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Antifungal activity of volatile compounds produced by *Staphylococcus sciuri* strain MarR44 and its potential for the biocontrol of *Colletotrichum nymphaeae*, causal agent strawberry anthracnose

Zahra Alijani[a], Jahanshir Amini[a,*], Morahem Ashengroph[b], Bahman Bahramnejad[c]

[a] Department of Plant Protection, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran
[b] Department of Biology and Biotechnology, Faculty of Sciences, University of Kurdistan, Sanandaj, Iran
[c] Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

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ABSTRACT

A nonpathogenic endophytic bacterial isolate, recovered from *Fragaria* × *ananassa* stolon, and its antifungal activity against *Colletotrichum nymphaeae* was evaluated under *in vitro*, *in vivo*, and greenhouse conditions. Bacterial isolate was identified as ***Staphylococcus sciuri* MarR44 (Strain ID: WDCM 891 = CCSM-B 00640)** using phenotypic and biochemical properties and molecular phylogenetic analysis of the 16S rDNA gene sequences. The living cells of strain MarR44 inhibited mycelial growth of *C. nymphaeae* (52.46%) using dual-culture method. The volatile compounds (VOCs) produced by MarR44 inhibited mycelial growth and conidial germination of *C. nymphaeae* by 34.52% and 82.81%, respectively. However, inhibition percentage of mycelial growth of pathogen by culture filtrates of the strain MarR44 was lower (23.07%) than that for the two dual culture and volatile compounds assay tests. Moreover, the cell-free-culture filtrates of this strain reduced the biomass and conidial germination of pathogen by 91.89% and 41.10%, respectively. Also, the strain MarR44 was capable of producing protease, chitinase, HCN, siderophore, IAA, gibberellin, and biofilm. The living cells and volatile compounds of the strain MarR44 reduced anthracnose disease at post-harvest on fruit by 52.45% and 72.17%, respectively. Furthermore, disease severity of strawberry anthracnose was reduced using drenching soil and inoculated plants methods by 77.77 and 72.22%, respectively, 60 days after inoculation. The VOCs released by strain MarR44 were analyzed by Gas chromatography-mass spectroscopy (GC–MS). Out of 24 identified VOCs, Mesityl oxide (81.436%), Acetic acid, 2-methylpropyl ester (3.442%), 4-Methyldecane (1.837%), 4-Penten-2-one,4-methyl- (1.739%), Toluene (1.248%), and o-Xylene (1.24%) were the major components. The mode of action of *S. sciuri* MarR44 on the *C. nymphaeae* was through the production of antifungal volatile compounds (Antibiosis), which inhibited mycelial growth and conidial germination of pathogen *in vitro* and fruit decay development *in vivo*. To the best of our knowledge, this is the first report of *S. sciuri* having antifungal activity against causal agent strawberry anthracnose. These results indicated that the VOCs of *S. sciuri* strain MarR44 are promising biofumigant for management of strawberry anthracnose.

1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most delicious and fragrantly sweet flavored fruits of the world, very popular in many countries (Natsheh et al., 2015). In Iran, strawberry is generally produced in the western part of the country, Kurdistan province. Strawberry anthracnose is a major limiting factor in strawberry production. Anthracnose is a devastating disease of strawberry worldwide (Freeman and Katan, 1997). The disease is caused by *Colletotrichum* species (Howard et al., 1992). The species *Colletotrichum nymphaeae* is associated with strawberry anthracnose in Iran. It is pre- and post-harvest disease, which causes yield loss in Kurdistan Province (Karimi et al., 2017). Fruit rot and flower blight are the common symptoms in fruiting fields, whereas lesions on stolons, petioles, and leaves are particularly damaging in nurseries. Traditional methods, including practical control, breeding for resistant plant cultivars have been inefficient to control anthracnose and none of these have individually provided complete control. However, application of fungicides against anthracnose disease may occasionally be ineffective due to the appearance of resistant pathogen population and the environmental

* Corresponding author.
E-mail address: jamini@uok.ac.ir (J. Amini).

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pollution. Therefore, alternative potential environmentally-friendly method need to be developed. The use of biocontrol agents to control phytopathogens has recently attracted more attention, because they offer an effective and safe way to circumvent the drawbacks of chemical fungicides, and can reduce environmental risks associated with their pollution, and prevent the development of resistance to fungicides (Huang et al., 2015; Naing et al., 2014; Nguyen et al., 2012). Using endophytic bacteria as biocontrol agents is a complementary method for solving the contamination problems that are effective, favorable and environmentally safe (Li et al., 2011; Urena-Padilla et al., 2002). Endophytic bacteria suppress disease development in plants by inducing the plant defense systems, producing inhibitory compounds (antibiosis), production of fungal cell wall-degrading enzymes or Fe^{3+}-chelating siderophores, competition for space and nutrition, and colonization of plant inner tissues (Compant et al., 2005; Compant et al., 2010; Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011; Seo et al., 2012; Wicaksono et al., 2017). Many investigations have been carried out with endophytic bacteria as potential biological control agents (BCAs) of some fungal disease (Ben Abdallah et al., 2016; Hidayati et al., 2014; Huang et al., 2015; Yang et al., 2013). *Staphylococcus* spp. are Gram-positive, aerobic, coccoid, endospore-forming bacteria that inhabit different ecological niches such as animals, humans, plants, rhizosphere, soil, and water (Bodhankar et al., 2017; Nemeghaire et al., 2014). Several *Staphylococcus* species were characterized as having salt-tolerance potential (Roohi et al., 2012) and they mitigated the deleterious effects of salinity in strawberry (Karlidag et al., 2013) and radish (Yildirim et al., 2008). They are capable of colonization of the rhizosphere, penetrating the plant roots and triggering plant salinity-tolerance mechanisms by influencing plant physiology using releasing growth regulators (Akram et al., 2016). Sagar et al. (2012) indicated that *Staphylococcus arlettae* strain Cr11 promoted plant growth *via* the reduction of hexavalent chromium. In this study, we isolated the antagonistic endophytic bacterium *Staphylococcus sciuri* MarR44 (MH161571) from stolon of healthy strawberry plant at the flowering stage. Then, the effectiveness of this endophytic bacterium against *Colletotrichum nymphaeae* was investigated under *in vitro*, *in vivo* and greenhouse conditions. To our knowledge, this is the first report of *Staphylococcus sciuri* as biological control agent and its effects on strawberry anthracnose.

