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PLANT PATHOLOGY | ORIGINAL ARTICLE

Isolation and characterization of bacterial endophytes from weeds against *Pseudomonas syringae* pv. *syringae* causing bacterial canker of stone fruit trees

Nikoo Javadi-Dodaran[1], Reza Khakvar[1*], Nasser Aliasgarzad[2]

[1]Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Iran
[2]Department of Soil Science, Faculty of Agriculture, University of Tabriz, Iran

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smonjil@yahoo.com

**Corresponding Author*
Reza Khakvar
khakvar@tabrizu.ac.ir

ABSTRACT

This research was conducted to evaluate the inhibitory effect of endophytic bacteria isolated from some common weeds of stone fruits orchards on *Pseudomonas syringae* pv. *syringae* (Pss). Weeds samples were collected from stone-fruit orchards in the northwest of Iran during the year 2017-2019. Bacterial strains were isolated from the plant samples using different culture media then chloroform test was applied to evaluate the antagonistic properties of bacterial isolates. Among 112 bacterial isolates, 34 strains showed inhibitory effects against Pss. Subsequently, three isolates with higher inhibitory capability were selected for supplementary assay. 16s rDNA sequencing results indicated that the selected endophytic bacteria with 99% probability could belong to *Bacillus simplex*, *Bacillus mycodies*, and *Arthrobacter* sp. Study of the effective mechanisms of these three bacterial isolates showed that *Bacillus simplex* with 181.76 mg/L auxin production had the highest auxin production capability in comparison to the other species but in hydrogen cyanide production assay, *Bacillus mycoides* were the only one which showed producing hydrogen cyanide.

Keywords: Antagonistic bacteria, biological control, endophytic bacteria

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1 Introduction

Biological control is used by applying beneficial agents against harmful living organisms. Biological control can be used in combination with other control methods to reduce the consumption of used pesticides. Among different biocontrol agents, endophytic microorganisms can have significant competitive potential to control pathogens. Many studies have represented that most endophytic populations are beneficial to plants and stimulate growth through nitrogen fixation (Hurek et al., 1994; Azabou et al., 2020). They cause biocontrol of plant pathogens in the root, through the production of antifungal and antibacterial agents, phytohormones and siderophore production, food competition, systemic induction of host resistance, or an increase in mineral availability (Moslehi et al., 2021; Sturz and Nowak, 2000). The effect of endophytes as biocontrol agents depends on many factors, some of which are host characteristics, population dynamics, host colonization pattern, the capability of moving into the host and capability of having systemic induction (Backman and Sikora, 2008; de Almeida Lopes et al., 2018). Bacterial canker disease caused by *Pseudomonas syringae* pv. *syringae* is one of the serious and limiting problems of stone fruit trees (Sulikowska and Sobiczewski, 2008). *Pseudomonas syringae* pv. *syringae* can be pathogenic in more than 180 plant species from different genera (Bradbury, 1986) and can cause significant damage of 10 to 75% to stone fruit trees such as peach, nectarine, plum, cherry, apricot, almond, and several diseases in a variety of crops products (Agrios, 2005). Weeds in stone fruits orchards can be a good source for finding effective endophytes against this bacterium; previ-

ous studies have shown that weeds in all types of orchards are a rich source of a variety of biocontrol agents such as endophytes. These endophytic microorganisms are collected from the same place and with the same growth conditions, so they have the ability to establish and stabilize on the surrounding trees (Duman and Soylu, 2019). This study was conducted, to evaluate the efficiency of endophytic bacteria isolated from weeds in control of bacterial canker disease of stone fruit trees in laboratory conditions

2 Materials and Methods

2.1 Sampling and isolating endophytic bacteria

During the years 2017-2019, weeds samples were randomly collected from stone-fruit orchards in northwestern Iran. In total five different weed species were totally collected and selected to isolate endophytic bacteria, including *Malva sylvestris*, *Soforaalo pecuroides*, *Trifolium repens*, *Plantago major* and *Chenopodium album*. Different parts of weed plants including root, stem and leaves were divided into small pieces and washed under tap water. After disinfection, the disinfected tissues were divided into small pieces and dried under a laminar hood airflow and placed at different culture mediums such as casein agar, king-b and nutrient agar with 0.3% sucrose in addition to 50 mg/L nystatin and 200 mg/L streptomycin and were kept at 28 °C for one week to one month (Gholami et al., 2014, 2013; Schaad et al., 2001).

