55%), Mesitylene (4.17%), and Phenylethyl Alcohol (3.33%).

Table 3. Inhibitory effect of *Tetrapisispora* sp. 111A-NL1 on strawberry fruit decay development *in vivo* after 7 days using two methods treated fruit with living cells and volatile organic compounds.

Treatments	Living cells		Volatile compounds	
	Disease severity	Efficacy %	Disease severity	Efficacy %
strain 111A-NL1	2.86 ± 0.68 b	47.61	1.28 ± 0.18 b	74.05
Control	5.46 ± 0.51 a	-	4.94 ± 0.37 a	-
LSD (5%)	2.09	-	0.84	-

Mean followed by different letters within the column represents significant differences according to the LSD test ($p \leq 0.05$). Data are the mean of four replicates with ± standard deviation (SD).

Figure 3. Effect volatile organic compounds of *Tetrapisispora* sp. 111A-NL1 (A, B and C) on disease severity of strawberry grey mold disease under *in vivo* in comparison with control (D, E and F).

Effects of Tetrapisispora sp. 111A-NL1 on storage quality of strawberry fruits

Tetrapisispora sp. 111A-NL1 decreased the WL more than the control significantly ($p \leq 0.05$) after 2 and 4 days of storage (Fig. 4). The WL rate for treatment after 2 and 4 days of storage was 0.26% and 1.35%, respectively, while the WL rate for untreated control after 2 days was 0.75% (Fig. 4). The fruit treated with the *Tetrapisispora* sp. 111A-NL1 was significantly firmer than untreated fruit after storage. Also, the firmness of the fruit treated with selected yeast strain slightly increased after 2 days and decreased at 4 days. Fruit firmness of yeast treatment was 6 N and 4.81 N after 2 and 4 days, respectively (Fig. 4). The SSC content in treated fruit with the *Tetrapisispora* sp. 111A-NL1 was 4.83% at harvest time and decreased to 4.30% at the end of storage. The treatment significantly ($p \leq 0.05$) preserved the SSC content during storage compared with the untreated control (Fig. 4). During storage, the pH and TA of strawberry fruits decreased in both treated and untreated fruits, but this reduced rate of TA in untreated control was more than that of treated fruits. The losses of TA content in strawberry fruit were delayed obviously by selected yeast strain during storage. The pH and TA of the treated fruit were 3.20 and 0.52% after 4 days, respectively (Fig. 4). *Tetrapisispora* sp. 111A-NL1 significantly ($p \leq 0.05$) decreased decay incidence in artificially inoculated fruits up to 55.55% after storage at 25 °C for 4 days (Fig. 4). The decay incidence was 90% and 40% for the untreated and treated fruits, respectively (Fig. 4).

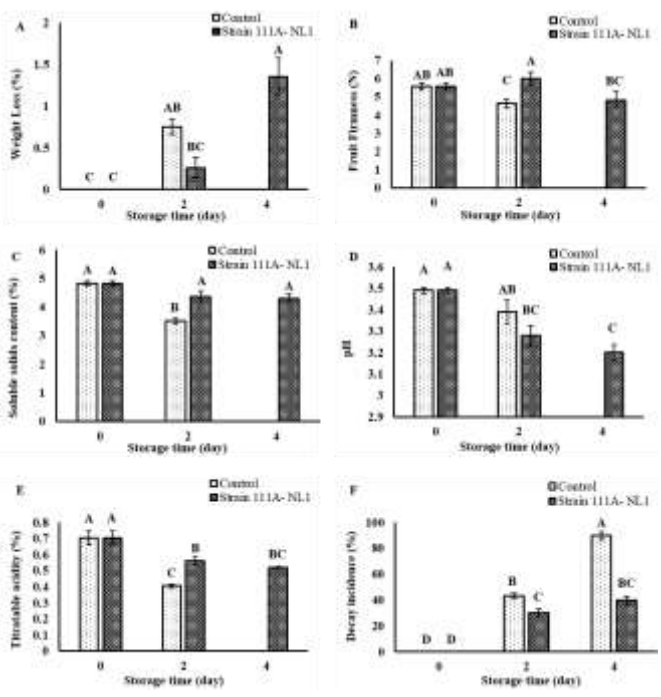

Figure 4. Effect of *Tetrapisispora* sp. 111A-NL1 on strawberry fruit quality after four days storage. A) Weight loss, B) Fruit firmness, C) Soluble solids content, D) pH, E) Titratable acidity, F) Decay incidence. Fruits were incubated at 25 ± 2 °C, humidity (75%) under a regime of 12 h of light and 12 h of darkness for 4 d. Values are the mean ± standard deviation of three replicates with 10 fruits in each replicate. Within the same figure, different letters represent a significant difference test ($p \leq 0.05$) among the control and the selected strain according to the LSD test.

