Analysis of strawberry metabolic profiling in interaction with *Bacillus* spp. strain DM12 using Gas Chromatography–Mass Spectrometry and Determination of its Antifungal Activity

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Abstract

Colletotrichum nymphaeae infects the roots, leaves and fruits of the plant at different developmental stages and is one of the most important diseases of strawberry. The principal problems to strawberry cultivation are fruit rot caused by anthracnose that affect the quality and quantity of production and marketing in the field, and post–harvest. An endophytic bacterial strain, isolated from *Fragaria* × *ananassa*, and antifungal properties against *Colletotrichum nymphaeae* was assayed under *in vitro*, *in vivo*, and greenhouse experiments. Bacterial strain was identified as *Bacillus* spp. DM12 (MH161581) using phenotypic, biochemical and molecular phylogenetic analysis of the *16S rDNA* gene. DM12 strain inhibited mycelial growth of fungal pathogen (64.03%) using dual–culture test. The cell–free culture compounds produced by DM12 prevented mycelial growth and conidial germination of *C. nymphaeae* by 32.86% and 73.98%, respectively but, inhibition percentage of mycelial growth of pathogen by volatile compounds was less (9.82%). As well as, protease, chitinase, pectinase, siderophore, IAA, gibberellin, and phosphate solubilization tests for this strain were positive. Anthracnose disease at post–harvest on fruit suppressed by the strain DM12 (90.87%). Also, biocontrol efficacy on strawberry plants by drenching soil and spraying methods were 72.22% and 94.44%, respectively, 60 days after inoculation. PCR amplification represented the presence of genes of *surfactin*. In addition, metabolite profile of strawberry was changed on presence of bacterial strain that a number of metabolites in control treatment with maximum area percent were Acetoglyceride (19.418%), Acetic acid, butyl ester (4.734%) and Ribitol (4.349%), in treatment inoculated with DM12 strain alone were Tetramethyl–2–hexadecen (21.35%), Ethylene glycol monoisobutyl ether (18.688%) and Myrtenol (8.75%), in treatment inoculated with fungal pathogen alone were Acetoglyceride (18.089%) and Acetic acid, monoglyceride (17.96%) and in treatment inoculated with *C. nymphaeae* and DM12 strain together were tert–Butanethiol (36.153%), Ethoxy triethyl silane (14.126%), 5–(Methylamino)–1,2,3,4–thiatriazole (9.53%) and 2,3–Butanediol (7.795%). Some of these compounds, such as Butanediol, Lactose and Tetradecane, which were produced in DM12 strain alone and *C. nymphaeae* and DM12 strain together treatments, have antifungal properties. These results showed that strain DM12 has able to decrease mycelial growth, conidial germination, fruit decay development and disease severity of strawberry anthracnose under *in vitro*, *in vivo* and greenhouse conditions. Further works are required in order to understanding the interaction of DM12 with the plant and others phytopathogens and its effects on the plant defense system.

Keywords: Antifungal metabolites, Bacillus spp, GC–Mass, Strawberry anthracnose

Introduction

In natural environment, plants have to overcome multiple stressors, such as pathogens. To survive, they must respond to these agents with fast and effective mechanisms. Endophytic bacteria with biological attributes inhabit plant internal tissues and may collate with the phytopathogens (Hallmann *et al*., 1997; Berg *et al*., 2005). They compete with the pathogens by production of effective compounds with antimicrobial and antifungal properties, produce of fungal cell wall–degrading enzymes, competition for space and nutrition and inducing the plant defense systems (Mengistu, 2020). Numerous researches have been done on the useful effects of endophytic bacteria as biological control agents on phytopathogenic fungi (Yang *et al*., 2013; Hidayati *et al*., 2014; Huang *et al*., 2015; Ben Abdallah *et al*., 2016). *Bacillus* species are gram positive, rod–shaped, sporulating bacteria that which are considered as beneficial agents for disease control through a variety of mechanisms, such as antibiosis, inducing of defense responses in the host plant, competition for nutrient sources, and space (Mora *et al*., 2011). Several studies have been performed to investigation of mechanisms involved in biocontrol of plant diseases by *Bacillus* species (Yu *et al*., 2011; Cao *et al*., 2012; Li *et al*., 2013). *B. subtilis* suppress main phytopathogens, including *Sclerotium rolfsii* (De Curtis *et al*., 2010), *Fusarium* sp. (Cao *et al*., 2011; Zhao *et al*., 2013), *Rhizoctonia solani* (Kumar *et al*., 2012), and *Verticillium dahliae* (Li *et al*., 2013). *Colletotrichum nymphaeae* is one of the members of *C. acutatum* complex species that causes strawberry anthracnose (Embaby & Abd–Ellatif, 2013; Karimi *et al*., 2019). In Iran, strawberry is

generally cultivated in the western part of country, Kurdistan province. The pathogen infects the roots, leaves and fruits of the plant at different developmental stages and is one of the most important diseases of strawberry (Garrido *et al*., 2011). The principal problems to strawberry cultivation are fruit rot caused by anthracnose that affect the quality and quantity of production and marketing in the field, and post–harvest (Embaby & Abd–Ellatif, 2013).

In this research, we isolated the endophytic bacterium *Bacillus* spp. strain DM12 from healthy sepals of strawberry plants and studied antagonistic properties against *C. nymphaeae* and investigated *in vitro*, *in vivo* and greenhouse tests to suppress the growth of fungal pathogen. Also, we evaluated strawberry metabolic profiling in interaction with anthracnosis agent and endophytic bacterial strain. These results suggest that antifungal metabolites derived from DM12 are important in the emergence of beneficial properties. Therefore, we analyzed the chemical profiles of metabolites produced by DM12 using gas chromatography–mass spectrophotometer (GC–MS) system. Collectively, our results suggest this bacterial strain decreases the growth of fungal pathogen by multiple mechanisms including, producing antifungal metabolites.

Materials and methods

Fungal pathogen

Colletotrichum nymphaeae (GenBank accession no. MK372221; Strain ID; IRAN 3427C) was obtained from the collection culture of University of Kurdistan in Iran (Alijani *et al*., 2019).

Bacterial isolation and identification

The endophytic bacterial strain was isolated from the healthy sepals of strawberry in Kamyaran, Kurdistan province. The samples (3–5 mm) were sterilized in 70% ethanol (v/v) for 1 min, then in 2.5% sodium hypochlorite (v/v) for 3 min and then in 70% ethanol for 30 s. The sterilized pieces rinsed three times and dried. Then the samples were squashed in 10 mL sterile distilled water and abandoned for 30 min. Finally, 50 µL of suspension was inoculated on nutrient agar (NA) and after 24 h, a single colony of bacterial isolate was cultured on NA media to get pure culture. For evaluation of sterilization efficiency, 50 µL of the water from the last rinse of samples was cultured on NA medium. The pure culture in nutrient broth (NB) medium supplemented with 20% (v/v) glycerol was retained at 20˚C. Morphological, physiological and biochemical properties was performed (Bergey & Holt, 1994; Grimont & Grimont, 2006). The bacterial strain was identified based on *16S rRNA* gene sequencing. Genomic DNA was extract and PCR reaction was done with the universal primers (Alijani *et al*., 2019). The purified PCR products were 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) and the BLASTN algorithm was performed. Also, the bacterial strain was analyzed for the biosynthetic lipopeptide genes including *srfAA*, *FenD*, and *ItuD*. The PCR amplification of *FenD* and *ItuD* genes were done with an initial denaturation for 5 min at 95˚C followed by 30 cycles denaturation for 1 min at 94˚C, annealing for 1 min at 55˚C, extension for 1 min at 72˚C and final extension at 72˚C for 10 min. The primers used for lipopeptide genes are FenD1f (5′–TTTGGCAGCAGGAGAAGTTT–3′), FenD1r (5′–GCTGTCCGTTCTGCTTTTTC–3′), and ItuD1f (5′–GATGCGATCTCCTTGGATGT–3′) and ItuD1r (5′–ATCGTCATGTGCTGCTTGAG–3′) with 964 and 647 bp product size, respectively (Gond *et al*., 2015). The PCR conditions for the amplification of *srfAA* gene was initial denaturation for 4 min at 95˚C followed by 40 cycles of denaturation for 1 min at 94˚C, annealing for 1 min at 55˚C, extension for 1 min at 70˚C and final extension at 70˚C for 5 min. The primers for this gene are SRFAF (5′–TCGGGACAGGAAGACATCAT–3′) and SRFAR (5′–CCACTCAAACGGATAATCCTGA–3′) with 201 bp product size (Mora *et al*., 2011).