2.2 Evaluating antibacterial activity of isolates

In order to evaluate the antagonistic effect of bacterial isolates, chloroform test was applied using Khodakaramian and Zafari (2010) methods with some modification. To determine the inhibitory of endophytic bacteria *in vitro*, the bacteria strains were cultured on NA culture medium in a completely randomized design with three replications. Then, the colony of antagonistic bacteria cultured by an ethanol 96%-imbibed sterile cotton was removed from the culture medium and three drops of chloroform were poured into each Petri dish and the Petri dishes were kept upside down for 20 minutes. Pss suspension was then cultured in Petri dishes and stored at 28 °C for 48-72 hours the inhibition zone of each bacterial strain was measured. The obtained data were used in a statistical form, they were analyzed and finally, they were evaluated between the maximum and minimum inhibition degree and some representatives were selected among them. In the control sample, sterile distilled water was used instead of antagonistic bacteria or suspension of pathogenic bacteria.

2.3 DNA extraction and PCR

For extracting DNA, the bacteria were transferred to a micro-tube containing 600 µL of lysing buffer (Tris 1 M, pH = 7.5; NaCl 5 M; EDTA 0.5 M, pH = 8; SDS = 2%) (Vasebi et al., 2019). The microtubes were first immersed in liquid nitrogen and immediately transferred to a bain-marie at 65 °C and then vortexed (this was repeated for 7 times). After adding another 300 µL of the lysing buffer, the microtubes were centrifuged in a centrifuge at 12000 g, 10 °C for 10 minutes. The supernatant was removed and chloroform-isoamyl (1:24) solution was added according to its equal volume and then, it was centrifuged at 12000 g, 10 °C for 10 minutes. The supernatant was transferred to a new microtube and cold isopropanol was added according to its equal volume and kept in the freezer at −20 °C for one night and then centrifuged again at 12000 g, 10 °C for 10 minutes, and the plate was washed with 300 µL of alcohol 70% and then, it was centrifuged at 1200 g, 10 °C for 10 minutes. 50 µL of deionized water was added to the resulting sediment and kept in a freezer at −20 °C. PCR was performed using universal primer 8F (5′ TAGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′) with initial denaturation at 94 °C for 5 minutes, denaturation at 94 °C for 45 seconds, annealing at 48 °C for 45 seconds and extension at 72 °C for 1 minute and 45 seconds with 35 cycles and consequently, the final extension at 72 °C for 5 minutes. PCR products were electrophoresed then resulting bands were sent to Pishgam Co. for sequencing. After obtaining the sequencing results, cconsensus sequences generated from raw data files belonging to forward and reverse primers were made using MEGA-X software. In BLAST search, consensus sequences were used as queries for finding high similar subjects in GenBank database hosted by NCBI. Subjects with the highest similarity were downloaded and aligned with sequences generated in this study with the Muscle software implemented in MEGA-X. Phylogenetic analysis was performed using the UPGMA method implemented in MEGA-X.