2. Material and methods

2.1. Plant and fruit

Healthy strawberry plants cv. Paros (5-weeks old) were used in this study. Plants were grown in greenhouse conditions at 24 to 27 °C, 60–70% RH, 16 h light, and 8 h darkness for greenhouse experiments. Healthy strawberry fruits (c.v. Paros) of uniform size, shape, maturity and free from any injury and pathogenic infection were collected and washed with distilled water, and air-dried under the laminar hood for 15 min for *in vivo* experiments.

2.2. Pathogen

Strain used in this study isolated from infected strawberry fruit in Kurdistan Province. The aggressive isolate was identified as *C. nymphaeae* (GenBank Acc. No. MK372221; Strain ID; IRAN 3427C). The PCR reactions were performed using the primers ITS-1 (5′-TCCGTAGG TGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Then sequences were edited using the software BioEdit Sequence Alignment Editor 7.0.5.3 (Hall, 1999) and deposited in Genbank and compared against those sequences already found in the databases using BLAST search. The pathogen cultured on Potato dextrose agar (PDA) medium for 14 days at 25 ± 2 °C. Afterwards, the spores were harvested by flooding the surface of the culture with sterile distilled water and it was filtered with three layers of sterile gauze to remove mycelial debris to collect spores. Then, the concentration of conidial suspension was adjusted to 1×10^6 CFU/mLusing a hemocytometer.

2.3. Endophytic bacterium isolation, characterization and identification

The selected endophytic bacterium was isolated from stolon of healthy strawberry c.v Paros in Marivan (N 35° 15 09.2; E 46° 25 34.2) in September 2016. Samples were washed by flowing water until they were clean and dried with tissue paper. The small pieces of samples (3–5 mm) were sterilized in 70% ethanol (v/v) for 1 min, and then soaked in 2.5% NaOCl (v/v) for 3 min, again in 70% ethanol for 30 s. They were rinsed 3 times in sterile-distilled water and air-dried on sterile filter papers. After that, the sterilized pieces were macerated in 10 mL of sterile distilled water for 30 min and 50 μL of final suspension was cultured on nutrient agar, king's B agar and tryptic soy agar media. To check the efficiency of sterilization, 50 μl of the water from last rinse of samples was cultured on nutrient agar (NA) plate as a negative control. *Re*-isolation was done to assay the colonization ability of MarR44 isolate inside the meristem culture of strawberry. Bacterial suspension (1×10^8 CFU/mL) was inoculated to roots of meristem culture and after 96 h were washed by distilled water and sterilized by same method that previously described. The sterilized roots were grinded and extract cultured on NA medium and after 24 h, bacterial colonies were observed. A single colony of bacterial isolate was cultured on NA medium at 27 ± 2 °C to get pure cultures. The isolate was maintained in NB medium supplemented with 20% (v/v) glycerol at −20 °C. Fresh bacterial cultures were active on NA medium and incubated for 24 h at 26–28 °C before using. In order to get bacterial suspension, the bacterial culture was suspended in sterilized distilled water (SDW) and the concentration was subsequently adjusted to 1×10^8 CFU/mL ($OD_{600nm} = 0.2$). The initial characterization of strain MarR44 was done based on morphological, physiological and biochemical tests. The tests used to identify the strain included Gram staining, catalase, oxidase, motility, nitrate reduction, Simons citrate, H_2S production, oxidative fermentative, gelatin hydrolysis, starch hydrolysis, KOH, gas production from glucose, Urease test as well as nitrogen fixation, phosphatase, lipase and levan production were calculated according to the standard methods (Gerhardt et al., 1994; Hugh and Leifson, 1953; Schaad et al., 2001). Cell morphology of selected bacterium was examined under scanning electron microscope (FE-SEM TESCAN MiRA3, Czech Republic) with cells from exponentially growing cultures. For molecular identification of the strain MarR44, genomic DNA was first extracted. Polymerase chain reaction (PCR) amplification of 16S rDNA gene fragment was performed with the universal primers FD_2 (5′-AGAGTTTGATCATGGCTCAG-3′) and RP_1 (5′-ACGGTTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). PCR were performed in 25 μL reaction as follows: initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, then annealing at 52 °C for 1 min, and extension at 72 °C for 2 min, and final extension at 72 °C for 7 min in Thermal Cycler (BIO RAD T100™). The purified PCR product was sequenced in both directions with the ABI3730xl DNA sequencer by Macrogen Company (Seoul, Korea). Then sequences were edited using the software BioEdit Sequence Alignment Editor 7.0.5.3 (Hall, 1999). The BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST) was used to examine the resulting sequences. Then, the phylogenetic tree was constructed using similar sequences that were taken from NCBI with MEGA7 based on the Neighbor Joining method; and the bootstrap test was done with 1000 times to confirm the reliability of the phylogenetic tree (MH161571). *Macrococcus caseolyticus* ATCC 13548 (NR_119262) sequence was used as outgroup (Kumar et al., 2016).

2.4. Hypersensitive response test

Hypersensitive response test was carried out by using tobacco

(*Nicotiana tabacum* L.) as indicator of whether endophytic bacterium had potential as pathogen for plant. Bacterial isolate with density of 1×10^8 CFU/mL in sterile-distilled water suspension was injected using 1 mL syringe to 3-month old tobacco leaf and the observation was made until 48 h (Ben Abdallah et al., 2016).

2.5. In vitro assays

2.5.1. Dual culture method

Bacterial isolate was tested for *in vitro* biocontrol activity toward *C. nymphaeae* on Potato Dextrose Agar (PDA) medium using a dual-culture technique. The mycelial disc (5-mm diameter) from 7-day-old culture of *C. nymphaeae* was placed on one side of the plate (9-cm diameter) containing PDA medium, and then endophytic bacterium isolate was streaked on the opposite side of the plate. The plates inoculated with the pathogen alone were used as control. Four replications were performed for each treatment. The plates were incubated at 25 ± 2 °C in 12 h light/12 h dark for 7 days. The diameter of *C. nymphaeae* colony was measured (Moreira et al., 2014). Then, the inhibition percentage (I) was calculated using the following formula:

$$I(\%) = [(Rc - Rt)/Rc] \times 100$$

where Rc is radial growth of pathogen in control treatment, Rt is radial growth of pathogen in dual plate culture (treatment).