Plant growth promotion features

Several antifungal and plant growth promoting traits were done. Siderophore and HCN production was estimated for this strain (Alijani *et al*., 2019). The ability of lytic enzymes production including protease, chitinase and pectinase was performed by culturing bacterial strain onto sterilized skim milk agar, chitin–agar (0.5% w/v) and Vincent's agar media, respectively (Shanmugaiah *et al*., 2008; Tiru *et al*., 2013; Aaisha and Barate, 2016). IAA and gibberellic acid (GA) produced by bacterial strain were investigated according to previous studies (Holbrook *et al*., 1961; Ben Abdallah *et al*., 2016) and comparison with a standard curve (Alijani *et al*., 2019). Also, the ability of phosphate solubilization was performed by Pikovskaya's agar medium (Dias *et al*., 2009). Hypersensitive response test was done with tobacco (*Nicotiana tabacum* L.) to investigate the pathogenicity of bacterial strain (Ben Abdallah *et al*., 2016). Three replicates per each treatment were used.

Evaluation of antifungal traits *in vitro*

For dual culture assay, bacterial strain was cultured on one side of plate comprising potato dextrose agar (PDA) medium and at one side of the plate, a 5–mm diameter disc of a 7–day–old culture of fungal pathogen was placed (Moreira *et al*., 2014). The effect of volatile compounds was assayed by double–plate method that, a fresh bacterial inoculated on NA medium and 5 mm disc

of a 7–day–old culture of the fungal pathogen inoculated on the other plate with PDA medium. Then, the plate containing pathogen culture was placed inversely on the plate containing NA medium and covered with Parafilm (Alijani *et al*., 2019). For evaluated effect of volatile compounds on biomass of fungal pathogen, two section plates were used. The bacterial strain was cultured in one section and incubated at 24 ± 2˚C for 24 h and plate was weighed in control and treatments plates. Then, a 5–mm diameter disc of a 7–day–old culture of fungal pathogen was placed on other section of plate (Alijani *et al*., 2019). After 7 days, plates It was weighed again and compared with the control. In non–volatile metabolites test on mycelial growth of pathogen, the bacterial strain was inoculated into PDB medium (45 mL) and placed on a rotary shaker at 150 rpm at 27 ± 2˚C for 48 h. After that, the bacterial cultures were centrifuged at 5000 rpm for 15 min and supernatants filtered by 0.22 µm Millipore filter. Then, in each plate containing PDA medium two wells (5 mm) were created and 150 µl of the culture filtrates loaded to each and the fungal pathogen (5 mm diameter disc) cultured in the between of two wells (Jangir *et al*., 2018). PDA media containing fungal pathogen alone were used as controls. In three experiments, the plates were maintained at 25 ± 2˚C for 7 days and then, the diameter of the fungal pathogen colony was measured and inhibition percentage was calculated using the following formula:

I (%) = $[(d_c - d_t)/d_c] \times 100$, where d_c is radial growth of pathogen in control, d_t is radial growth of pathogen in treatments (Islam *et al*., 2016).

For investigation the effects of non–volatile metabolites on conidial germination, 200 µl of a conidial suspension (1×10^6 CFU/mL), 1 mL of bacterial culture filtrates and 5 ml of PDB medium were mixed. The media with spore suspension of pathogen alone were considered as controls (Alijani *et al*., 2019). The results were assayed after 24 h by use an optical microscope and 50 conidia were investigated for each replication. The inhibition percentage of conidial germination was tested following Alijani *et al*. (2019). Three replication was used for all treatments.

Evaluation of antifungal traits *in vivo*

The effects of the bacterial strain on fruit decay were performed using ripe and healthy strawberry (*Fragaria* × *ananassa* Duch.) fruit c.v Paros with same size and colour and without symptoms of diseases. The fruits were disinfected with 70% ethanol for 30s and rinsed. Then, fruits were dipped in bacterial suspensions (1×10^8 CFU/mL) for 5 min. twenty µl of conidial suspension of fungal pathogen (1×10^6 spore/mL) was loaded on the inoculated fruits after 24 h. As negative and positive controls, fruits inoculated with sterile distilled water and conidial suspension of the fungal pathogen respectively. The experiment was performed with 12 replications. The fruits were placed into boxes and stored at 25 ± 2˚C, 75–80% relative humidity (RH) (Essghaier *et al*., 2009). After 5 days, the height and base radius of each fruit were recorded. Then, disease severity (DS) was measured by AutoCAD software (version 2012) using the following formula:

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

Where, (A) is infected area, (H) and (r) are fruit height and the base radius of the fruit respectively (Alijani *et al*., 2019).

Evaluation of antifungal traits in greenhouse conditions

The ability of bacterial strain against strawberry anthracnose was investigated under greenhouse conditions on strawberry plant c.v Paros. Two methods were performed, in method of soil drench, the roots of seedlings (4–week–old) were inoculated with 25 mL of a 1×10^8 CFU/mL of bacterial suspension. In other method, five mL of bacterial suspension (1×10^8 CFU/mL) were sprinkled on the foliage of seedlings (4–week–old). In both methods, five mL of the conidial suspension of fungal pathogen (1×10^6 spore/mL) was inoculated to each plant after 24 h. Plants were sprayed with sterilized distilled water and conidial suspension of fungal pathogen as negative and positive controls, respectively (Rakotoniriana *et al*., 2013). Then, plants were maintained at 24 to 27˚C, 60–70% RH, 16 h light, 8 h darkness. The disease severity was assayed two months after inoculation using the following scale: 0=healthy petiole without lesions; 1=petiole with lesions<3mm 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 & Milholland 1980). Biocontrol efficiency was calculated by [(disease index of control) – (disease index of treated)]/ disease index of control ×100 formula. Four replication was considered for each treatment. After the test, the fungal pathogen was re–isolated.

Metabolite extraction from bacterial strain

Production of secondary metabolites

Bacterial strain was grown at 30˚C on a shaker at 150 rpm. After 48 h, ethyl acetate was added in 1:1 ratio and mixed on magnetic stirrer for six hours. Then, the bacterial culture was centrifuged at 5000 rpm for 20 minutes and debris removed. Then, ethyl acetate phase was collected and dried in a rotary evaporator at 50˚C and the residual was maintained at 4˚C (Al–Imara *et al*., 2020).

Thin layer chromatography (TLC)

A thin layer chromatography (TLC) test was done using silica gel 60 GF–254 as the solid matrix with a moving phase consisting of (ethyl acetate: acetic acid: methanol) by ratios of (v: v: v

17:38:50) and retention factor (Rf) was calculated (Al–Imara *et al*., 2020).