2.4 Evaluating effective mechanisms in antagonistic properties

Due to the significant rule of Auxin, Siderophore, and hydrogen cyanide production in antagonistic properties of biocontrol agents, these three capabilities were evaluated. In order to evaluate auxin production capacity, 50 µL of each strain was first transferred to a 20 mL TSB medium with/without 50 mg/mL of L-tryptophan and placed on a shaker for 72 hours. These suspensions were centrifuged at 10,000 g for 15 minutes then 1 mL of the supernatant were mixed with 4 mL of salkowski reagent (including 75 mL of concentrated sulfuric acid, 250 mL of distilled water,

and 7.5 mL of 0.5 M ferric chloride ($FeCl_3$: $6H_2O$)). The resulting mixture was kept at room temperature for 20 minutes and then the absorption of light was read at a wavelength of 535 nm using a spectrophotometer. IAA production by any strain was calculated by comparing its light absorption with the standard curve obtained at 0, 5, 10, 15, 20, 30, 50 mg/L of indoleacetic acid (Bent et al., 2001).

For Siderophore production assay, Fe-CAS solution was first prepared then this mixture was added to 40 ml of distilled water containing 72.8 mg of HDTMA (1.82 mg/L). The resulting dark blue solution was autoclaved and cooled to 50 °C. Then, the buffer solution (30.24 g PIPES in 750 mL of the saline solution including 0.3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl) was prepared and the pH of this solution was adjusted to 6.8 and autoclaved. Then, the reagent and buffer solutions were gently mixed together and spread on the Petri dishes and after cooling and freezing, a small hole was created in them by a cork borer. The bacteria were cultured for 40 hours at 27 °C in the mentioned culture medium. The bacterial suspension was centrifuged at 100 g for 10 minutes and the supernatant was removed and 50-70 µL of it was poured into the holes and the petri dishes were kept at 27 °C for 8 hours then the diameter of the orange halo around the hole was measured.

In the end, evaluation of the hydrogen cyanide production was performed with the method described by Hasanzadeh (1995) with some modifications. To do so, 100 µL of bacterial suspension was spread on the NA culture medium. The reagent-imbibed filter paper (with 2% of sodium carbonate and 0.5% of picric acid) was placed inside the Petri dish and was sealed with a parafilm tape to prevent the escape of hydrogen cyanide. The Petri dishes were stored upside down at 28 °C for one week. According to a color change in filter paper from initial yellow (no production) to cream (low production), orange (relatively low production), light brown (relatively high production), and brick red (high production), the amount of hydrogen cyanide production was determined. Eventually, some biochemical tests including Gram staining, OF test, catalase, citrate, and starch hydrolysis tests were performed as supplementary assays for detection of the isolates (Schaad et al., 2001).

3 Results

A total of 112 bacterial strains were isolated from different parts of collected weeds. Among 112 endophytic bacterial isolates, 34 strains were showed the inhibitory regions against Pss (Fig. 1). Among these 34 isolates, only three isolates with the highest inhibition effect were chosen for further investigations. These three bacterial strains showed between 15-25 mm inhibition zone which were up to 50% higher than the other bacterial isolates. Results demonstrated that in the inhibition degree of Pss pathogenic bacterial growth, there is a significant difference between antagonistic isolates at a probability level of 5% (Fig. 2).

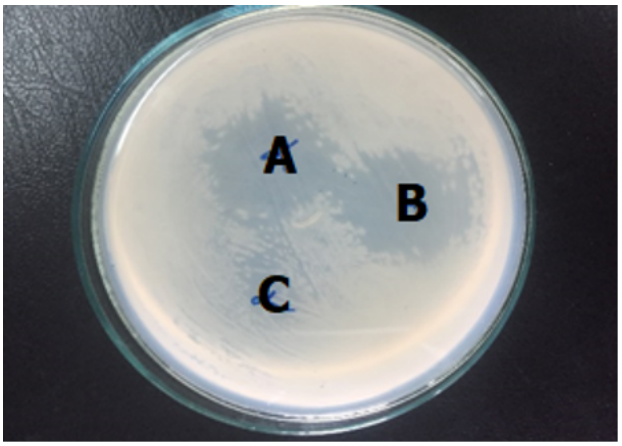

Figure 1. Inhibitory halo of three selected endophytic bacterial isolates against *Pseudomonas syringae* pv. *syringae* using chloroform test