2.5.2. Effect of endophytic bacterium on fungal biomass

The effect of endophytic bacterium on the biomass of *C. nymphaeae* was determined using method described by Kim (2005) with some modifications. In so doing, the selected endophytic bacterium was cultured into NB medium and incubated on a rotary shaker at 26 ± 2 °C for 72 h. Then it was centrifuged at 5000 rpm for 15 min and filtered by 0.22 μm Millipore filter. The cell-free culture filtrates of strain MarR44 at 10% (v/v) was added to potato dextrose broth (PDB) medium in Erlenmeyer flask (100 mL). The Erlenmeyer flask was inoculated with one 5- mm diameter plugs of mycelial disk of 7-day-old culture of *C. nymphaeae* and was incubated with shaking (150 rpm) at 25 ± 2 °C for 14 days. Cultures of the fungus grown without culture filtrates were used as the control. Subsequently, the mycelium was separated from the medium by filtration on filter paper (No 1. Whatman), dried in an oven at 95 °C until constant weight, and the dry mass was determined. Three replications were performed for each treatment.

2.5.3. Volatile compounds antifungal assay

The volatile antifungal activity of selected bacterial isolate was investigated for inhibition of mycelial growth and conidial germination of *C. nymphaeae* on PDA medium and water agar (WA), respectively. For inhibition of mycelial growth of pathogen, the two-sealed-base plates method was used to test the antifungal activity of volatile compounds(s) (VOCs) of selected bacterial isolate. One base plate contained NA medium, and another plate contained PDA medium. The bacterial isolate was streaked onto the NA plate, and a mycelial disc (5-mm diameter) from 7-day-old culture of *C. nymphaeae* was placed on the PDA plate, so that the plates of pathogen could be placed inversely over a bottom plate containing bacterial isolate. These two base plates were sealed tightly using parafilm. The plates inoculated with the pathogen alone were served as control. Three replications were performed for each treatment. All plates were incubated at 25 ± 2 °C in 12 h light/12 h dark for 7 days (Gao et al., 2017). The inhibition percentage of mycelial growth of pathogen for selected bacterial isolate was measured using the formula as described in section 2.5.1.

For inhibition of conidial germination of *C. nymphaeae*, a total of 50 μl of the conidial suspension of *C. nymphaeae* (1×10^6 CFU/mL) were pipetted and plated on WA medium in plates (90-mm-diameter). Then, bacterial isolate was cultured on NA medium. The plates containing conidial suspension of pathogen were placed upside of the bacterial culture plates. The double dishes were sealed using parafilm and incubated at 25 ± 2 °C for 72 h. Plates containing sterile water (without bacterium isolate) were used as control (Huang et al., 2011). The inhibition percentage of conidial germination (I) was measured according to the following formula:

$I(\%) = [(N_c - N_t)/N_c] \times 100$, where N_c is number of germinated conidia in control, N_t is number of germinated conidia in treatment. A conidium was adverted to have germinated if the length of the germ tube was larger or equal to the conidial diameter. Four replications were performed for each treatment.

2.5.4. Non-volatile compounds antifungal assay

In order to evaluate non-volatile metabolites of endophytic bacterium, strain MarR44 was inoculated into PDB medium (45 mL) in a 100-mL Erlenmeyer flask and incubated on a rotary shaker at 150 rpm at 27 ± 2 °C for 48 h. The bacterial culture was centrifuged at 5000 rpm for 15 min to get cell-free filtrate solution and then filtered using 0.22 μm Millipore filter. The plates (90-mm diameter) were filled with 25 mL of PDA medium, and 5-mm-diameter mycelial plug of a 7-day-old culture of pathogen was placed in the center of the culture plates. For each plate, two 5-mm-diameter plugs were dug out, 25 mm away from the plug of fungal pathogen. To each hole, 150 μL of cell-free culture filtrates of selected strain MarR44 was added. Plates inoculated only with pathogen and medium broth without culture filtrates of bacterial isolate were applied as control. There were three replicates per each treatment. The plates were incubated at 25 ± 2 °C in 12 h light/12 h dark for 7 days (Jangir et al., 2018). The inhibition percentage of mycelial growth of pathogen for selected bacterial isolate was measured using the formula as described in section 2.5.1.

2.5.5. Effects of culture filtrates on conidial germination

The inhibitory effects of bacterial culture filtrates on the conidial germination of pathogen was investigated using methods described by Mohammadi et al. (2017) and Saechow et al. (2018) with some minor modifications. 200 μl of a pathogen spore suspension (1×10^6 CFU/mL) was mixed with the 1 mL of bacterial culture filtrates in sterile tube containing 5 mL of potato dextrose broth (PDB). The control treatment was treated only with pathogen spore suspension under the same conditions. Three replicates were used for each treatment. All tubes were incubated on a rotary shaker (120 rpm) at 26 °C for 24 h in dark. The conidial germination was determined under an optical microscope (Olympus BX51, Japan; 60 × magnification using micrometer). For each treatment, 50 conidia were assessed for germination and morphology of conidia. The inhibition percentage of conidial germination of pathogen for selected bacterial isolate was measured using the formula as described in section 2.5.3.

2.5.6. Secondary metabolites and enzymes

The hydrogen cyanide (HCN) production ability was assessed qualitatively following the method described by Alstrom and Burns (1989). Selected endophytic bacterium was inoculated individually on NA medium and Whatman paper soaked in solution including 2% Na_2CO_3 and 5% picric acid, placed inside the upper lid of the plate (90-mm-diameter). Change in the color from yellow to light-reddish brown was marked as positive production of HCN by the selected bacterium. Endophytic bacterial isolate was assessed for its ability to produce proteolytic enzymes on skim milk agar (3% v/v) medium. The formation of the clear zone around the bacterial colony was measured after 4 days of incubation at 28 ± 2 °C (Tiru et al., 2013). Bacterial isolate was inoculated on the Vincent's agar medium in plates (90-mm-diameter) to assay the pectinase activity using spots inoculation method. The plates were kept at 28 ± 2 °C for 4 days. Then, the results were observed for the formation of clear zone around the bacterial colonies by flooding Iodine solution on the plate (Aaisha and Barate, 2016). For chitinase production assay colloidal chitin was prepared from chitin flakes (Sigma Chemicals Co.) by the method of Mathivanan et al. (1997). The bacterial isolate was deposited onto sterilized chitin-agar medium

(0.5% w/v). After incubation at 28 ± 2 °C for 4 days, the presence of clearing zones around bacterial colony was taken as evidence of the chitinase production (Shanmugaiah et al., 2008). Three replicates were used per each experiment.