Antifungal activity test of secondary metabolites

The antifungal activity test was carried out by agar diffusion method. The PDA media were inoculated by conidial suspension (1×10^6 CFU/mL) of *C. nymphaeae* and three wells created on medium and filled with 100 µL metabolite extraction of DM12 that filtered by 0.22 µm Millipore filter. In the control plates, the wells were filled by ethyl acetate. Plates were incubated at 25˚C. After 72 h inhibition zone around agar plug was assayed (Espinel–Ingroff *et al*., 2007; Ibrahim *et al*., 2014).

Metabolite extraction from meristem culture

Meristem culture of strawberry

Strawberry seedlings c.v Paros were collected from field and washed 4–5 times. Surface sterilization was performed inside the laminar air flow by dipping the parts of plant in 70% ethanol for 10 s, and then in 1% sodium hypochlorite for 10 min. The sterilized pieces rinsed three times with sterile distilled water. The media supplemented with MS salts (pH: 5.8) loaded in test tubes and separated meristems under sterile conditions cultured into tubes (Murashige and Skoog, 1962). The cultures were incubated in a growth chamber under 16/8 h light/dark cycle at 25±2˚C under continuous illumination with cool white fluorescent tubes at an intensity of 2000 lux. The seedlings that were obtained after three months were cultured into jar with MS media and proliferated. These seedlings were used for subsequent experiments.

Interaction tests

In order to, jars containing MS broth media were prepared and covered with aluminum foils. One hole was created on the center of aluminum foil and strawberry seedlings obtained from the previous stage were cultured in the holes. The door of jar was placed and the cultures were incubated in a growth chamber under 16/8 h light/dark cycle at 25±2˚C under continuous illumination with cool white fluorescent tubes at an intensity of 2000 lux for 48 h in order to adapt to the conditions. Then, 1 mL of bacterial suspension (1×10^8 CFU/mL) sprayed on foliage and the seedlings were placed in the growth chamber. In order to assessment of successful entry of bacterium into the plant, after 72 h, several seedlings were collected and after surface sterilization and maceration, final suspension cultured on NA medium. Afterward, 1 mL, conidial suspension of fungal pathogen (1×10^6 spore/mL) were sprayed on aerial parts of seedling and incubated on growth chamber with previous conditions. For interaction test four treatment were considered including, A) negative control that sprayed with sterile distilled water, B) positive control that sprayed with conidial suspension of fungal pathogen, C) seedlings that sprayed with bacterial suspension alone and D) seedlings that sprayed with bacterial suspension and conidial suspension of fungal pathogen together (Vahabi *et al*., 2014).

Metabolite extraction

After 96 h, aerial parts of seedlings from each treatment were harvested (100 mg) and pulverized to a fine powder in a mortar with a pestle under liquid nitrogen. Metabolite extraction was done as previously described (Aliferis *et al*., 2014). Briefly, 1 ml of a mixture of methanol: ethyl acetate (50:50, v/v) was added to the pulverized parts of seedlings followed by sonication in an ultrasonic bath for 25 min. Then, samples were placed to an orbital shaker for extraction under 150 rpm agitation for 2 h at 24˚C. Extracts were filtered with 0.2 mm filters (Millex–FG, Millipore, MA, USA) to eliminate debris.

Gas chromatography–mass spectrometry diagnosis of secondary metabolites of plant extracts

The secondary metabolites were determined using GC–MS. 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.

Statistical analysis

The completely randomized block design was used in all experiments. All experiments were repeated twice and performed with at least three replications. One representative experiment is presented in the results. After testing for homoscedasticity and normal distribution, the tests were designed as a completely randomized design and data were subjected to a one–way analysis of variance (ANOVA) using SAS software (version 8.2; SAS Institute, US). All data are presented as mean values ± standard deviation (SD). Statistical significance between different treatments were assessed using LSD test ($\alpha = 0.05$).

Results

Identification of endophytic biocontrol bacterium

Endophytic bacterial, *Bacillus* spp. strain DM12 was isolated from sepal the healthy strawberry fruits. Biochemical assays indicated that bacterial strain was positive for catalase, oxidase, and lipase and was able to hydrolyze starch, phosphate solubilization and production of protease, chitinase, pectinase and siderophore. The endophytic bacterial strain did not cause hypersensitive response in the inoculated tobacco plants (Table 1). Blast of the

sequences of DM12 in the GenBank databases of the NCBI represented identity with *Bacillus* spp. The PCR amplification of the lipopeptide genes showed that this bacterial strain was able to production of surfactin.

Table 1. Chemical characteristics of bacterial strains

Taxonomic/Functional traits	DM12
Secondary metabolites and enzymes	
Protease	+
Chitinase	+
Pectinase	+
HCN	–
Lipase	+
Plant growth promoting characters	
Indole–3– acetic acid (IAA)	+
Gibberellin	+
Phosphate solubilization	+
Siderophore production	+
Nitrogen fixation	–
General features	
Gram test	+
Oxidase	+
Catalase	+
Motility	+
Simon citrate	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Phosphatase	–
Nitrate reduction	+
H_2S production	–
Gas from glucose agar	–
Hypersensitive Response Test	–

+ represent production, – represent no production; +, ++, +++ and ++++: isolates showing very low, low, high and very high activity respectively.

Inhibition effects of bacterial strains on *C. nymphaeae in vitro*, *in vivo* and greenhouse conditions

The data of the *in vitro* experiments represented that strain DM12 prevented the hyphal growth of *C. nymphaeae* by dual culture test with (64.03%). The results of culture filtrates tests indicated that active metabolites from bacterial strain inhibited the mycelial growth with the percentage 32.86% and on conidial germination with 73.98%. Also, effects of volatile metabolites on growth of mycelium showed that minimum inhibition zone with the percentage 9.82% (Fig. 1A, B). The effects of volatile metabolites decreased of fungal biomass and efficiency was measured (82.37%) (Table 2). The results under *in vivo* conditions showed that, after inoculation of fruits by DM12 suspension, fruit decay development significantly decreased with the biocontrol efficacy 90.87%. Under greenhouse conditions, two methods were performed and indicated that this bacterial strain significantly reduced appearance of symptoms on petioles of strawberry plants by soil drenching (72.22%) and foliar spraying (94.44%) (Fig. 1C, D).

Table 2. Efficacy of volatile metabolites of *Bacillus* spp. strain DM12 on biomass of *C. nymphaeae*

Treatments	VOC on biomass	
	Biomass weight change	Inhibition (%)
DM12	0.092 ± 0.01 b	82.37%
Control	0.522 ± 0.06 a	–
LSD (5%)	0.0687	–

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).