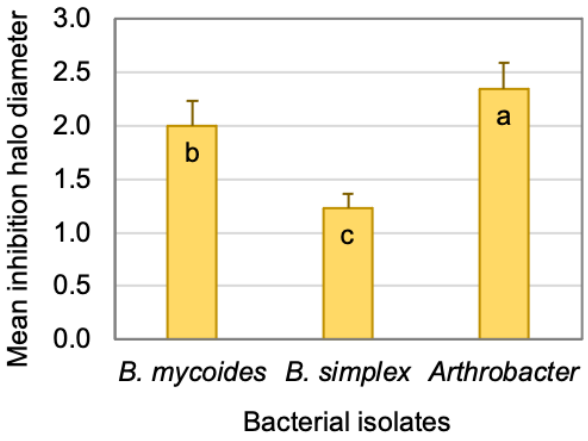

Figure 2. Mean inhibition zone diameter of *Pseudomonas syringae* pv. *syringae* by three selected endophytic bacterial strains isolated from weeds of stone fruits orchards in northwest of Iran

Polymerase chain reaction with universal 16srDNA primers 8F and 1492R formed a1500-bp single band for three selected bacterial strains on the agarose gel (Fig. 3).

The result analysis of 16S rDNA sequencing data were done by MEGA-X software and their comparison to standard isolate sequencing in the GeneBank (NCBI) after phylogenetic tree plotting represented that the selected bacterial strains isolated from different weed organs are probably similar to Bacillus and *Arthrobacter* species (Fig. 4).

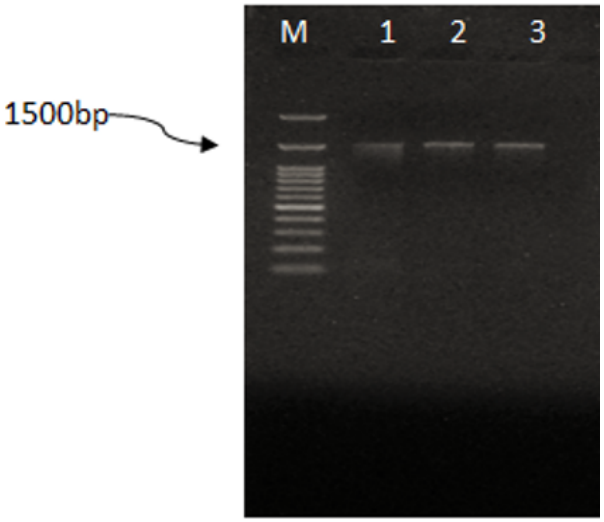

Figure 3. Amplification of desired 1500-bp DNA fragments using 8F and 1492R primers from selected bacterial strains

The results of biochemical assays including aerobic and anaerobic tests, starch hydrolysis test, catalase test, citrate and gram tests are presented in the Table 1.

The results for evaluation of IAA production indicated that all three selected bacterial isolates were capable of producing auxin in TSB mediums both with/without l-tryptophan. Nevertheless, the highest auxin production was related to *Bacillus simplex* isolate, where the value was 181.759 mg/L that the culture medium was with the addition of L-tryptophan bacteria and the lowest auxin production was related to *Bacillus mycodies* isolate where the value was 27.696 mg/L that the culture medium was without the addition of l-tryptophan bacteria.

The results of this test represented that none of the selected isolates were capable of inhibition in the siderophore production test. To ensure the correct performance of the assay, some EDTA was added to the well-free medium as a positive control. After one hour, the environment color changed from blue to purple, which shows the great performance of this work.