Table 4. Volatile organic compounds (VOC) released from *Tetrapisispora* sp. 111A-NL1, identified by GC-MS analysis

Number	Compounds	Chemical formula	Retention time (min)	Area (%)	CAS #
1	1,3-Benzenediol, 4-ethyl-	$C_8H_{10}O_2$	3.130	0.88	2896-60-8
2	Benzene, (1-methylethyl)-	C_9H_{12}	3.201	1.11	98-82-8
3	Cyclohexane, propyl-	C_9H_{18}	3.260	1.40	1678-92-8
4	Nonane, 3-methyl-	$C_{10}H_{22}$	3.314	2.99	5911-04-6
5	Octane, 2,3-dimethyl-	$C_{10}H_{22}$	3.412	2.33	7146-60-3
6	Cyclohexane, 1,1,2,3-tetramethyl-	$C_{10}H_{20}$	3.599	2.71	6783-92-2
7	Benzene, 1-ethyl-3-methyl-	C_9H_{12}	3.700	5.55	620-14-4
8	Nonane, 2-methyl-	$C_{10}H_{22}$	3.726	3.67	871-83-0
9	Benzene, 1,2,3-trimethyl-	C_9H_{12}	3.799	7.67	526-73-8
10	Benzene, 1-ethyl-2-methyl-	C_9H_{12}	3.955	1.91	611-14-3
11	Cycloheptane, methyl-	C_8H_{16}	4.016	2.06	4126-78-7
12	Mesitylene	C_9H_{12}	4.146	4.17	108-67-8
13	Decane	$C_{10}H_{22}$	4.234	12.79	124-18-5
14	Nonane, 2,6-dimethyl-	$C_{11}H_{24}$	4.559	5.69	17302-28-2
15	Cyclohexane, (1-methylpropyl)-	$C_{10}H_{20}$	4.683	1.28	7058-01-7
16	Decane, 3-methyl-	$C_{11}H_{24}$	4.791	1.37	13151-34-3
17	Benzene, (1-methylpropyl)-	$C_{10}H_{14}$	4.982	1.84	135-98-8
18	Heptane, 4-ethyl-	C_9H_{20}	5.067	1.94	2216-32-2
19	Decane, 4-methyl-	$C_{11}H_{24}$	5.111	1.25	2847-72-5
20	Decane, 2-methyl-	$C_{11}H_{24}$	5.163	1.93	6975-98-0
21	3a,4,5,6,7,7a-Hexahydro-4,7-methanoindene	$C_{10}H_{14}$	5.203	1.28	4488-57-7
22	Decane, 3-methyl-	$C_{11}H_{24}$	5.257	1.34	13151-34-3
23	Undecane	$C_{11}H_{24}$	5.689	7.98	1120-21-4
24	Phenylethyl Alcohol	$C_8H_{10}O$	5.915	3.33	60-12-8
25	Dodecane	$C_{12}H_{26}$	7.134	2.75	112-40-3
26	Tetradecane	$C_{14}H_{30}$	9.842	2.13	629-59-4
27	Heneicosane	$C_{21}H_{44}$	11.046	0.62	629-94-7
28	Hexadecane	$C_{16}H_{34}$	12.278	2.05	544-76-3
29	Octacosane	$C_{28}H_{58}$	13.522	1.17	630-02-4
30	Octadecane	$C_{18}H_{38}$	14.479	1.31	593-45-3
31	Hexadecane, 2-methyl-	$C_{17}H_{36}$	15.741	0.93	1560-92-5
32	Eicosane	$C_{20}H_{42}$	16.477	1.00	112-95-8
33	Squalene	$(C_5H_8)_6$	23.469	9.60	111-02-4

