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A study on endophytic bacteria isolated from wild legumes against *Xanthomonas phaseoli*

SH. ROSTAMI[1], N. HASANZADEH[1]✉, S. RAJAEI[2], A. GOLNARAGHI[1,3], R. AZIZINEZHAD[4]

1. PhD student, Associate Professor and Assistant Professor respectively, Department of Plant Protection, Faculty of Agricultural Sciences and Food Industries, Science and Research Branch, Islamic Azad University, Tehran, Iran; 2. Assistant Professor, Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran; 3. Researcher, Department of Biodiversity, Boom Zista Institute, Vancouver, British Columbia, Canada; 4. Assistant Professor, Department of Plant Breeding and Biotechnology, Faculty of Agricultural Sciences and Food Industries, Science and Research Branch, Islamic Azad University, Tehran, Iran
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Abstract

Common bacterial blight disease (CBB) of bean (*Phaseolus vulgaris*) caused by *Xanthomonas phaseoli* (Xp) is considered as one of the most deleterious pathogens for bean production in the world. In this study, 105 samples were collected from asymptomatic wild fabaceous plants, i.e. *Astragalus ovinus*, *Vicia villosa* and *Vicia lutea*, grown in Zagros forests of Iran. The plant samples were cultured on nutrient agar and purified. The isolates were then screened for some important criteria for biological control such as phosphate solubilization, protease activity, IAA and H_2S production, and antagonistic effect. Three endophytic bacterial isolates were found as potential biocontrol agents against Xp. Based on key biochemical tests and comparative analysis of the partial 16S rDNA sequences, the isolates were identified as *Pseudomonas fluorescens* and two *Bacillus* species. Under greenhouse conditions, all the three strains significantly increased shoot and root lengths in bean plants at the 5% level ($P < 0.05$) and decreased disease severity above 70%. This is the first report on the presence and capabilities of endophytic bacteria from wild leguminous plants in the Zagros Mountain steppe forests of Iran.

Keywords: Bean common blight, biological control, endophytic bacteria, wild legumes, Zagros Mountain steppe forests

بررسی نقش باکتری‌های اندوفیت جداسازی شده از حبوبات وحشی در کنترل *Xanthomonas phaseoli* عامل بیماری بلایت باکتریایی لوبیا

شریعه رستمی[۱]، نادر حسن‌زاده[۱]✉، سعیده رجائی[۲]، علیرضا گل‌نراقی[۱،۳]، رضا عزیزی نژاد[۴]

۱- به‌ترتیب دانش آموخته مقطع دکتری، دانشیار و استادیار گروه گیاه‌پزشکی، دانشکده علوم کشاورزی و صنایع غذائی، واحد علوم و تحقیقات دانشگاه آزاد اسلامی، تهران، ایران؛ ۲- استادیار پژوهشکده زیست فناوری صنعت و محیط زیست، پژوهشگاه ملی مهندسی ژنتیک و زیست فناوری، تهران، ایران؛ ۳- پژوهشگر گروه تنوع زیستی، مؤسسه بوم‌زیستا، ونکوور، کانادا؛ ٤- استادیار گروه بیوتکنولوژی و به نژادی، دانشکده علوم کشاورزی و صنایع غذائی، واحد علوم و تحقیقات دانشگاه آزاد اسلامی، تهران، ایران

چکیده

بیماری بلایت باکتریایی لوبیا یکی از مخرب ترین بیماری در زراعت‌های لوبیا در سراسر جهان است. تعداد ۱۰۵ نمونه از گیاهان وحشی تیره لگومینوز شامل *Astragalus ovinus*، *Vicia villosa* و *Vicia lutea* که فاقد علائم آشکار بیماری بودند از جنگل‌های زاگرس ایران جمع‌آوری و تعداد ۳٦ جدایه باکتریایی جداسازی شدند. بر پایه برخی صفات مانند توانایی حل نمودن فسفات، فعالیت پروتئازی، تولید اکسین و سیانید هیدروژن و نیز قابلیت آنتاگونیستی، سه اندوفیت برتر از بین آن‌ها انتخاب و با انجام آزمون‌های فنوتیپی و تکثیر ژن 16S rDNA، یک سویه *Pseudomonas fluorescens* و دو سویه از گونه های جنس *Bacillus* شناسائی شدند. هر سه سویه موجب افزایش معنی‌دار رشد گیاهان لوبیای آلوده در سطح پنج درصد و کاهش بیش از ۷۰ درصد بیماری گردیدند. این اولین گزارش از وجود و قابلیت‌های باکتری‌های اندوفیت از گیاهان وحشی لگومینوز در جنگل‌های زاگرس ایران می‌باشد.