The results of hydrogen cyanide production represented that only *Bacillus mycoides* isolate was capable of producing hydrogen cyanide at a low level. The amount of hydrogen cyanide production is determined by a color change in filter paper from initial yellow (no production) to cream (low production), orange (relatively low production), light brown (relatively high production) and brick red (high production). In order to final verification of the endophytic bacteria identification, biochemical tests including aerobic and anaerobic tests, starch hydrolysis test, catalase test, citrate and gram tests were performed that the outcomes of these tests are re-confirmed the 16srDNA sequencing.

4 Discussion

Studying the antagonistic capability of endophytic bacterial strains against Pss, represented that the endophytic bacteria have significant inhibitory capability. In similar study, Khodakaramian and Zafari (2010) showed that antagonistic bacteria on *Pectobacterium carotovorum* can created a significant inhibition halo against *Pectobacterim caratovorum* that the reason for this inhibition may be the production of antibiotics and toxic metabolites, which are considered as biocontrol mechanisms. This feature has also been observed in many other isolates investigated by other researchers (Swadling and Jeffries, 1996; Duman and Soylu, 2019). Antagonistic bacteria use various mechanisms to control diseases. One of the recently reported mechanisms is the neutralization of pathogen secreted signals which play a role in the host-pathogen interaction (Dong et al., 2004). Naturally, the capability of producing these metabolites and toxins without providing a negative effect on other microorganisms and plant tissue, can be effective in controlling such a disease agent and preventing the disease progression. Some enzymes like proteases and metabolites such as hydrogen cyanide are considered as the most important compounds with the antagonistic property of bacteria (Castric and Castric, 1983). In this investigation, a low level of hydrogen cyanide was observed in one of the strains, which can be considered as one of the reasons for its antagonistic properties. Shoda (2000) showed that the *Bacillus* strains on the many plant species can be considered as significant biocontrol options since they have some properties like high-temperature tolerance, rapid growth in a liquid environment and having a resistant state in the form of spore. In a study, Popović et al. (2012) entitled as antagonistic activity of the soil bacteria *Pseudomonas* and *Bacillus* for controlling *in vitro* bacterial canker of stone fruits (Pss), represented that these isolates were capable of forming the inhibitory zones more than 10 mm.

Bacillus isolates can control the disease by producing antibiotics (Asaka and Shoda, 1996; Liu et al., 2007). These bacteria by producing growth stimulants and hormones as well as plant stimulation to absorb nutrients or the conversion of nutrients in organic matters to absorbable materials (i.e. phosphorus) in addition to their antimicrobial effects (El-Barougy et al., 2009). Keshavarz Zarjani et al. (2013) reported that some strains of *Bacillus megaterium* and *Arthrobacter* are capable of producing organic acids and siderophores. Organic acids and siderophores can play an important role for releasing some elements like potassium, iron and phosphorus. The bacteria *Bacillus* and *Arthrobacter* can stimulate plant growth. These bacteria have the capability of colonizing the root surface and penetrating into the root tissue and therefore, they can increase plant growth

Table 1. Results of biochemical assays on selected endophytic bacterial strains isolated from the weeds of stone fruits orchards

Isolate name	Starch hydrolysis assay	Gram staining	Catalase assay	OF assay	Citrate assay
Bacillus mycoides	−	G+	+	FA	+
Bacillus simplex	−	G+	+	FA	+
Arthrobacter sp.	+	G+	+	FA	+