2.5.7. Plant growth promoting characters

The bacterial isolate was examined for production of Indole-3-acetic acid (IAA) and bacterial isolate was inoculated in Luria-Bertani broth (LBB) medium supplemented with 0.2% (v/v) of L-tryptophan and put in shaker with 200 rpm for 10 days at 28 ± 2 °C. Bacterial biomass was separated with centrifuge at 5000 rpm for 12 min. One mL of the obtained Supernatant was mixed with 2 mL of Salkowaski reagent (150 mL H_2SO_4, 250 mL distilled water, 7.5 mL $FeCl_3.6H_2O$) and then incubated in the dark for 30 min. Appearance of a pink color was expressed as IAA production. The absorbance of pink pigmentation was read using spectrophotometer (SPECORD® PC/210, Analytik jena, Germany) at 530 nm. Uninoculated media supplemented with Salkowski reagent alone was used as blank. The concentration of IAA produced by the selected bacterial isolate was measured using a standard curve according to that prepared from IAA dilution series (Merk, Frankfurt, Germany) to the following equation: $Y = 0.0292\times$, $R^2 = 0.9788$ (Ben Abdallah et al., 2016). Production of gibberellin by selected bacterial isolate was determined using the method of Holbrook et al. (1961). In brief, overnight culture of bacterial isolate was inoculated into Jenson broth media and incubated at 26 ± 2 °C on shaker at 200 rpm for 5 days, following centrifugation at 5000 rpm for 2 min. The pellet was discarded and the pH of the supernatant was adjusted to 1–2 with 0.1 M HCl and transferred to a separation funnel and extracted three times in equal volumes of ethyl acetate. Then, extraction yield was mixed with phosphate buffer and absorbance was measured at 254 nm. The concentration of GA produced by the selected bacterial isolate was measured using a standard curve prepared using gibberellic acid (GA) (Merk, Frankfurt, Germany) with the equation of: $Y = 0.0234\times$, $R^2 = 0.7312$ (Holbrook et al., 1961). Phosphate solubilization activity was investigated according to Dias et al. (2009). The bacterial isolate was inoculated on Pikovskaya agar medium containing tricalcium phosphate and incubated at 26 ± 2 °C for 7 days in dark. Uninoculated plates were used as control. The clearing zone observed around the bacterial colonies was indicated as phosphate solubilization by the selected bacterial isolate. Bacterial isolate was detected for siderophore production using the universal Chrome Azurol Sulphonate (CAS, Sigma–Aldrich, USA) assay with small modifications (Schwyn and Neilands, 1987). CAS agar medium was prepared by mixing 100 mL CAS-HDTMA solution in 900 mL sterilized LB agar medium. The CAS agar medium was added into the plates (15 mL for each plate). After solidification, the CAS was cut into two parts, one of which was replaced with LB agar. Isolate was streaked on the boundary between CAS-LB agar medium and the plates incubated at 26 ± 2 °C for 7 days. The production of yellowish orange halo around endophytic bacterium colonies was taken as evidence of the siderophore production (Arora and Verma, 2017; Louden et al., 2011). For estimation of biofilm formation cell culture of selected bacterium was suspended in LB broth medium to final concentrations (OD600 = 0.2; 1 × 108 CFU/mL). The experiment was performed in 96-well polystyrene microliter dishes containing of LB broth medium. The wells were inoculated with overnight culture 1:100 for biofilm assays. For quantitative assays, we typically used 4 replicate wells for each treatment and incubated the microliter plate for 24–48 h at 28 ± 2 °C. Planktonic bacteria were discarded, and the biofilms, which formed on the wall, were washed three times with distilled water. Into each well, 125 μL of 0.1% crystal violet were added, and the microliter plate was incubated at 28 °C for 15 min. Excess crystal violet was then discarded, and stained biofilms were washed three times with distilled water. Finally, 95% ethanol was added to the stained biofilms, and the OD at 570 nm was read to assess the strength of biofilm formation (Sun et al., 2017).

2.6. Efficacy of bacterial strain on strawberry fruit decay development

Effects of selected endophytic bacterium were investigated on fruit decay development *in vivo* by two methods using living cells and volatile metabolites of strain MarR44.

In living cells, freshly harvested non symptomatic strawberry fruits were gently washed with tap water. The fruits were sterilized by 70% ethanol for 30 s, followed by washing 3 times with sterile-distilled water and air-dried under a laminar flow hood for 10 min. Then the fruits were dipped in a cell suspension (1×10^8 CFU/mL; OD = 0.2) of bacterial strain for 5 min. After that, the fruits were wounded (one mm wide and deep) using a sterilized scalpel. Then, 20 μl conidial suspensions of *C. nymphaeae* (1×10^6 conidia/mL) were added to each wound site on fruits. The fruits inoculated with sterile distilled water and conidial suspension of pathogen were used as negative control and positive control respectively. All fruits were placed into a sterile polyethylene boxes and maintained at 26 ± 2 °C under a regime of 12 h of light and 12 h of darkness for 7 days (Essghaier et al., 2009). 12 replicates were used per each treatment.

In volatile metabolites, the experiment was done in closed glass desiccators (24 by 18 cm, diameter by height, ~6 l in volume). The bacterial isolate was streaked onto the NA plates (14 cm in diameter) and incubated at 26 ± 2 °C for 24 h. The covers of the plates were removed and the plates with the cultures of selected bacterium isolate were placed at the bottom of the desiccators (one plates per desiccators). Uncovered plates without bacterial colonies were used as control. Similarly, mature and healthy strawberry fruits were surface sterilized in 70% ethanol for 30 s, and inoculated with the conidial suspension of pathogen (10^6 conidia/mL) on the surface of strawberry fruit (20 μl per fruit). Then, the fruits were placed on the perforated ceramic clapboard above the uncovered dishes containing the selected bacterium cultures or uncolonized bacterium in a desiccator. There were three desiccators (replicates) for each treatment (23 fruit for each treatment). All desiccators were covered using parafilm and placed at 26 ± 2 °C under a regime of 12 h of light and 12 h of darkness for 7 days (Huang et al., 2011).