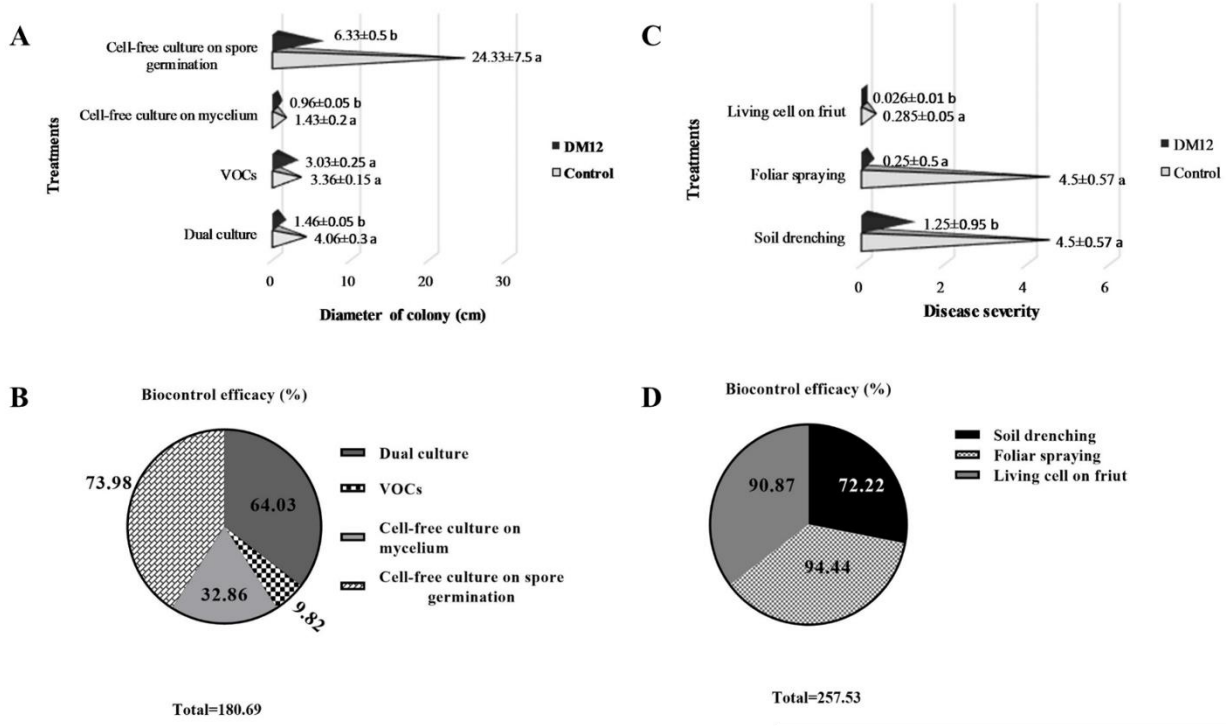

Fig. 1. A) Antifungal activity DM12 strain against *C. nymphaeae in vitro* conditions. B) Biocontrol efficacy percentage under *in vitro* conditions on mycelial growth and conidial germination of *C. nymphaeae*. C) The diagram of effects of DM12 strain on disease severity of strawberry anthracnose in greenhouse conditions, 60 days after inoculation and fruit decay development under *in vivo* conditions after 7 days. D) Biocontrol efficacy percentage under *in vivo* and greenhouse conditions on disease severity of *C. nymphaeae*. Mean followed by different letters within the column represents significant differences according to the LSD test ($P \leq 0.05$). Data are mean with ± standard deviation (SD)

Effects of antifungal activity of secondary metabolite production by DM12

Metabolite compounds extraction from strain DM12 in agar diffusion test prevented growth of *C. nymphaeae* around the wells. RF value under UV light was calculated 0.81 on TLC sheet.

Effects of interaction tests on metabolites profile of strawberry plants

In this study, DM12 strain was selected and the interaction tests in control treatment, seedlings inoculated with endophytic bacterial strain alone, inoculated with fungal pathogen alone and inoculated with endophytic bacterium and fungal pathogen together were investigated. The results showed that, DM12 strain is able to enter and colonize of strawberry seedlings after 72 h. Also, the symptoms of the disease in the treatment of inoculation with the fungal pathogen alone, were appeared 72 hours after inoculation while in treatments inoculated with endophytic bacterium and fungal pathogen together at this time no symptoms were observed and symptoms developed in this treatment in more time and with less severity. The results represented that, the presence of endophytic bacterium in seedlings strawberry before the fungal pathogen reduces the severity of the disease and time of the appearance of symptoms.

Gas chromatography–mass spectrometry analysis

Secondary metabolites production by DM12 strain

The GC–MS analysis of metabolite extracted of strain DM12 with ethyl acetate represented 36 compounds that, decane (14.64%), benzene, 1,2,3–trimethyl– (9.14%), and undecane (7.88%) were the most abundant components (Table 3 and Fig. 2).

Table 3. Major bioactive metabolites from ethyl acetate extract produced by DM12 strain identified by GC–MS analysis with details on their retention time and area %.

Number	Compounds	Retention Time (min)	Area (%)
1	Methane, isothiocyanate–	3.221	0.61
2	2,2,6–Trimethyl–4–methylene–2H–pyran	3.291	2.55
3	Benzene, (1–methylethyl)–	3.361	1.54
4	Cyclohexane, propyl–	3.425	2.02
5	Octane, 2,6–dimethyl–	3.477	4.14
6	Octane, 2,3–dimethyl–	3.576	3.38
7	1–Hexadecanol, acetate	3.641	0.90
8	Benzene, propyl–	3.763	3.38
10	Benzene, 1–ethyl–2–methyl–	3.862	6.08
11	Nonane, 2–methyl–	3.891	4.78
12	Benzene, 1,2,3–trimethyl–	3.967	9.14
13	Menth–2–ene	4.183	3.14
14	Mesitylene	4.317	4.24
15	Decane	4.398	14.64
16	Nonane, 2,5–dimethyl–	4.596	1.16
17	Decane, 4–methyl–	4.725	5.64
18	Decane, 2–methyl–	4.800	1.20
19	Cyclohexane, (2–methylpropyl)–	4.853	1.38
20	Eicosane	4.894	1.40
21	Decane, 3–methyl–	4.958	1.50
22	2–Tolyloxirane	5.150	1.90
23	Decane, 5–methyl–	5.226	1.78
24	Tricyclo[5.2.1.0(2,6)]dec–3–ene	5.372	1.42
25	Undecane, 3,4–dimethyl–	5.418	1.55
26	Undecane	5.850	7.88
27	Ethenyloxy	6.100	1.05
28	Tetracosane	6.246	0.96
29	Cyclohexane, pentyl–	6.345	0.60
30	Nonadecane	6.654	0.91
31	Undecane, 2–methyl–	6.776	0.80
32	Undecane, 3–methyl–	6.870	0.43
33	Dodecane	7.295	2.97
34	Tetradecane	10.000	1.08
35	Hexadecane	12.436	1.33
36	Octadecane	14.628	1.07