FA: Facultative anaerobic

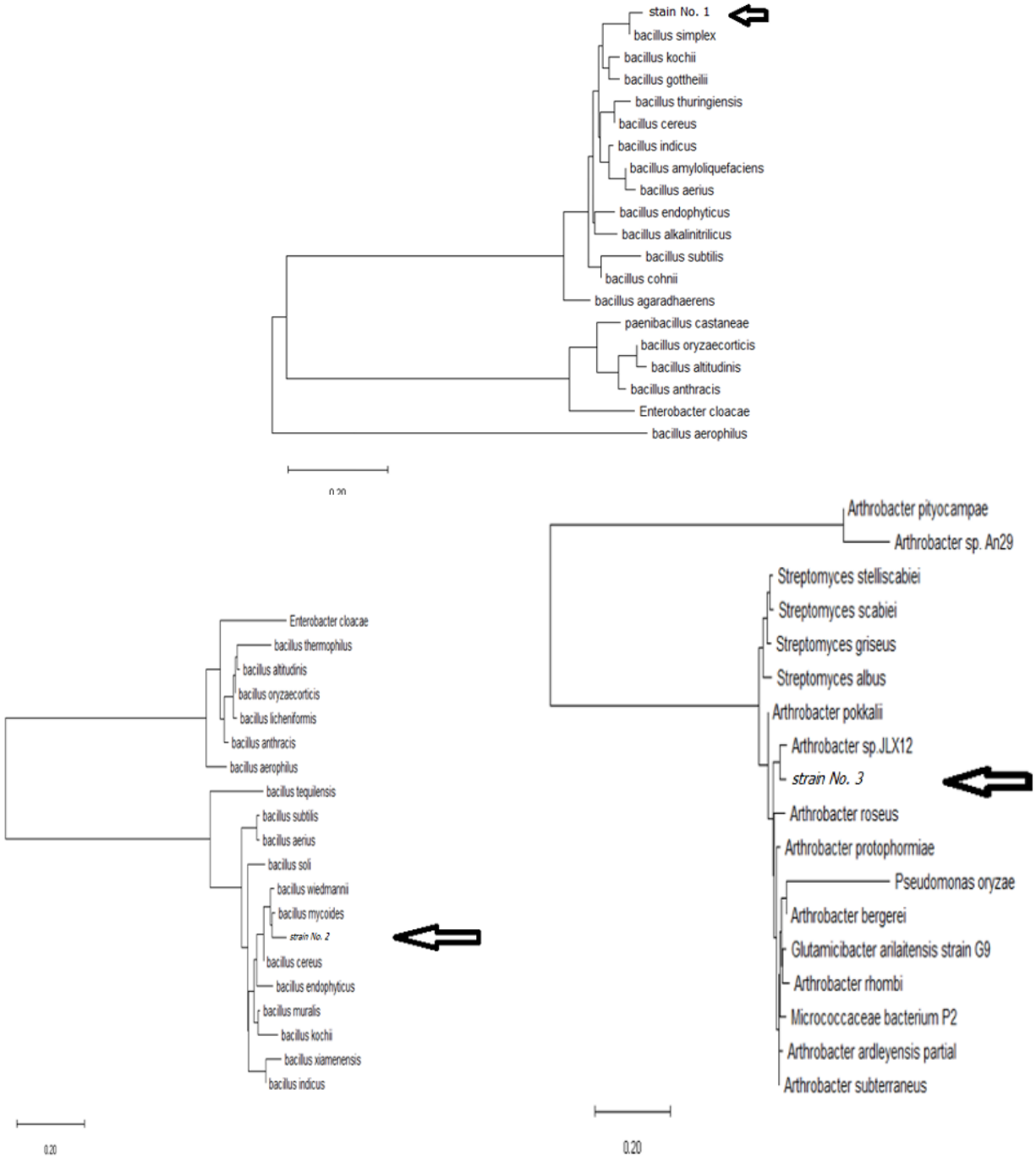

Figure 4. UPGMA phylogenetic trees obtained from the 16s RNA gene sequence data of *Bacillus mycoides*, *Bacillus simplex* and *Arthrobacter* sp. isolates