In both bioassays, seven days after inoculations, strawberry fruits showing symptoms of sunken necrotic lesion were recorded. Each strawberry fruit was imagined as having a conical shape. Afterwards, the total surface area by measuring the lateral and base area was measured. Then, disease severity (DS) was calculated by AutoCAD software using the following formula:

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

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

2.7. Greenhouse experiments

The effectiveness of bacterial strain to control anthracnose was determined using a pot assay under greenhouse conditions by two methods soil drench and spraying on leaves. Bacterial suspension from 48 h-old NA cultures adjusted to 1×10^8 CFU/mL (OD = 0.2) with sterilized distilled water was used for inoculation. Two groups of strawberry cv. Paros seedlings (5- weeks old) were treated with bacterial isolate A: by drenching, 25 mL of a bacterial cell suspension (1×10^8 CFU/mL) was added around the root and crown of strawberry plants and B: other groups of seedling were sprayed on leaves with 5 mL of same concentration of bacterial cell suspension. After 24 h, 25 mL of the conidial suspension (1×10^6 CFU/mL) of *C. nymphaeae* were also sprayed to all plants in both groups of A and B. The positive control plants were inoculated with pathogen and negative control only with sterile distilled water. Each treatment was replicated four times (Freeman et al., 2001; Rakotoniriana et al., 2013). Disease severity was measured 60 days after inoculation. Disease severity ratings were performed using the following scale: 0 = healthy petiole without lesions;

1 = petiole with lesions < 3 mm in length; 2 = petiole with lesions from 3 to 10 mm; 3 = petiole with lesions from 10.1 to 20 mm; 4 = petiole with lesions from > 20 mm; 5 = entirely necrotic petiole and plant is dead (Delp and Milholland, 1980).

Biocontrol efficacy%

= [(disease index of control) − (disease index of treated)]

/disease index of control × 100.

2.8. Volatile compounds extraction and GC–MS analysis

In order to extract the volatile compounds, bacterial isolate was inoculated on NA medium in plates and the lid was replaced with a bottom plate that contained 3 g of sterile activated charcoal (Sigma). Then, the plates were sealed with parafilm and incubated at 27 °C ± 2 for 4 d at darkness. Next, the activated charcoal was collected and washed with 5 mL of ethyl acetate, centrifuged at 5000 rpm for 10 min to extract all trapped volatile compounds (Tenorio-Salgado et al., 2013). The chemical analysis of volatile compounds was performed on an Agilent 7890A gas chromatography (GC) coupled with Agilent 5975C mass spectrometry (MS) (Agilent Technologies, USA), using a HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The analytical conditions were reported by Amini et al. (2016) previously.

2.9. Statistical analysis

All experiments were designed in completely randomized design (CDR) and repeated two times. Data were subjected to analysis of variance (ANOVA) using SAS software (version 8.2; SAS Institute, Cary, NC, USA, 2013). All data are presented as mean values ± standard deviation (SD). Statistical significance between different treatments was assessed using LSD test at $P \leq 0.05$.

3. Results

3.1. Identification of endophytic bacterial strain

The endophytic bacterial strain MarR44 was isolated from healthy strawberry stolon, and identified as *Staphylococcus sciuri* based upon phenotypic characterization coupled with *16S rRNA* gene sequencing. The strain was Gram positive, non-motile, oxidase catalase positive and was able to fix the nitrogen. Cells spherical, 0.45–0.75 μm in diameter that tend to *occur* in irregular clusters resembling bunches of *grapes* (Fig. 1). It could liquefy gelatin, hydrolyze starch, soluble phosphate, formation of biofilm, reduce nitrate to nitrite, and produce hydrogen cyanide, protease, chitinase, siderophore, IAA and gibberellin. It was negative for lipase reaction and pectinase production (Table S1). To confirm its phylogenetic relationship with *Staphylococcus* spp., genomic DNA was extracted and gene coding for 16S rDNA was amplified by PCR using the universal 16S rRNA primers FD2 and RP1. Blast of this sequence in the GenBank databases of the NCBI showed 98.99% identity with *Staphylococcus sciuri* DSM 20345 (GenBank accession No: AJ421446). Phylogenetic tree constructed by neighbor-joining method showing the position of the strain MarR44 within the genus *Staphylococcus* (Fig. 2).

3.2. In vitro assay

The results of antagonistic tests *in vitro* showed that the strain MarR44 had an effective antagonistic activity against *C. nymphaeae* of strawberry anthracnose. Strain MarR44 showed *in vitro* antifungal activity on PDA medium against *C. nymphaeae* by dual culture and volatile metabolite tests with (52.46%) and (34.52%) inhibition respectively, while this strain had low effect (23.07%) on mycelial growth of *C. nymphaeae in vitro* by non-volatile metabolites test (Table 1). Culture filtrates of strain MarR44 decreased conidial germination of pathogen (41.1%), but there was no significant difference between the treatment and control (Table S2). Volatile metabolites of strain MarR44 significantly decreased conidial germination of *C. nymphaeae* by 82.81% (Table S3). Also, the biomass of *C. nymphaeae* was reduced significantly (91.89%) by cell-free-culture filtrates of this strain at 10% (v/v) concentration (Table S2). Furthermore, the strain MarR44 was capable of forming biofilm in LB liquid medium (Table S1).

3.3. In vivo assay

The efficacy of strain MarR44 in controlling strawberry post-harvest anthracnose on fruit is shown in Table 2. Seven days after inoculation fruit by bacterial cell suspension, the average disease index (DI) of the strain MarR44 treatment was 0.136 which was significantly lower than that of positive control (0.286). Therefore, the endophytic bacterium reduced disease severity (52.45%) compared to control. In addition, volatile metabolites of strain MarR44 decreased fruit decay development (72.17%) compared to control (Table 2).

3.4. Pot experiments

Efficacy of strain MarR44 on disease severity of strawberry anthracnose under greenhouse was evaluated using drenching soil and inoculated plants (spraying on areal organs of plant) methods 60 days after inoculation. Results indicated that strain MarR44 significantly suppressed strawberry anthracnose disease. The disease severity in the MarR44 treatment was much lower compared to control. The biocontrol efficacy of MarR44 was (77.77%) and (72.22%) in soil drenching and plant inoculation, respectively (Table 3).

3.5. Chemical analysis of volatile compounds using GC–MS

The GC–MS analysis of VCOs produced by strain MarR44 revealed various compounds. The results of GC–MS analysis and major compounds have been shown in Table 4. Six compounds with highest area percent were Mesityl oxide (81.436%), Acetic acid, 2-methylpropyl ester (3.442%), 4-Methyldecane (1.837%), 4-Penten-2-one,4-methyl- (1.739%), Toluene (1.248%), and o-Xylene (1.24%). The GC–MS chromatogram of the compounds detected was shown in Figs. S1 to S25.