واژه‌های کلیدی: باکتری‌های اندوفیت، بلایت لوبیا، جنگل‌های زاگرس، کنترل بیولوژیکی، لگوم‌های وحشی

✉ **Corresponding author: hasanzadehr@yahoo.com**

Introduction

The common bean (*Phaseolus vulgaris* L.) is considered as an economical legume and necessary food crop in the world which contribute to a balanced and healthy diet by providing proteins, carbohydrates, fibers, vitamins and minerals such as phosphorous and potassium (Graham and Ranalli 1997; Yu *et al.* 2000; Schulz 2004; Popovic *et al.* 2012; Schmutez *et al.* 2014). Various diseases are limiting the cultivation of this strategic crop. Among them, common bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *phaseoli* (Xap), is known as one of the most destructive seed-borne disease of bean crops all through the world (Sultana *et al.* 2018), including mid-Eurasian region of Iran where the disease has been reported from different geographical locations (Lak and Dorri, 2009). *X. axonopodis* is a gram-negative bacterium which grows on leaves, stems, pods and seeds, and induces typical water-soaking symptoms. These symptoms can be observed on leaves within 4 to 10 days post-infection (Goodwin and Sopher 1994).

Management of CBB disease is a challenging task since spraying bactericides and chemical inhibitors have often negligible efficiency (Zanatta *et al.* 2007). Biological control of the pathogens is an alternative way to suppress the pathogen in the plants. In some cases, biological agents like plant growth-promoting bacteria (PGPB) reduce the environmental stresses of plants by enhancing plant nutrition or through other protective anti-stress activities (Sessitsch *et al.* 2002). Endophytic bacteria, which reside asymptomatically within a plant, have the potential to be candidate for biocontrol applications (De Silva *et al.* 2019), and for promoting plant growth and yield (Lodewyckx *et al.* 2002; Compant *et al.* 2005; Ryan *et al.* 2008). The beneficial effects of bacterial endophytes on their host plant seem to occur through similar mechanisms described for plant growth promoting rhizobacteria (PGPR) (Lodewyckx *et al.* 2002).

Endophytes (endo=within, phyte=plant) address microorganisms that live within plants without causing apparent disease. In recent years, many endophytic bacteria have been isolated from different hosts, including agronomic crops, rangeland plants, plants growing in extreme environments, wild plants and perennial plants (Yuan *et al.* 2014). Biocontrol of plant pathogens, particularly by using endophytic microorganisms, has been a challenging subject in sustainable agriculture (De Silva *et al.* 2019). Some bacterial isolates like strains belong to *Bacillus* and *Pseudomonas* genera have been offered as biocontrol agents of some pathogens (Liu *et al.* 2007; Liu *et al.* 2020). Other studies have shown that bacterial endophytes isolated from fabaceous crops or wild plants may act as bio controllers against plant pathogens or as plant growth enhancers (Zinniel *et al.* 2002; Mark *et al.* 2006; Zanatta *et al.* 2007; Costa *et al.* 2012). *Rhizobium* and *Bacillus* strains were specifically introduced as antagonistic agents against Xap and *X. citri* subsp. *citri* (Zanatta *et al.* 2007; Daungfu *et al.* 2019).

Natural environments like steppe forests are valuable sources of biodiversity. There are limited reports on endophytic bacterial communities of wild plants in forest areas (Zinniel *et al.* 2002; Zanatta *et al.* 2007; Costa *et al.* 2012; Etminani and Harighi 2018). Zagros Mountain steppe forests ecoregion is a wide habitat of various species in Iran. Considering high biological richness and the diversity of wild plants, this ecosystem plays an important role to conserve sustainability (Akhani *et al.* 2010; Heidari *et al.* 2010). There is also some information that shows a high diversity of microbial endophytes in wild plants in these forests (Tashi-Oshnoei, Harighi *et al.* 2017; Ghorbani and Harighi 2018; Etminani and Harighi 2018). Considering exclusive diversity of wild legumes in Zagros Mountain steppe forests, this study aimed to isolate some beneficial bacterial endophytes from three wild fabaceous species, hoping get some antagonists against the causal agent of the CBB disease of beans.

Material and methods

Collection of samples

All plant specimens were collected from Zagros Mountain steppe forest located in Kermanshah province in the spring of 2010. A total of 105 asymptomatic wild legumes, including *Astragalus ovinus*, *Vicia villosa* and *V. lutea* species were collected from five geographical regions. Sampling locations were determined by Global Positioning System (GPS) (Figure 1). Each sample, comprised of whole plant (root and shoot), was placed in a separate plastic bag and transported to the laboratory under cold conditions.