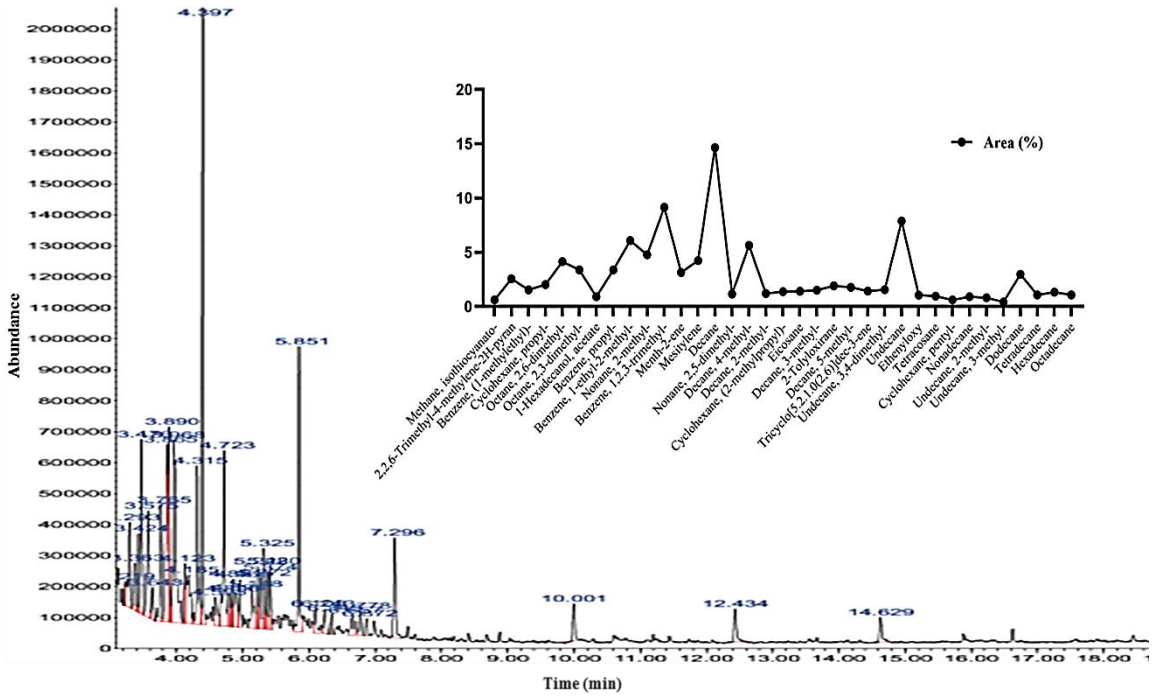

Fig. 2. Chromatogram of major bioactive metabolites from ethyl acetate extract produced by DM12 strain identified by GC–MS system.

Metabolic profiles of strawberry in various treatments

Representative chromatograms of the metabolome of strawberry plants in various treatments are shown in Figure 3–6. A total of 10 peaks in control treatment, 23 peaks in treatment inoculated with DM12 strain alone, 10 peaks in treatment inoculated with *C. nymphaeae* alone and 14 peaks in treatment inoculated with *C. nymphaeae* and DM12 strain together were detected (Table 4–7). A number of compounds in control treatment with maximum area percent were acetoglyceride (19.418%), acetic acid, butyl ester (4.734%) and Ribitol (4.349%), in treatment inoculated with DM12 strain alone were tetramethyl–2–hexadecen (21.350%), ethylene glycol monoisobutyl ether (18.688%) and myrtenol (8.75%), in treatment inoculated with *C. nymphaeae* alone were acetoglyceride (18.089%) and acetic acid, monoglyceride (17.96%) and in treatment inoculated with *C. nymphaeae* and DM12 strain together were tert–butanethiol (36.153%), ethoxytriethylsilane (14.126%), 5–(Methylamino)–1,2,3,4–thiatriazole (9.53%) and 2,3–Butanediol (7.795%).

Table 4. Metabolites profile of strawberry plants identified by GC–MS analysis with details on their retention time and area % in control treatment.

Number	Compounds	Retention Time (min)	Area (%)
1	Acetic acid, butyl ester	6.515	4.734
2	Decane	12.789	2.294
3	Acetoglyceride	18.336	19.418
4	Ribitol	19.437	4.349
5	D–Arabinitol	21.590	1.237
6	Tetradecane	24.894	12.330
7	Isosorbide Dinitrate	27.415	0.437
8	Maltose	28.636	1.671
10	Dodecyl fluoride	30.270	1.897

Table 5. Metabolites profile of strawberry plants identified by GC–MS analysis with details on their retention time and area % in DM12 strain alone treatment.

Number	Compounds	Retention Time (min)	Area (%)
1	3–Methylthietane	5.973	3.368
2	Ethoxytriethylsilane	7.391	1.914
3	2,3–Butanediol	7.502	5.713
4	Ethylene glycol monoisobutyl ether	7.635	18.688
5	3,5–Dimethyloctane	12.758	0.540
6	Myrtenol	19.442	8.750
7	4–Hydroxy–4–methylhex–5–enoic acid, tert.–butyl ester	21.569	0.698
8	Kasugamycin	22.183	0.966
10	Acetoxyacetic acid, nonyl ester	22.380	1.165
11	Methionine sulfoxide	22.559	1.046
12	Lactose	22.856	0.744
13	3–Allyl–2–methoxyphenol	24.304	3.307
14	Cyclopropane, 1–cyclopropylethynyl–2–methoxy–3,3–dimethyl–	24.646	2.139
15	Methyl 2–methyl–3–oxobutyldithiocarbamate	24.847	3.139
16	7–[.beta.–d–Ribofuranosyl]imidazo[4,5–d][1,2,3]–triazin–4–one (2–azainosine)	25.428	1.570
17	Methoxyacetic acid, 4–tridecyl ester	27.350	2.096
18	4–Hydroxylamino–6–methylpyrimidin–2(1H)–one	27.915	1.766
19	Phenol, 2,4–bis(1,1–dimethylethyl)–	28.221	3.552
20	Tetradecane, 2,6,10–trimethyl–	32.897	1.261
21	3,7,11,15–Tetramethyl–2–hexadecen–1–ol	36.800	21.350
22	2–Dodecyloxirane	37.554	4.257
23	10–Methyl–E–11–tridecen–1–ol propionate	38.174	6.267

Table 6. Metabolites profile of strawberry plants identified by GC–MS analysis with details on their retention time and area % in fungal pathogen alone treatment.

Number	Compounds	Retention Time (min)	Area (%)
1	Formic acid, 2–methylpropyl ester	6.501	1.472
2	Cyclotrisiloxane, hexamethyl–	7.264	0.143
3	D–Mannoheptulose	17.429	0.143
4	d–Glycero–d–ido–heptose	17.543	0.160
5	Acetoglyceride	18.250	18.089
6	Butanetetrol	20.999	0.596
7	Acetic acid, monoglyceride	21.490	17.960
8	D–Glucose, cyclic 1,2–ethanediyl mercaptal, pentaacetate	27.536	0.502
10	Phenol, 2,4–bis(1,1–dimethylethyl)–	28.429	0.190

Table 7. Metabolites profile of strawberry plants identified by GC–MS analysis with details on their retention time and area % in DM12 strain and fungal pathogen together treatment.

Number	Compounds	Retention Time (min)	Area (%)
1	5–(Methylamino)–1,2,3,4–thiatriazole	6.008	9.530
2	1,1,1,3,5,5,5–Heptamethyltrisiloxane	6.346	2.526
3	Ethoxytriethylsilane	7.404	14.126
4	2,3–Butanediol	7.496	7.795
5	tert–Butanethiol	7.707	36.153
6	1,2,3–Butanetriol	12.777	2.862
7	Cyclotetrasiloxane, octamethyl–	12.972	1.829
8	Cyclopentasiloxane, decamethyl–	18.041	0.800
9	Acetoxyacetic acid, nonyl ester	19.306	2.503
10	tert–Nonyl mercaptan	21.577	3.189
11	Tetradecane, 2,6,10–trimethyl–	27.368	5.055
12	2H–1–Benzopyran, 3–(2,4–dimethoxyphenyl)–3,4–dihydro–7–methoxy–	30.063	1.303
13	2–Dodecenal, (E)–	31.065	0.947
14	Oxirane, tetradecyl–	36.838	4.296