4. Discussion

Strawberry anthracnose was prevalent in Kurdistan Province, Iran. In this research, an endophytic bacterial strain was isolated from healthy strawberry stolon and its antagonistic ability was evaluated against *C. nymphaeae* causal agent of strawberry anthracnose. On the basis of the cultural, morphological, physiological, biochemical properties and analysis of the 16S rDNA gene sequences, strain MarR44 was identified as *Staphylococcus sciuri* MarR44 (GenBank accession number: MH161571). Previous studies have also reported that *S. sciuri* colonizes the root of plants to improve the growth parameters and increases healthy development of plants (Akram et al., 2016; Dutta et al., 2017). To our best knowledge, there are not studies about role of *S. sciuri* as biocontrol agents against causal agent of strawberry anthracnose. However, this is the first time that *S. sciuri* has been used as biocontrol agents against *C. nymphaeae* causal agent of strawberry anthracnose. The *S. sciuri* MarR44 was shown to be able to manage strawberry anthracnose under *in vitro*, *in vivo* and greenhouse conditions. Culture filtrates of selected endophytic bacterium significantly decreased fungus biomass and conidial germination of pathogen. In addition, mycelial growth, conidial germination of pathogen, and fruit decay development were significantly reduced by volatiles compounds (VOCs) of strain MarR44 (Huang et al., 2015). These results suggest

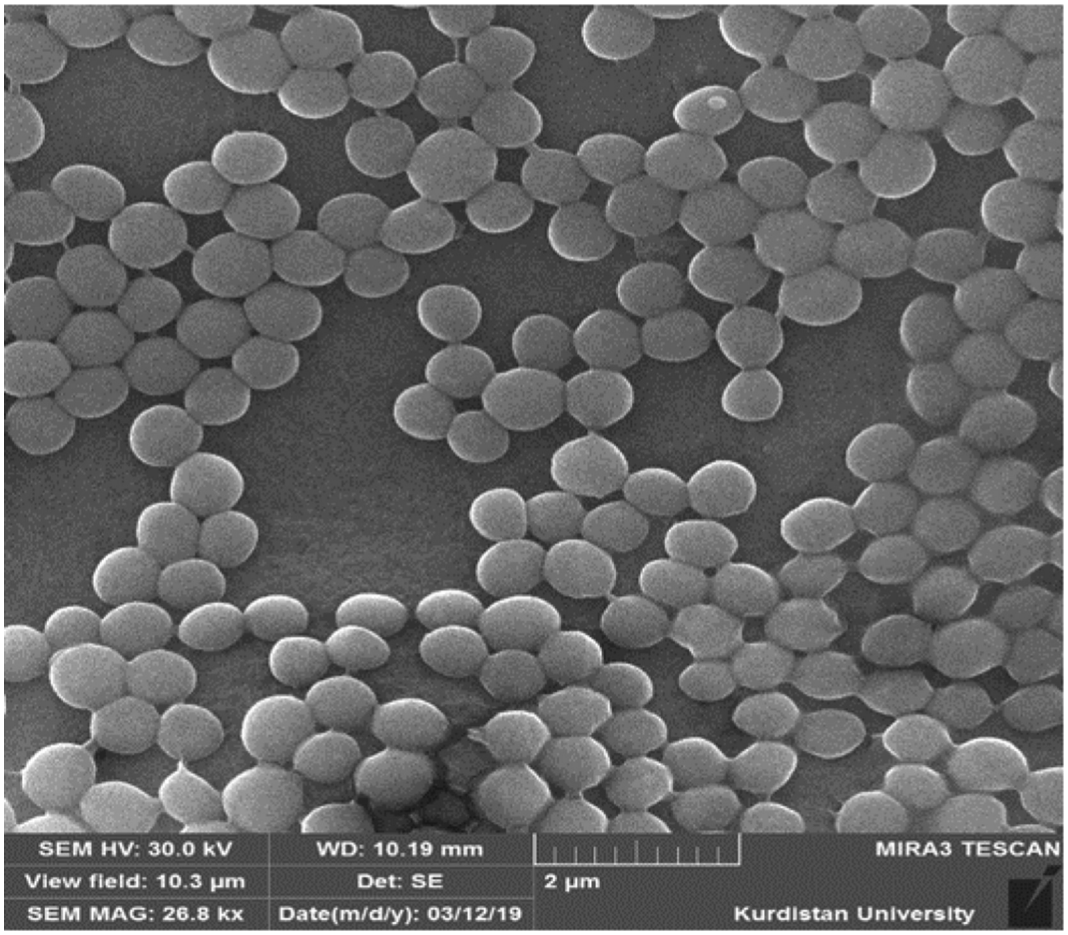

Fig. 1. Scanning electron micrograph of cells of *S. sciuri* strain MarR44 from an exponentially growing culture. Bar, 2 μm.

that production of antifungal VOCs by *S. sciuri* MarR44 is an important mechanism for reduction of infection of strawberry plant tissues by *C. nymphaeae* under airtight conditions. Previous studies have indicated that antifungal VOCs produced by bacteria or fungi are effective biofumigation for control of plant diseases under airtight conditions (Huang et al., 2011; Koitabashi, 2005; Mercier and Jiménez, 2004; Mercier and Smilanick, 2005; Schnabel and Mercier, 2006). Also, the strain MarR44 was efficient in production of siderophore, chitinase,