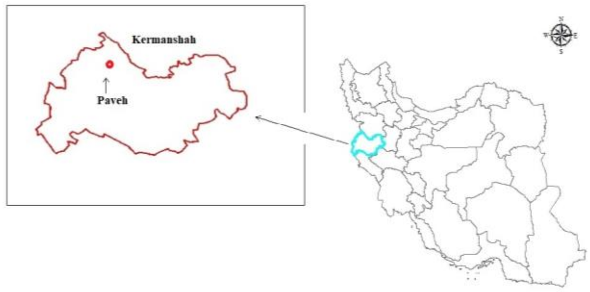

Fig 1. The geographical location of the sampling area in the Zagros Mountain steppe forests.

Surface sterilization and endophyte isolation

In order to isolate endophytes, all legume tissues were washed thoroughly with tap water (for 10 min) to remove soil and then separated into stems, roots and nodules. Subsequently, legume tissues were cut into 2-3 cm pieces, rinsed in 70% ethyl alcohol for 30 seconds, sterilized with 0.2% $HgCl_2$ (3 min for roots and 5 min for stems), and washed thoroughly with sterile water. Macerated tissues were serially diluted to 10^{-8} in sterile distilled water. A 100-µL of the diluent was spread on nutrient agar (NA) medium; the plate was then incubated at 28 °C for five days (Phetcharat and Duangpaeng 2012). The emerged bacterial colonies with different morphologies were subsequently picked and purified three times using a single-colony culture method (Schaad *et al.* 2001).

Source of Xap culture and pathogenicity test

Xap culture used in this study was provided from the culture collection center of Agricultural and Natural Resources Research Center (ANRRC) in Kermanshah, Iran. To obtain pure cultures, the Xap strain was streaked onto NA medium and incubated for 48 h at 28 °C. A single colony was re-suspended in distilled water and cultured on NA medium. Ultimately, a suspension was prepared to a concentration of 10^8 CFU mL^{-1} (colony forming unit). Plates were incubated at 27 °C for 3–5 days and examined for colony development. The pathogenicity test was performed on one-month old bean seedlings using leaf sectioning inoculation method. The inoculum was prepared from early log-phase cells which were obtained by growing the bacteria in nutrient yeast extract broth incubated on a rotary shaker at 25 ^{o}C and 200 rpm for 24 h. Bacteria were subsequently pelleted by centrifugation at 15,000 rpm for 5 min; the pellet was washed by 0.1% saline solution. The concentration of bacterial cells was then adjusted to 10^8 CFU mL^{-1} to achieve OD_{600} equal to 0.2. The middle leaf vein was injected by 0.1 mL of the bacterial suspension. Control plants were treated similarly with 0.1% bacteria-free saline solution. Inoculated plants were kept in a greenhouse under normal light conditions for 48 h at 25–27 ^{o} C, and development of typical disease symptoms was checked two weeks after inoculation (Sallam, 2011).

***In vitro* antagonistic activity assay**

All 36 bacterial isolates obtained in this study were screened against Xap. A fresh-overnight suspension of each isolate was streaked as a perpendicular line to the Xap culture in three replicates. The antagonistic activity was recorded by quantitative measuring of the growth inhibition zone around Xap after 48 h in a dual culture assay (Aquino-Martinez *et al.* 2008).

Plant growth promoting assays

To evaluate indole-3-acetic acid (IAA) production, eight bacterial isolates including A33, Z73, Z51, A11, A41, Z5-1, Z73-2 and Z73-1 showing the largest growth inhibition zones were inoculated in nutrient broth (NB) containing L-tryptophan and incubated at 28 ^{o}C for 10 days with vigorous shaking. The concentration of IAA in the culture supernatant was estimated by mixing 4 mL of Salkowski reagent in 1 mL of each supernatant. The optical absorbance of the solution was measured at 535 nm using a spectrophotometer (Bio-Tek, ELX808IU, USA) at least 30 minutes after adding the reagent, when the color of the mixture turned to pink. A standard curve of various concentrations of pure IAA in the range of 0-250 µg mL^{-1} was prepared by plotting IAA concentration to optical density (at 530 nm). The concentration of IAA for each isolate was then determined by using standard curve according to the equation, $Y = 0.0071X+0.1108$ (Etminani and Harighi 2018). The isolates were also evaluated for Hydrogen cyanide (HCN) production. To this end, 50 µL of each bacterial suspension were streaked on NA medium with Whatman paper soaked in 0.5% picric acid solution placed inside the plate's lids. Plates were sealed and incubated at 28 °C for 4 days. HCN production was indicated by the color change of the Whatman paper from brown to red (Alstrom and Burns1989). For phosphate solubilization assay, Pikovskaya (PVK) agar medium (Pikovskaya 1948) was utilized. A single colony for each isolate was placed on the medium containing (g L^{-1}): yeast extract (0.5), dextrose (10.0), $Ca_3(PO_4)_2$ (5.0), $(NH_4)_2SO_4$ (0.5), $MgSO_4$ (0.1), KCl (0.2); $MnSO_4.7H_2O$ (0.002) and $FeSO_4.7H_2O$ (0.002), Agar-agar (20.0); pH-7.2. The plates were incubated at 28 °C for one week. The phosphate solubilizing efficiency was measured by this formula: SE= $\frac{solubiliztion\ diameter}{growth\ diameter} \times 100$ Protease assay tests were performed according to earlier recommendations (Sgroy *et al.* 2009). Plates containing skim milk agar (SMA) medium (g L^{-1}): pancreatic digest of casein (5), yeast extract (2.5), glucose (1), skim milk (7) and agar (15) were inoculated with 10 µl of bacterial suspension and incubated at 28 °C until the formation of clear zones around the bacterial colonies.