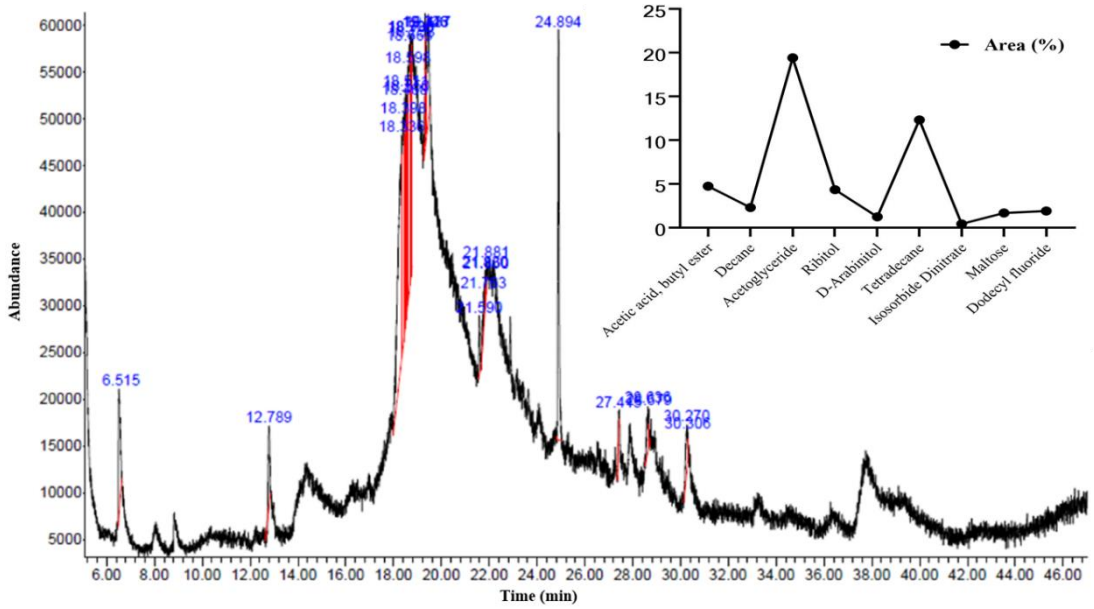

Fig. 3. GC–MS total ion chromatogram registered for the strawberry plants (cv. Paros) in control plants.

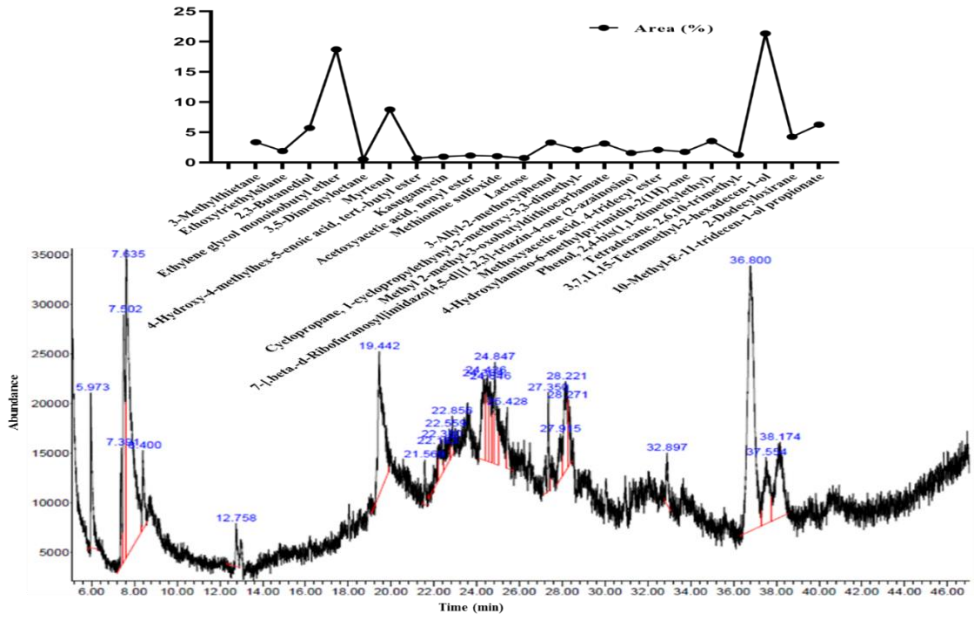

Fig. 4. GC–MS total ion chromatogram registered for the strawberry plants (cv. Paros) after inoculation with DM12 strain alone.

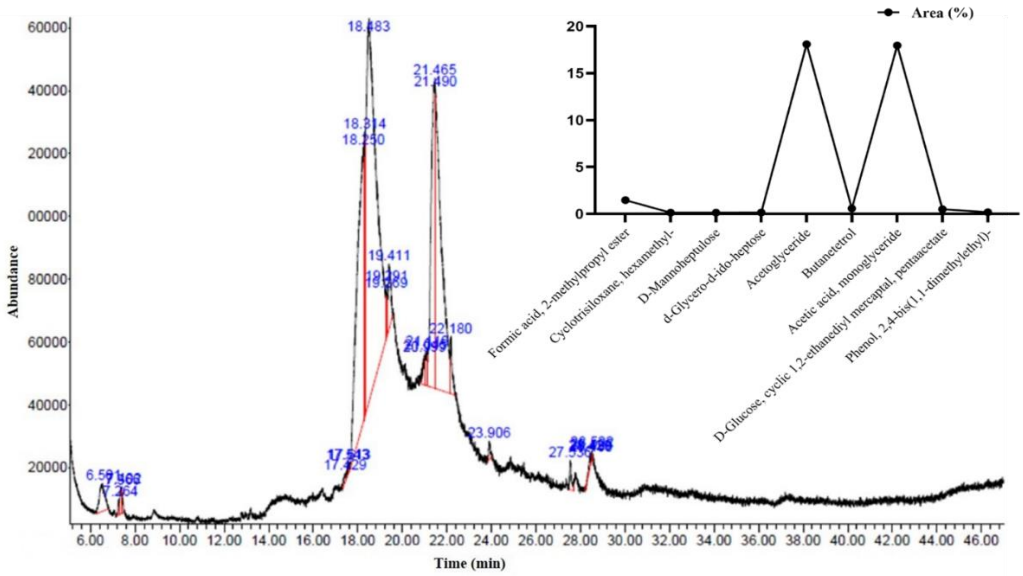

Fig. 5. GC–MS total ion chromatogram registered for the strawberry plants (cv. Paros) after inoculation with fungal pathogen alone.

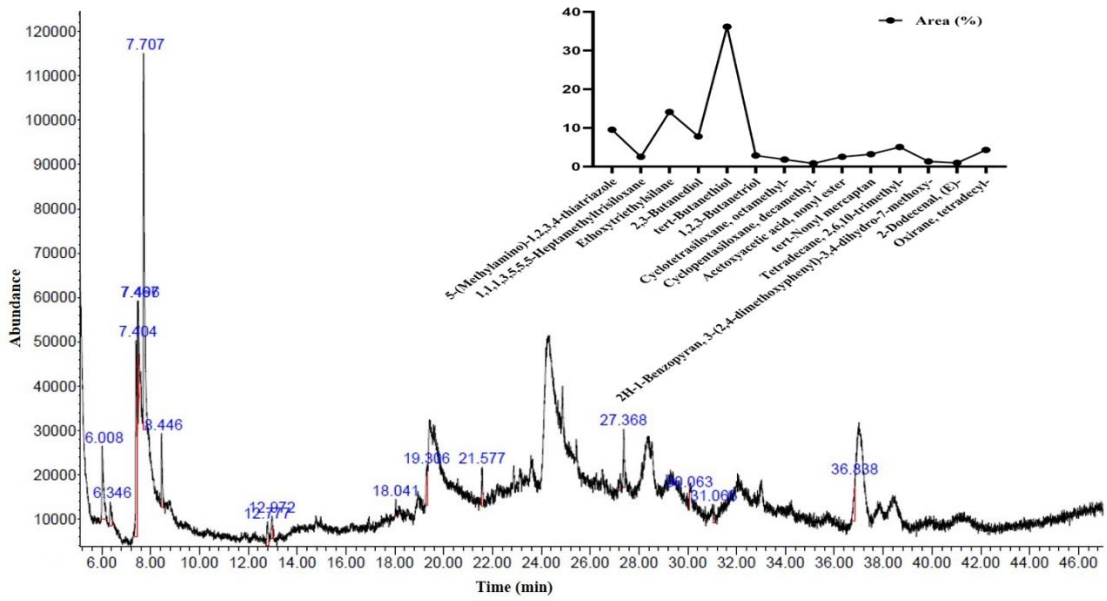

Fig. 6. GC–MS total ion chromatogram registered for the strawberry plants (cv. Paros) after inoculation with DM12 strain and fungal pathogen together.