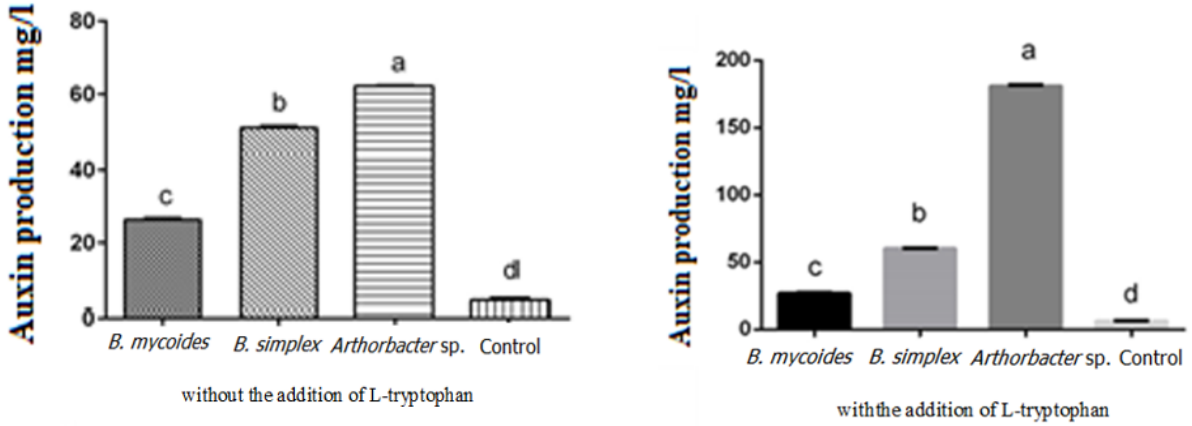

Figure 5. Auxin production by three endophytic bacterial isolates in TSB medium a) without l-tryptophan, b) with l-tryptophan

through various mechanisms (Dimkpa et al., 2009; Kollakkodan et al., 2020). These mechanisms include the plant access increment to nutrients (via nitrogen fixation, phosphorus dissolution and siderophore production), an increase in plant hormone production (e.g. auxin, cytokinin, gibberellin), antibiotic production, plant resistance increment against biotic stresses through induced systemic resistance and tolerance increment against abiotic environmental stresses through induced systemic tolerance. The outcomes of studying the antagonistic effects of bacteria used in this study represented that they have a better potency in producing antibiotics. Many rhizosphere bacteria contain a direct positive effect on plant growth and can directly enhance plant health. Reviewing resources demonstrated that the indole acetic acid (IAA) production by beneficial bacteria could accelerate the growth and development of its host root system (Patten and Glick, 2002). *Bacillus* bacteria can produce many antifungal proteins, metabolites and enzymes such as fungi maysin, surfactin, protease, hydrogen cyanide, bacillin and toxin maysin with antagonistic potency against pathogens affecting the in-vitro and farm growth of pathogens (Li et al., 2009; Panjehkeh et al., 2021; Trung et al., 2021).

One of the most important biological control mechanisms of plant pathogens by PGPRs is the hydrogen cyanide production (HCN) (DeCoste et al., 2010). Hydrogen cyanide of plants is produced as an accompanying material in the ethylene biosynthesis pathway. In this reaction, the enzyme ACC Oxidase can convert the compound 1- Aminocyclopropane-1- carboxylicacid to ethylene and HCN. Unlike ethylene that almost all stress studies have addressed the role of this hormone in opposing against the stress, the hydrogen cyanide role as a by-product in the plant ethylene production pathway, intracellular settings in response to environmental stresses has rarely been noticed. However, hydrogen cyanide along with ethylene is produced equally and in response to biotic and abiotic stresses of the plant. Hydrogen cyanide in the plant is the amino acid precursor to asparagine and a stimulant of ethylene production in plants in the non-toxic concentrations (Oracz et al., 2008), in addition to cyanide and pentose phosphate resistant airways induction in plants (Bogatek and Lewak, 1991). In the present research, only the bacterial isolate *Bacillus mycoides* was capable of producing low level of hydrogen cyanide.

5 Conclusion

The results of this study showed that there was inhibitory effect of antagonist isolates separated from weeds and it can be used as an *in vitro* biocontrol agent. However, further investigation of this hypothesis required to prove the performance of these isolates with the purpose of their application in the farm.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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