Identification of endophytes

For the determination of the phenotypic features of endophytic isolates A33, Z51 and Z73, standard bacteriological methods were employed. These tests were Gram staining, aerobic or anaerobic growth, fluorescent pigment production on King's B medium, as well as oxidase and catalase activity (Schaad *et al.* 2001). Moreover, the three isolates with maximum scores in developing inhibition zones and *in vitro* plant-growth potentials were further studied and their partial 16S rDNA sequences were determined. To this end, bacterial DNAs were extracted according to a CTAB method (Young *et al.* 2004) and tested by polymerase chain reaction (PCR) using universal primer pair 27F (5′AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′TACGYTACCTTGTTACGACTT3′) to amplify 1.4 kb DNA fragments (Zhu *et al.* 2016); these primers were synthesized by Taligene Pars Co. (Isfahan Science and Technology Town, Isfahan, Iran). The 25 µl PCR reaction mixture contained 2.5 µL 10xbuffer, 2 µL dNTPs (2.5 mmol L^{-1}), 1 µL of each primer (5 µmol L^{-1}), 0.2 µL *Taq* DNA polymerase (5u μL^{-1}), 17.3 µL ddH2O, and 1.0 µL template DNA. The thermal cycling condition was 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1.5 min and a final extension at 72 °C for 10 min. PCR products were separated by 1.2% agarose gel electrophoresis, visualized using ethidium bromide staining, and photographed with ultraviolet-illumination. To determine the nucleotide sequences, the PCR products were purified using a QIA quick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's directions. DNA sequencing was done by direct Sanger sequencing in both directions using the aforementioned amplification primers. The editing and assembling sequence data in this study were done using Bio-Edit Version 7.2.5 (Hall, 1999). The GenBank non-redundant nucleotide database was subsequently searched using BLASTN (Altschul *et al.* 1997) to determine the bacterial genus according to maximum homology (Kepczynska and Karczynski 2020).

Phylogenetic relationships of the rDNA sequences were investigated by using MEGA X software. The sequences were first aligned with other representative sequences, prepared from the GenBank based on homology and nucleotide BLAST analysis, using MUSCLE algorithm (Kumar *et al.* 2018). Phylogenetic trees were constructed by maximum likelihood (ML) algorithm using Kimura 2-parameter model (K2) and gamma distributed with invariant sites (G+I) (Kimura, 1980) that found as the best nucleotide substitution model. The goodness-of-fit of the model was measured by the Bayesian information criterion (BIC) and corrected Akaike information criterion (AICc) (Tamura, 2011). To assess the reliability of a phylogenetic tree, the Bootstrap test was conducted with 1000 replicates (Kumar *et al.* 2018).

Seed germination tests

This experiment was done under light conditions. The three endophytic bacterial isolates A33, Z51 and Z73, with the highest plant growth promoting activities, which were considered as plant growth promoting bacteria (PGPBs), were selected for further greenhouse experiments. To prepare inoculants, these isolates were sub-cultured and diluted to the concentration of 10^8 CFU mL^{-1} (Sallam, 2011). Meanwhile, Mexican red beans (*P. vulgaris* cultivar KS31169) were soaked in water for 24 h and subsequently rinsed with 2.5% NaOCl solution (Sodium hypochlorite) for 3 min; NaOCl was thoroughly removed by using sterile distilled water. The seeds were placed on two layers of Whatman No. 1 filter papers and then incubated at 24 ± 1 °C for 14 days. After that, the germinated seeds were counted and expressed as percentage. Vigor indexes were measured as described earlier (Shalini *et al.* 2017). The experimental design included seeds without pathogens and PGPB inoculations (Xap^-, $PGPB^-$), seeds without pathogens but inoculated separately with PGPBs (Xap^-, $PGPB^+$), Xap-infected seeds without PGPB (Xap^+, $PGPB^-$), and infected seeds that separately inoculated with PGPBs (Xap+, $PGPB^+$). Inoculations were carried out under vacuum conditions (20 lb inch^{-2}) in three replicates. Treatments were placed on presoaked filter paper and incubated at 24 °C for 14 days.