The utilization of biocontrol agents for the control of fruit postharvest disease is determined to be a safe strategy (8), and biocontrol agents including yeasts have demonstrated apparent usefulness against main postharvest decays of fruit including postharvest grey mold (44). Yeasts possess numerous features that make them alternative BCAs of post-harvest decay (45) and have low technological constraints for industrial-scale production (1). The current research aimed to isolate and to determine antagonistic yeast from strawberry fruit and to assess its potential ability for biological control of *B. cinerea* causal agent of strawberry grey mold disease. Based on sequencing of the D1/D2 region of the 26 rDNA, this strain was identified as *Ttetraposispora* sp. 111A-NL1. To our best knowledge, this is the first time that *Ttetraposispora* sp. 111A-NL1 has been used as a biocontrol agent against *B. cinerea* causal agent of strawberry grey mold disease. This strain was shown to control *B. cinerea in vitro* and *in vivo*. The extant research has also shown that yeasts as biocontrol agents play an important role in controlling some fruit postharvest pathogens and in improving the healthy development of fruit (14,18,19,23). Also, other studies showed that selected fungal species such as *Candida intermedia* (14), *Candida oleophila* (46), *Aureobasidium pullulans* (46), *Metschnikowia fructicola* (47), *Pichia guillieemondii* (48), and *Rhodotorula glutinis* (49) are useful agents for suppression of Botrytis fruit rot of strawberry under preharvest or postharvest conditions. We calculated VOCs produced by *Tetrapisispora* sp. 111A-NL1 and tested their antifungal activity against *Botrytis cinerea in vitro* and *in vivo*. The volatile organic compounds (VOCs) produced by yeast strain 111A-NL1 exhibited antifungal activity against *B. cinerea* and prevented mycelial growth and conidial germination of *B. cinerea*. Previous research has revealed that VOCs produced by *Bacillus pumilus* (50), *Bacillus subtilis* CF-3 (51), and *Aureobasidium pullalans* (23) inhibited the mycelial growth of *Colletotrichum gloeosporioides*. In addition, volatile compounds produced by *Staphylococcus sciuri* (31) and *Stenotrophomonas maltophilia* (52) inhibited the mycelial growth and conidial germination of *Colletotrichum nymphaeae*. Gao et al. (2018) (51) have also reported a positive correlation between these VOCs and the inhibition of *M. fructicola in vitro*. In addition, the *Ttetraposispora* sp. 111A-NL1 strain produced chitinase, pectinase, and siderophore. The mechanisms of action of yeast biocontrol agents include the competition for nutrients and physical space, parasitism, and eliciting host defense pathways (12), and the production of antifungal metabolites including volatile organic compounds (24). These results indicated that the production of antifungal VOCs by yeast strain 111A-NL1 is an essential mechanism for the reduction of Botrytis fruit rot in strawberries (14,22,23). These results have suggested that the mechanism of VOCs on *B. cinerea* may be via