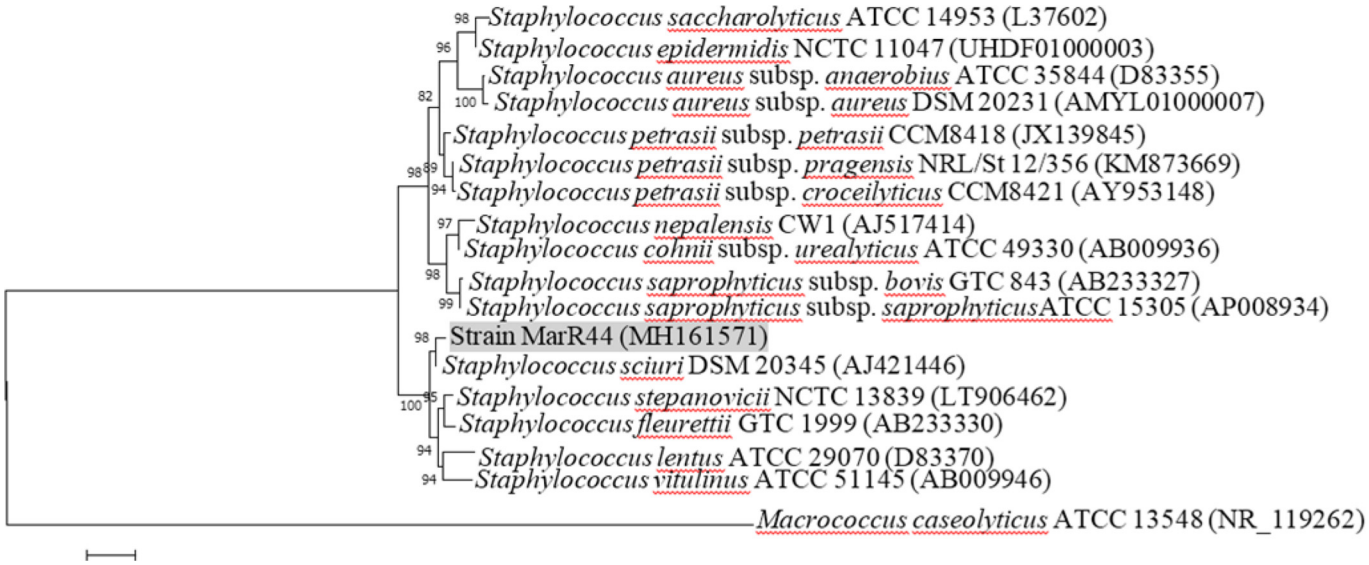

Fig. 2. Phylogenetic tree of *S. sciuri* strain MarR44 based on 16S rRNA gene encoding region, which show relationship between sequences this strain and other *Staphylococcus* species. These phylogenetic analyses were performed by neighbor-joining method implemented in MEGA7. Bootstrap values (expressed as percentages of 1000 replicates) > 50% are shown at the branch points *Macrococcus caseolyticus* was used as outgroup. Scale bar represents 0.02 substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

Table 1
Inhibitory effect of *S. sciuri* strain MarR44 on mycelia growth of *C. nymphaeae* under *in vitro* tests after 7 days.

Treatments	Dual culture		Volatile metabolite		Non-volatile metabolite	
	Colony diameter (mm)	Inhibition%	Colony diameter (mm)	Inhibition%	Colony diameter (mm)	Inhibition%
MarR44	1.93 ± 0.05 b	52.46	2.20 ± 0.85 b	34.52	1.10 ± 0.00 b	23.07
Control	4.06 ± 0.25 a	–	3.36 ± 0.12 a	–	1.43 ± 0.17 a	–
LSD (5%)	0.3106	–	1.054	–	0.208	–

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

Table 2
Effect of *S. sciuri* strain MarR44 on fruit decay development *in vivo* after 7 days using two methods treated fruit with living cells and volatile compounds.

Treatments	Living cell		Volatile compounds	
	Disease severity	Biocontrol efficacy (%)	Disease severity	Biocontrol efficacy (%)
MarR44	0.136 ± 0.073 b	52.45	0.069 ± 0.04 b	72.17
Control	0.286 ± 0.053 a	0	0.248 ± 0.05 a	–
LSD (5%)	0.0561	–	0.0274	–

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

Table 3
Effect living cells of *S. sciuri* strain MarR44 on disease severity of strawberry anthracnose in greenhouse condition, 60 days after inoculation.

Treatments	Soil drench		Plant inoculation	
	Disease severity	Biocontrol efficacy (%)	Disease severity	Biocontrol efficacy (%)
MarR44	1.00 ± 2.00 b	77.77	1.25 ± 1.89 b	72.22
Control	4.50 ± 0.58 a	–	4.50 ± 0.58 a	–
LSD (5%)	2.5468	–	2.4213	–

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

protease, HCN, IAA and gibberellin. Many such metabolites showed significant inhibitory effects on biomass, radial growth, microsclerotia and play significant role in biocontrol of phytopathogens (Das et al., 2008; Dowling and O'Gara, 1994). Several genera of endophytic bacteria such as *Bacillus*, *Burkholderia*, *Serratia*, *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Micrococcus*, and *Microbacteruim* were positive for chitinase, and protease involved in cell wall degradation of various pathogens (Ben Abdallah et al., 2016; Jangir et al., 2018). Production of HCN is well-documented among prokaryotes and acts as general inhibitor to avoid competition and predation between pathogen and biocontrol agent (Paramanandham et al., 2017). The HCN as secondary metabolite is considered to be toxic against plant pathogens, and decrease the pathogens growth in the rhizospheric environment (Jangir et al., 2018). Siderophore producing bacteria as antagonists have major role in limiting the iron available for the plant pathogens (Ilyas and Asghari, 2012). Also, siderophore is an iron-healing compound (iron chelators) that has an immense role in microbial interaction, especially in the rhizosphere (Eisendle et al., 2004). On the other hand, production of siderophore and HCN by strain MarR44 in the present work is one of the major mechanisms of bacteria that are involved in biocontrol of *C. nymphaeae* in pot experiments.

Furthermore, we identified 24 VOCs produced by strain MarR44 including two ketones, two esters, three alcohols, five benzene, eleven alkanes, and one cycloalkene. Ketones (83.175%), alkanes (6.703%), esters (4.175%) and benzene (3.604%) were the main compounds types, and these four types accounted for 97.657% of the 24 detected compounds. Based on the size of the areas, we concluded that Mesityl oxide; acetic acid, 2-methylpropyl ester; 4-Methyldecane and 4-Penten-

Table 4
Major VCOs produced by *S. sciuri* strain MrR44 identified by GC–MS analysis with details on their retention time, area %, chemical formula and CAS#.