Greenhouse experiments

These experiments were conducted in a completely randomized design with three replicates for every treatment. For seed inoculation tests, presoaked sterile seeds were first infested with both endophytic bacterial strain and pathogen in the same ratio (10^8 CFU mL^{-1}) according to the information in table 1. The mixture of pit-perlite-vermiculite, seeds and bacterial suspensions (10^8 CFU mL^{-1}) were transferred in plastic bags filled with Peat-perlite-vermiculite (2:1:1). The negative control received no treatment or neutral treatment. Plastic bags were kept two weeks under greenhouse conditions (24 °C, 60% soil moisture and 80% relative humidity). For seedling inoculation tests, briefly, seeds were presoaked and sterilized as mentioned above. Sterile seeds were planted in 600 mL volume pots filled with autoclaved pit-perlite-vermiculite (2:1:1). The plastic bags were kept in the greenhouse for 14 days; after that, the newly grown seedlings were sprayed with Xap cell suspension at 10^8 CFU mL^{-1}. The seedlings were subsequently sprayed with each of the PGPR isolates (A33, Z51 and Z73) in the same method three weeks after inoculation. While negative controls received no treatment or a neutral treatment (non-infected controls) (Xap^-, $PGPB^-$), positive controls or infected controls were inoculated by Xap but not by the endophytic isolates understudy (Xap^+, $PGPB^-$). Treated seedlings were kept under greenhouse conditions for 30 days.

Disease index reduction and plant growth measurement

Disease index (DI) was determined two weeks subsequent to PGPB inoculation (Dhanya and Mary, 2007; Sallam, 2011). Considering the intensity of symptoms, plants were assigned to five grades, including lack of symptoms (0), lesions at pinpricks (1), lesions at pinpricks along with yellowing of 1-2 leaves (2), lesions with size of 1.2 x 0.5 cm along with yellowing of 1-2 leaves (3), yellowing of all leaves with blackening of petiole of leaves (4), complete death of the plant (5). DI was calculated using this formula: DI= [sum of individual scores/(total leaves observed × maximum score)] x 100. The impact of PGPBs on plant growth as well as fresh and dry weight were also measured (Zinniel *et al.* 2002).

Statistical analysis

Statistical analysis of the data was performed using SPSS statistical computer package (version 19.0 SPSS Inc., Chicago, USA). Data were compared with the control or among treatments by analysis of variance (ANOVA) to discriminate significant differences at $P < 0.05$ followed by Duncan's test.

Results

Isolation and screening of potential antagonists

A total of 36 morphologically different isolates were obtained from samples of three species: *A. ovinus*, *V. villosa*, and *V. lutea*. Among them, eight isolates (A33, Z73, Z51, A11, A41, Z5-1, Z73-2 and Z73-1) exhibited clear inhibition zone. The maximum inhibition zones (>8-10 mm) were recorded for A33, Z51 and Z73 (Table 1).

Table 1. Antagonistic and plant growth promoting potential of endophytic bacterial isolates from wild legumes in Zagros Montain steppe forests.

Bacterial code	Inhibition zone against *Xap* (mm)	IAA production (µg/ml)	Hydrogen cyanide production	Phosphate solubilization	Protease activity
A33	**	7.26	-	+	+
Z73	***	6.48	+	+	+
Z51	***	7.85	-	+	+
A11	*	-	-	-	-
A41	*	-	-	-	-
Z5-1	*	-	-	-	-
Z73-2	*	-	-	-	-
Z73-1	*	-	-	-	-
Control	*	5.5	-	-	-

*, poor inhibition (>2 mm); **, moderate inhibition (>8 mm); and ***, high inhibition (>10 mm); Xap: *Xanthomonas axonopodis* pv. *phaseoli*; +, positive result; -, negative result.

Plant growth promoting assays

Three isolates, i.e., A33, Z51 and Z73, were able to produce IAA in the range of 6.48-7.85 µg mL^{-1}. Among them, the isolate Z51 with 7.85 µg mL^{-1}, Z73 with 6.48 µg mL-^{1} and A33 with 7.26 µg mL^{-1}, produced the highest to the lowest amount of IAA, respectively. The level of IAA production for isolates A33, Z51 and Z73 was statistically higher than that of controls. Moreover, isolate Z73 was the only HCN producing bacterium, *in vitro*. The isolates A33, Z51 and Z73 developed a clear zone around the inoculation spot in Pikovskaya medium, indicating their phosphate solubilization activities. These isolates also showed remarkable protease activity as well (Table 1).

Table 2. Phenotypic properties of three representative endophytic bacterial isolates.