the diffusion of gas in limited space (53). Numerous yeast agents including *Wickerhamomyces*, *Metschnikowia*, *Aureobasidium* or *Saccharomyces*, *Cryptococcus*, *Hanseniaspora*, *Candida*, *Rhodotorula*, *Debaryyomyces*, and *Pseudozyma* can produce VOCs against postharvest pathogens (23,54,55). These results agree with the present study since *B. cinerea* showed high susceptibility to the VOCs produced by *Ttetraposispora* sp. 111A-NL1. Microbial VOCs have antimicrobial activity (56), inhibit the growth of pathogenic fungi (57,58), improve plant growth (52), and induce systemic resistance in plants (59). The VOCs produced by microorganisms present several advantages; for example, they can diffuse through space more easily by reducing the distance of the infection of the pathogen (60). Furthermore, biogenic VOCs can coexist in the environment and decompose easily under natural conditions and they are effective bio-fumigation for the management of plant pathogens (17,52). The microbial VOCs are chemically classified into organic acids, phenols, esters, alcohols, olefins, ketones, pyrazines, terpenoids, alkanes, alkenes, alkynes, disulfides, nitrogen compounds, aldehydes, and ethers (31,17). Alcohols, amines, aldehydes, ketones, terpenes, and sulfur-based and chlorinated hydrocarbons are the main products of the primary and secondary microorganism metabolism (61). In the present study, the antagonistic VOCs produced by strain 111A-NL1 were detected and identified. The GC-Mass analysis of VOCs identified 33 compounds, such as twenty alkanes, seven benzenes, four cycloalkanes, one cycloalkene, and one phenol. Alkanes (64.84%), benzene (25.58%), and cycloalkanes (7.45%) were the main compound types, and these three types explained 97.87% of 33 detected compounds. Studies have indicated that the production of benzene, ketones and alcohol compounds by *Paenibacillus polymyxa* WR-2 in low quantity causes antifungal activity against *Fusarium oxysporum* (58). Yuan et al. (2012) have reported that various compounds, including nonane, decane, toluene, ethylbenzene, and undecane, evinced antifungal activity against *F. oxysporum* (62). Benzene compounds such as benzene, 1,3-bis (1,1-dimethyl ethyl), pentadecane, tetradecane, 1,3-dimethylbenzene, ethylbenzene had antifungal activities which could decrease the growth and virulence of plant pathogens (63,64). Also, volatile compounds such as decane, undecane and tetradecane could induce systemic resistance in plants against phytopathogens and improve the growth parameters in plants (60). Gao et al. (2017) indicated that Phenol-2,4-bis (1,1-dimethyl ethyl) showed high antifungal activities for controlling tomato fungal diseases such as early blight and grey mold (17). As far as postharvest conditions are concerned, VOCs can be simply employed to manage fruit and vegetable diseases infection during storage or long-term transport without the constraint of discharging them from the store or the container. Also, they can diffuse in the atmosphere of the store, ensuring appropriate protection of the agriculture products at the surface without penetrating inside them, which ensures more safety for the consumer (65).

Treatment of *Tetrapisispora* sp. 111A-NL1 maintained SSC and TA more than the untreated control during storage and the fruits treated with this strain were firmer than the control. Therefore, no significant adverse effect was found on storage quality parameters such as SSC, TA, and firmness of strawberry fruit during storage. The selected yeast strain prevents the loss of fruits moisture and decreases the natural decay incidence of strawberry fruits better than the untreated control. Similar results as broad-spectrum antifungal strain *Sporidiobolus pararoseus* Y16 were capable of declining the decay rate of table grapes and prolonged postharvest storage period (19). Studies have shown that yeasts can inhibit the growth of the pathogen and prevent the loss of fruit moisture (19). Also, the fruit firmness is considered an essential factor affecting the shelf life of postharvest fruits (66, 67). It is one of the most essential quality deterioration characteristics during storage time caused by the degradation of pectin in the fruit cell wall and hydrolysis of starch to sugar associated with fruit ripening (68). Identical values were reported by You et al. (2021), employing the impact of *Bacillus siamensis* on the preservation and maintenance of SSC in mango fruit during the late storage (69).

In conclusion, this research indicated that *Tetrapisispora* sp. 111A-NL1 could successfully repress postharvest infection of strawberry fruits by *B. cinerea*. The strain 111A-NL1 produced lytic enzymes, including chitinase and pectinase, as well as a secreted siderophore. VOCs produced by this strain

decreased mycelial growth and conidial germination of *B. cinerea*, fruit decay development, and disease severity of strawberry grey mold. In addition, strain 111A-NL1 preserved quality parameters of strawberry fruit such as weight loss, firmness, soluble solids content, titratable acidity, and decreased significantly decay incidence of strawberry fruits. The results of this study show that the production of antifungal VOCs by strain 111A-NL1 might be an essential mechanism for the suppression of infection of strawberry fruits by *B. cinerea* under *in vitro* and *in vivo* conditions. This study is the first report on the efficacy of *Tetrapisispora* sp. 111A-NL1 against *B. cinerea* which shows that the VOCs produced by this strain have excellent potential as biofumigant for the management of strawberry grey mold. Thus, further research is required to shed more light on the exact contribution of volatile compounds in the control of this pathogen, and these findings have initiated a new line of inquiry about *Tetrapisispora* sp. strain 111A-NL1 as a biocontrol agent of strawberry grey mold disease.

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Interest conflict

The authors declare that they have no conflict of interest. This research project was funded by the University of Kurdistan.

Availability of data and material

All data generated during this study are included in this published article

Authors' contribution

JA and SB designed and performed the experiments; JA, SB, MA and MKS analyzed the data; JA, SB and MA wrote the drafts of the article; JA supervised the manuscript. All authors revised and approved the final version of the manuscript.

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