4. Discussion

Endophytes are microorganisms isolated from sterilized plant organs that could constitute convenient biological control agents in the fight against several plant diseases (Manso and Nunes, 2011). Numerous researches have illustrated the potential of endophytic bacteria in the control of phytopathogens. Paul *et al*. (2013) showed that endophytic bacteria, isolated from chili pepper plants (*Capsicum annum* L.) belonged to *Pseudomonas*, *Bacillus* and *Burkholderia* have antifungal properties against *Alternaria panax*, *Botrytis cinerea, Colletotrichum acutatum*, *Fusarium oxysporum* and *Phytophthora capsici*. In the present study, evaluated biological control properties of select endophytic bacterium from the strawberry plant against *C. nymphaeae* under *in vitro*, *in vivo*, and greenhouse conditions. Bacterial strain was identified using phenotypic, biochemical properties and molecular phylogenetic analysis of the *16S rDNA* gene sequences. Based on the results of this research, DM12 strain is considered as biological control agents against *C. nymphaeae* and was able to control strawberry anthracnose under *in vitro*, *in vivo* and greenhouse conditions. A perfect perception of the mode of action of bacterial strain has a great impact in development of successful biological control agent. In this study, types of biological control mechanisms of DM12 strain against *C. nymphaeae* was investigated. The mechanisms of bacterial endophytes in controlling of phytopathogens depends on their competence. There are different mechanisms such as producing antifungal metabolites, siderophores, lytic enzymes, competing for nutrition and habitat and also, induce systemic resistance (Compant *et al*., 2005). Hydrolytic enzymes such as chitinase and protease produced by bacterial strains have long been reported of being able to cut the glycosidic and peptide bonds of fungal pathogens (Meliah *et al*., 2021). Research represented that *Serratia marcescens* was able to produce of chitinase that lysis of hyphal tips of *Sclerotium rolfsii* (Ordentlich *et al*. 1988). Also, pectinase production by bacterial endophytes facilitates colonization of plants and via releasing cell wall oligosaccharides and active pectic fragments of plant cell walls elicit defense reactions in plants (Davis *et al*., 1984; Ben Abdallah *et al*., 2016). It was shown that, pectinases from *Penicillium oxalicum* BZH–2002 induced local protection against *Cladosporium cucumerinum* on cucumber (Peng *et al*., 2004). Siderophore is an iron–healing compound that has an important role in the biological control of plant diseases. It has been represented that siderophores produced by *Bacillus subtilis* inhibited disease of *F. oxysporum* f. sp. *ciceri* and *Macrophomina phaseolina* in chickpea (Patil *et al*., 2014). Based on the results, production of chitinase, protease, pectinase and siderophore by DM12 strain was observed. Phytohormones of microbial origin promote plant growth, tolerance to abiotic stresses and resistance to pathogens. On the other hand, biological control agents enhance availability of nutrients by fixing nitrogen and solubilizing phosphate (Alori *et al*., 2017). Findings indicate that DM12 strain in this study was able to secret of IAA and GAs and capable to phosphate solubilization.

Bacillus species able to produce lipopeptides that are antibiotic compounds and have antifungal activity and stimulate defense pathways in plants (Ongena *et al*., 2007; Ongena and Jacques, 2008). It

was reported *B. subtilis* strain S499 produce surfactins, iturins and fengycins that suppresses *B. cinerea* on apple (Ongena *et al*., 2007). Also, *B. amyloliquefaciens* PPCB004 inhibited of postharvest fungal pathogen growth by producing various lipopeptides (Arrebola *et al*., 2010). The bacterial strain in this study was able to secret surfactin.

In this research the ability of production of antifungal compounds by DM12 strain *in vitro* conditions was evaluated. It has indicated that cell free supernatant from *B. atrophaeus* reduced mycelial growth and conidial germination of *C. gloeosporioides* (Guardado–Valdivia *et al*., 2018). Also has reported that ethyl acetate extract of *B. subtilis* suppressed mycelial growth of several fungal pathogens (Islam *et al*., 2012). Endophytic bacteria are capable to secret compounds that expose antifungal activity against pathogens, stimulate systemic resistance and inhibit the mycelial growth and spore germination of pathogen (Monte, 2001). Antifungal metabolites were secreted by DM12 strain and GC–MS analysis identified these compounds that inhibit growth of *C. nymphaeae*. Among the compounds identified in this study was eicosane which previous studies reported that this compound has antifungal properties (Karanja *et al*. 2012; Lata, 2015). Ahsan *et al*. (2017) showed that extracted eicosane produced by *Streptomyces* strain KX852460 controlled *R. solani* in tobacco. Furthermore, Geng *et al*. (2016) investigated antifungal activity of essential oil of bitter almond that 21 different components including, eicosane, mesitylene, dodecane, tetradecane and hexadecane identified. Also, DM12 strain suppress the mycelia growth of fungal pathogen in dual culture test which can be due to the production of lytic enzymes and volatile and non–volatile metabolites. It was reported that, *B. subtilis* produce antifungal metabolites that reduced the mycelial growth of *Gaeumannomyces graminis* var. *tritici* in wheat (Liu *et al*., 2010). In addition to, an antifungal protein secreted by *B. licheniformis* decreased the growth of *Aspergillus niger*, *Magnaporthe oryzae* and *R. solani* (Cui *et al*., 2012). Research has indicated that, culture filtrate of *B. subtilis* suppress conidial germination and mycelial growth of *Sclerotinia sclerotiorum* in rapeseed (Chen *et al*., 2014).

Fruit decay and disease severity of anthracnose under *in vivo* and greenhouse conditions were suppress significantly by strain DM12. These findings can due to the colonization of plant tissues, occupy of ecological niche, competence for the acquisition of nutrition, secretion of enzymes (protease, pectinase and chitinase) and antifungal compounds by this strain and induce of defense pathways (Mates *et al*., 2019). Previous findings showed that the antifungal protein extracted from *B. amyloliquefaciens* inhibited growth of fungal pathogen and fruit decay in loquats (Yan *et al*., 2021).

Also, in this study, was represented that each treatment including, endophytic bacterium, fungal pathogen each alone and endophytic bacterium and fungal pathogen together, have a different effect on metabolite profile of strawberry seedlings and production of different compounds were increased in endophytic bacterium alone and endophytic bacterium and fungal pathogen together treatments in comparison with the control. For example, 2,3–Butanediol is a signal that promotes growth in *Arabidopsis* and can induce resistance against pathogens (Schulz & Dickschat, 2007), and was produced in endophytic bacterium alone and endophytic bacterium and fungal pathogen together treatments. Also, Lactose that was produced in endophytic bacterium alone treatment, represented antifungal activity against *Alternaria solani* (Zhang *et al*., 2020). Various composition of Tetradecane, 2,6,10–trimethyl were showed antifungal activity against *Aspergillus parasiticus* TISTR 3276 and *Aspergillus flavus* PSRDC–4 (Boukaew & Prasertsan, 2020) that was produced on endophytic bacterium alone and endophytic bacterium and fungal pathogen together treatments. Research has shown that *R. solani* affects the metabolic profile of soybean, and more chemical compounds were detected in interaction with the pathogen than in controls (Aliferis *et al*., 2014). Previous study was indicated that, 2–3–butanediol accumulates in infected plant and acts as a signal for induce resistant on host (Effantin *et al*., 2011). Also, changes in the profile of volatile metabolites of tomato fruit were observed as a result of *A. alternata* infection (Encinas–Basurto *et al*., 2017). Research has shown that VOCs increased resistance of Arabidopsis against the necrotrophic fungal pathogen *B. cinerea* and represents that VOCs may induce disease resistance (Yi *et al*., 2009). Pandey *et al*. (2016) indicated that, the capsule endophyte *Acinetobacter* upregulated the expression of the key genes for the production of antifungal and antimicrobial alkaloids.