Tests	Bacterial isolates		
	A33	Z73	Z51
Gram staining	-	+	+
Oxidase	+	-	+
Aerobic growth	+	+	+
Fluorescence under UV	+	-	-
Catalase	+	+	+
Cell shape	Rod	Rod	Rod

+, positive result; -, negative result

Identification of bacterial isolates

Based on biochemical assays, the three representative isolates showed different phenotypic characteristics. The isolates were rod-shaped, aerobic growth and catalase positive; the isolate A33 was the only gram negative isolate among those tested (Table 2). The isolates A33, Z51 and Z73 produced the expected fragments of 1.4 kb in PCR (Zhu *et al.* 2016). The sequence of A33 was match to sequences from *Pseudomonas fluorescens*, and alignments generated by BLASTN searches showed identity score of 96%. Similarly, the sequences of isolates Z51 and Z73 were most closely related to sequences of *Bacillus pumilus* and *B. simplex* with identity scores of 99% and 98%, respectively. Phylogenetic analyses were in agreement with the databases searches results and showed a clustering of isolates A33, and Z51 and Z73 as sister groups of *P. fluorescens*, and *B. pumilus* and *B. simplex* isolates with high bootstrap values (Fig. 2-3). The genomic sequences determined in this study were deposited in the GenBank nucleotide under accession numbers MN886821 to MN886823.

Seed germination studies

Seedlings grown from seeds treated with the isolates showed the lowest symptom severity caused by Xap. Effect of treatments with the three endophytes on seedling length was statistically significant in compared to non-infected controls (Xap, PGPB^{-}). Results showed that treatments with the three endophytes significantly increased seedling length as compared with non-infected controls (Xap^{-}, PGPB^{-}). Isolates A33, Z51 and Z73 increased seedling length by 11.93, 15.23 and 10.83 cm, respectively. Isolate A33 also increased both fresh and dry seedling weight by 4.56 and 3.21 g, respectively. Isolate Z51 increased fresh and dry seedling weight by 3.36 and 2.33g, respectively. In case of Z73, this isolate increased both fresh and dry seedling weight by 4.5 and 3g, respectively (Table 3).

Table 3. Average values of plant growth indexes under various treatments.

Treatment	Plant			Seedling			Symptom incidence (%)	Disease Index reduction (%)
	Fresh weight (g)	Dry weight (g)	Height (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)		
A33, Xap^{+}	5.46^{a}*	3.46^{a}	20.00^{a}	3.06^{ab}	2.30^{ab}	4.46^{a}	20.52^{d}	70.71^{d}
A33, Xap^{-}	7.65^{b}	6.48^{b}	40.66^{c}	4.56^{b}	3.21^{b}	11.93^{c}	0.0^{a}	0.0^{a}
Z51, Xap^{+}	5.45^{a}	4.03^{a}	21.00^{a}	3.16^{ab}	2.65^{ab}	4.61^{a}	15.36^{b}	78.08^{b}
Z51, Xap^{-}	8.24^{b}	6.80^{b}	50.00^{d}	3.36^{ab}	2.33^{ab}	15.23^{d}	0.0^{a}	0.0^{a}
Z73, Xap^{+}	5.50^{a}	3.70^{a}	21.66^{a}	3.16^{ab}	2.43^{ab}	4.46^{a}	18.45^{c}	73.66^{c}
Z73, Xap^{-}	8.58^{b}	7.44^{b}	50.66^{d}	4.50^{b}	3.00^{b}	10.83^{bc}	0.0^{a}	0.0^{a}
Xap^{+}, PGPB^{-}	5.04^{a}	3.42^{a}	16.33^{a}	2.70^{a}	1.82^{a}	3.83^{a}	70.06^{e}	0.0^{e}
Xap^{-}, PGPB^{-}	8.52^{b}	6.10^{b}	32.66^{b}	3.43^{ab}	2.93^{b}	8.56^{b}	0.0^{a}	0.0^{a}

Xap^{+}: infected with *Xanthomonas axonopodis* pv. *phaseoli*, Xap: without *Xanthomonas axonopodis* pv. *phaseoli*, A33, Z51, and Z73: three plant growth promoting bacteria (PGPB) isolated from roots of wild legumes, PGPB: without PGPB inoculation.
Amounts show means and different letters beside each mean indicate significant differences under different Pvalue < 0.05