Number	Compounds	Chemical formula	Retention time (min)	Area (%)	CAS#
1	4-Penten-2-one, 4-methyl-	$C_6H_{10}O$	3.184	1.739%	3744-02-3
2	Toluene	C_7H_8	3.379	1.248%	108-88-3
3	Acetic acid, 2-methylpropyl ester	$C_6H_{12}O_2$	3.625	3.442%	110-19-0
4	Mesityl oxide	$C_6H_{10}O$	4.231	81.436%	141-79-7
5	Acetic acid, butyl ester	$C_6H_{12}O_2$	4.729	0.733%	123-86-4
6	5-Hexen-3-ol, 2,3-dimethyl	$C_8H_{16}O$	5.530	0.304%	19550-90-4
7	Ethylbenzene	C_8H_{10}	6.034	0.273%	100-41-4
8	m-Xylene	C_8H_{10}	6.291	0.498%	108-38-3
9	o-Xylene	C_8H_{10}	7.024	1.240%	95-47-6
10	Nonane	C_9H_{20}	7.315	0.889%	111-84-2
11	2,6-Dimethyloctane	$C_{10}H_{22}$	8.362	0.634%	2051-30-1
12	Heptane, 3-ethyl-2-methyl-	$C_{10}H_{22}$	8.563	0.524%	14676-29-0
13	Ethylmethylbenzene	C_9H_{12}	9.158	0.345%	611-14-3
14	4-Methylnonane	$C_{10}H_{22}$	9.204	0.491%	17301-94-9
15	2-Methylnonane	$C_{10}H_{22}$	9.284	0.401%	871-83-0
16	3-Methylnonane	$C_{10}H_{22}$	9.467	0.577%	5911-04-6
17	Cyclodecane	$C_{10}H_{20}$	9.787	0.278%	293-96-9
18	4-Methyldecane	$C_{11}H_{24}$	10.285	1.837%	2847-72-5
19	(1-Methylnonyl)cyclohexane	$C_{16}H_{32}$	10.892	0.684%	13151-73-0
20	2-Hexyl-1-decanol	$C_{16}H_{34}O$	11.040	0.450%	2425-77-6
21	2-Hexyl-1-octanol	$C_{14}H_{30}O$	11.298	0.307%	19780-79-1
22	Endo-8,9-dihydrodicyclopentadiene	$C_{10}H_{14}$	11.899	0.331%	2825-86-7
23	3-Methyldecane	$C_{11}H_{24}$	12.116	0.270%	13151-34-3
24	Undecane	$C_{11}H_{24}$	12.860	0.805%	1120-21-4

2-one, 4-methyl- were the main VOCs produced by strain MarR44. Bacterial endophytes have the ability to secrete volatile metabolites that defend the plants from pathogen, expose antifungal activity, induce systemic resistance, and prevent the mycelial growth and spore germination of pathogen (Monte, 2001). Studies have showed that volatile organic compounds produced by *B. amyloliquefaciens* decrease growth of *Ralstonia solanacearum* after 5 days (Raza et al., 2016). In sealed petri plate study, we found reduction in fungal mycelial growth (34.52%) and conidial germination (82.81%). Raza et al. (2015) have reported that production of benzenes, ketones and alcohols compounds by strain *Paenibacillus polymyxa* WR-2 in low quantity cause excellent antifungal activity (62–100%) against *Fusarium oxysporum*. Vapors of several common vinegars effectively prevented from conidial germination of *Monilinia fructicola*, *Botrytis cinerea,* and *Penicillium expansum* on stone fruit, strawberries, and apples, respectively (Sholberg et al., 2000). Also, several compounds such as toluene, ethylbenzene, nonane, decane, undecane showed antifungal activity against *F. oxysporum* (Yuan et al., 2012).

Disease severity of anthracnose in greenhouse conditions was reduced significantly by strain MarR44 using two soil drenching and plant inoculation methods. Growth inhibition level by strain MarR44 in greenhouse conditions was significantly more than those of the *in vitro* assays. These results were due to the colonization of plant tissues by endophytic bacterium, then production of antimicrobial metabolites including chitinase, protease, HCN and siderophore (Gholami et al., 2019; Jangir et al., 2018). According to the results, we expect that this bacterial isolate inhibit and inactivate the *C. nymphaeae* in the natural environments and aid in biocontrol of pathogen. Also, the strain MarR44 is capable of biofilm formation that helps during root colonization and acts as a barrier, thereby defending plants from penetration by soil-borne pathogens (Jangir et al., 2018). Furthermore, strain MarR44 was able to produce the plant growth hormone IAA, gibberellin, and solubilized phosphate, which may relate to the plant tissues growth regulation (Yasmin et al., 2017). Similar results were reported by Akram et al. (2016), and Dutta et al. (2017) who isolated endophytic bacterium *S. sciuri* from roots and rhizospheric soil of Kallar grass (*Leptochloa fusca* L.), which play an important role in the regulation of plant development by production of phytohormone and phosphate solubilization. *Staphylococcus* species can tolerate high salt concentration (Khan et al., 2015; Roohi et al., 2012) and exhibit plant growth promoting properties in plants (Yildirim et al., 2008). Zhou et al. (2015) indicated the growth increasing in treated sweet cherry plants with a PGPR strain *S. sciuri* subspecies *sciuri.* Gibberellins (GAs), a well-known plant growth promoting hormone and thereby playing a significant role in increasing the root surface area and number of root tips in many plants (Han et al., 2005). Moreover, Vassilev et al. (2006) reported that the phosphates solubilization by microbial activity usually induces the secretion of certain metabolites such as siderophore, lytic enzymes and phytohormone that are involved in the control of phytopathogens.

However, members of Firmicutes, are responsible for production of a wide range of antimicrobial and antifungal metabolites, enzymes and surfactants that play significant roles in plant growth promotion and avoid disease by colonizing the surface or inside of different parts of the plants (Bibi, 2017). Additionally, it has been documented that the synergistic effects of hydrolytic enzymes and antibiotics produced by the selected endophytic bacterium give a higher measure of biocontrol efficacy against *C. nymphaeae* than the antagonism obtained by either mechanism alone (Monte, 2001).

5. Conclusions

The strain MarR44 produced siderophore, chitinase, protease, HCN, IAA and gibberellin and was efficient in biofilm formation. Mycelial growth, conidial germination of pathogen, fruit decay development and disease severity of strawberry anthracnose were reduced by this strain. Potential modes of action may include antibiosis and production of antifungal metabolites (VOCs) by *S. sciuri* strain MarR44 against *C. nymphaeae*. To our best knowledge, this is the first report showing the antifungal activity of *S. sciuri* strain MarR44 against *C. nymphaeae* and its efficacy for reducing disease. Therefore, these results have established a new line of research about *S. sciuri* as biocontrol agent against plant pathogens and it can be a candidate for biological control. Further studies in order to determine other modes of action (*e.g.* colonization of plants, inducing plant resistance) and repetition of experiments under field conditions are required to confirm the ability of *S. sciuri* to control strawberry anthracnose disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108276.

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