Thereby, endophytic bacteria, have wide range of antimicrobial and antifungal metabolites, enzymes and surfactants that play important roles in plant growth promotion and inhibit phytopathogens by colonizing the surface or inner of different parts of the plants (Bibi, 2017). In our study, bacterial strain produces several lytic enzymes, lipopeptides and phytohormones that have biological control properties. Also, metabolic profile of strawberry seedlings in various interactions were different. As a result, it has been demonstrated that the synergistic effects of lytic enzymes and antifungal metabolites produced by DM12 strain give a higher level of biological control efficiency against *C. nymphaeae* (Monte, 2001).

Conclusion

In this paper, antifungal activity of *Bacillus* spp. strain DM12 against *C. nymphaeae* causal agent of strawberry anthracnose has been evaluated. The strain DM12 produced siderophore, protease, chitinase, pectinase, IAA and gibberellin and was capable to secret surfactin. This research showed that strain DM12 has able to decrease mycelial growth, conidial germination, fruit decay development and disease severity of strawberry anthracnose under *in vitro*, *in vivo* and greenhouse conditions. These results have established a new line of research about effects of various treatments including, endophytic bacterium, fungal pathogen each alone and endophytic bacterium and fungal pathogen together on metabolite profiling of strawberry. Further works are required in order to understanding the interaction of DM12 with the plant and others phytopathogens and its effects on the plant defense system.

Author contribution statements

Z.A. and J.A. designed and performed the experiments. Z.A. and J.A. contributed to data collection and sample preparation. Both Z.A and J.A. authors contributed to the final version of the manuscript. J.A. supervised the project. A.M. was involved in meristem culture of strawberry.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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بررسی پروفایل متابولیکی گیاه توت فرنگی در تعامل با باکتری اندوفیت *Bacillus* spp. سویه DM12 با استفاده از دستگاه کروماتوگرافی گازی– طیف سنجی جرمی و تعیین ویژگی های ضدقارچی سویه باکتری

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چکیده

قارچ *Colletotrichum nymphaeae* عامل بیماری آنتراکنوز توت فرنگی یکی از مهمترین بیماریهای این گیاه است که سبب آلودگی ریشه، برگ و میوه گیاه توت فرنگی در شرایط مختلف رشد میگردد. مشکل اصلی ارقام توت فرنگی در مقابل این بیماری پوسیدگی میوه است که کمیت و کیفیت و بازار پسندی میوه توت فرنگی را کاهش میدهد. در این تحقیق سویه¬ باکتریایی اندوفیت از گیاهچه¬های سالم توت¬فرنگی (Fragaria × ananassa) جداسازی و اثرات آنتاگونیستی این سویه¬ علیه قارچ عامل آنتراکنوز توت¬فرنگی *Colletotrichum nymphaeae* در شرایط آزمایشگاه، درون شیشه و گلخانه مورد بررسی قرار گرفت. سویه¬ جداسازی شده با استفاده از ویژگی¬های ریخت شناسی، بیوشیمیایی و آنالیز مولکولی ژن S rDNA۱۶ در جنس *Bacillus* .spp قرار گرفت. سویه DM12 در آزمون کشت متقابل باعث کاهش رشد قارچ بیمارگر (۶۴/۰۳٪) گردید. در آزمون تاثیر ترکیبات ضدقارچی تولید شده توسط باکتری اندوفیت میزان بازدارندگی از رشد میسلیوم و جوانه زنی اسپور قارچ بیمارگر به ترتیب ۳۲/۸۶٪ و ۷۳/۹۸٪ ارزیابی گردید. تاثیر متابولیت های فرار باکتریایی بر رشد میسلیوم قارچ بیمارگر ناچیز بود (۹/۸۲٪). با توجه به آزمایشات بیوشیمیایی این سویه قادر به تولید آنزیم های پروتئاز، کیتیناز و پکتیناز و همچنین تولید سیدوفور، هورمون ایندول استیک اسید و جیبرلین بوده و توانایی حلالیت فسفات را دارد. سویه باکتری اندوفیت در شرایط درون شیشه قادر به بازدارندگی بیماری بر روی میوه توت فرنگی به میزان ۹۰/۸۷٪ گردید. همچنین درشرایط گلخانه در روش خیساندن خاک به میزان ۷۲/۲۲٪ و در روش اسپری اندام های هوایی به میزان ۹۴/۴۴٪ قادر به جلوگیری از گسترش بیماری بر روی گیاهچه های توت فرنگی شد. همچنین در این تحقیق تغییرات متابولیت های گیاهی در تیمارهای مختلف با باکتری اندوفیت در مقایسه با شاهد بررسی گردید. نتایج نشان داد که در تیمار شاهد منفی میزان ترکیبات استوگلیسرید (۱۹/۴۱۸٪)، استیک اسید بوتیل استر (۴/۷۳۴٪) و ریبیتول (۴/۳۴۹٪)، در تیمار باکتری اندوفیت به تنهایی میزان ترکیبات تترامتیل دو–هگزامتان (۲۱/۳۵٪)، اتیلن گلیکول منوایزوبوتیل اتر (۱۸/۶۸۸٪) و میرتنول (۸/۷۵٪)، در تیمار قارچ بیمارگر به تنهایی ترکیبات استوگلیسرید (۱۸/۰۸۹٪)، استیک اسید منو گلیسرید (۱۷/۹۶٪) و در تیمار قارچ بیمارگر همراه با باکتری اندوفیت ترکیبات ترت–بوتانتیول (۳۶/۱۵۳٪)، اتوکسی تری اتیل سیلان (۱۴/۱۲۶٪)، ۵–متیل آمینو–۱–۲–۳–۴–تیاتریازول (۹/۵۳٪) و ۲–۳–بوتاندیول (۷/۷۹۵٪) بیشترین مقدار را داشتند. برخی از این ترکیبات مانند بوتاندیول، لاکتوز و ترادکان که در تیمار های باکتری اندوفیت به تنهایی و قارچ بیمارگر همراه با باکتری اندوفیت تولید شدند دارای خواص ضد قارچی می باشند. نتایج حاصل از این تحقیق نشان داد که سویه DM12 قادر به کاهش رشد میسلیوم، جوانه زنی اسپور، توسعه پوسیدگی میوه و شدت بیماری آنتراکنوز توت فرنگی در شرایط آزمایشگاه، درون شیشه و گلخانه می باشد. به منظور درک تعامل سویه DM12 با گیاه و سایر عوامل بیماریزای گیاهی و اثرات آن بر سیستم دفاعی گیاه، تحقیقات بیشتری مورد نیاز است.

واژه‌های کلیدی: آنتراکنوز توت فرنگی، متابولیت های ضد قارچی، *Bacillus* spp, GC–Mass