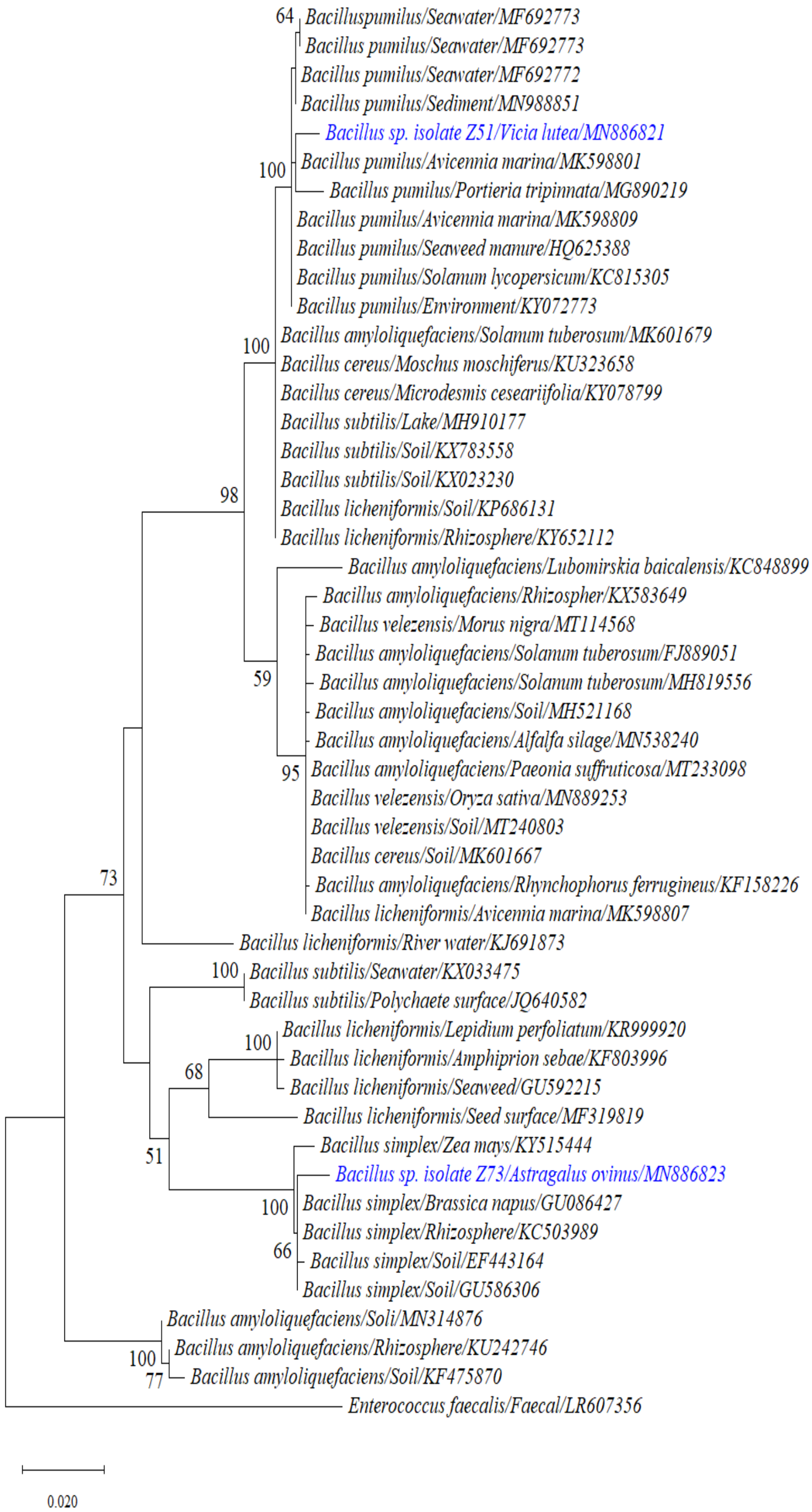

Fig. 2. Phylogenetic relationships of partial 16S rDNA gene sequence of the endophytic isolates Z51 and Z73, and corresponding regions of representative sequences from GenBank. This analysis was performed using the maximum likelihood (ML) algorithm and Tamura-Nei model in MEGA X software. Numbers at each node indicate the percentage of supporting bootstrap samples in ML method.

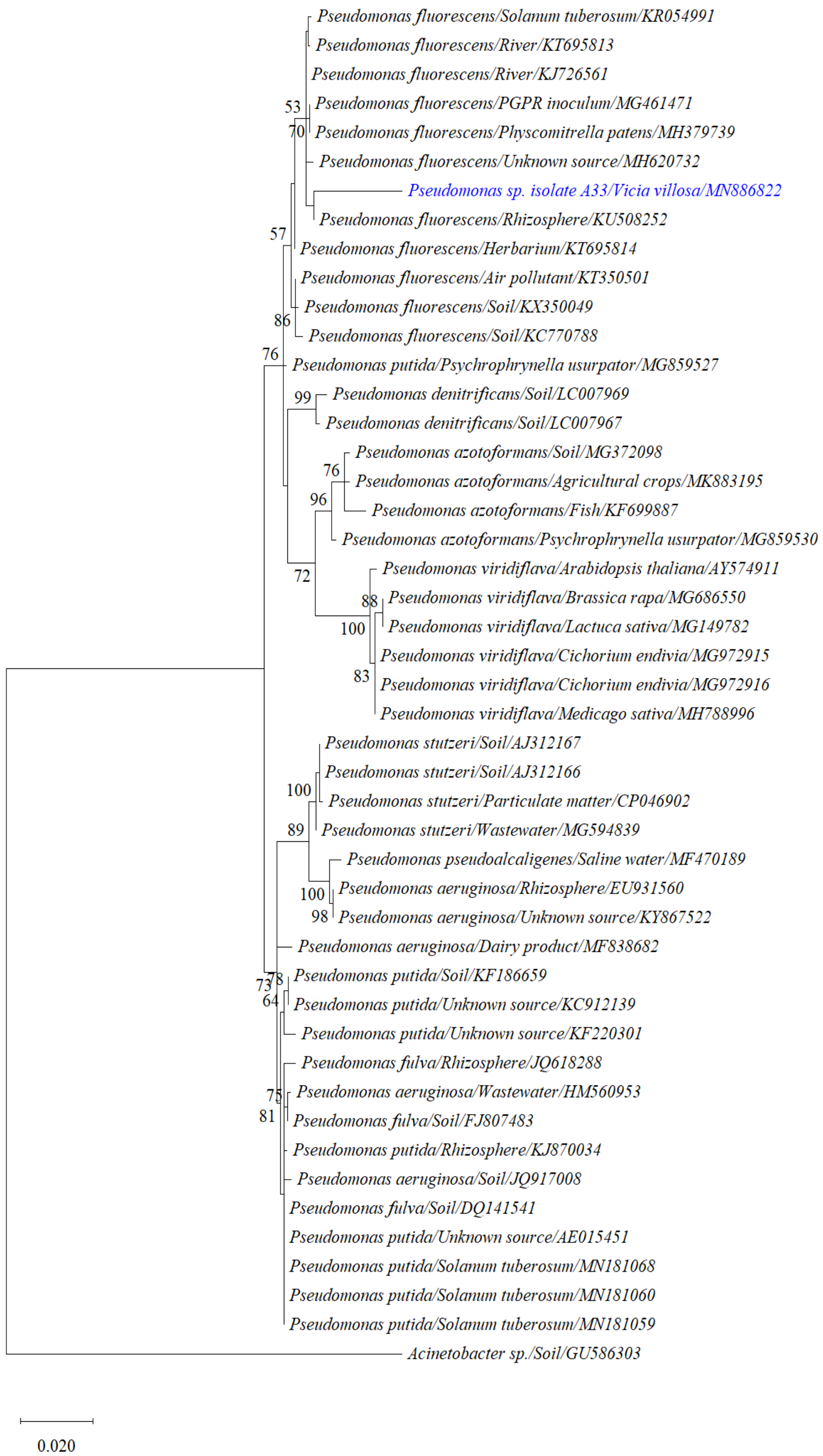

Fig. 3. Phylogenetic relationships of partial 16S rDNA gene sequence of endophytic isolate A33 and corresponding regions of representative sequences from GenBank. This analysis was performed by using the maximum likelihood (ML) algorithm and Tamura-Nei model in MEGA X software. Numbers at each node indicate the percentage of supporting bootstrap samples in ML method.

Greenhouse studies

The endophytic inoculation of Xap-infected plants had no significant effect on plant biomass (dry and fresh) as compared with non-infected controls (Xap^-, $PGPB^-$). However, the percent of symptom severity significantly decreased by more than 70% in plant inoculated with each of the three endophytes (Z51, Z73 and A33) and Xap (Xap^+, $PGPB^+$) as compared with those inoculated with Xap only (Xap^+, $PGPB^-$). The highest rate of disease reduction was belonged to Z51 (78.07%) and the lowest was related to A33 (70.71%). The disease index reduction by Z73 isolate was 73.66. Moreover, the heights of the healthy plants treated with endophytes only (Xap^-, $PGPB^+$) were remarkably higher than that of control plants; similar results were observed for seedling height without Xap- infection (Table 3).

Discussion

In contrast to incredible levels of endophytic microbial diversity of plants grown in Iranian forests (Etminani and Harighi 2018; Yazdani-Khameneh *et al.* 2019), limited studies have been performed in this area. In the current work, we isolated entophytic bacteria from wild legumes, i.e., *A. ovinus*, *V. villosa* and *V. lutea* and screened them for some characteristics for biocontrol and growth promotion activities. Amongst the isolates studied, three of them, namely A33, Z51 and Z73, showed plant growth promoting activities and had a significant growth inhibition against Xap under *in vitro* and greenhouse conditions. Although, the three endophytic isolates had no significant effect on plant biomass, they showed some positive effects on plant growth and efficiently controlled Xap infections under greenhouse conditions. According to the molecular analysis, the three isolates A33, and Z51 and Z73 were belonged to Pseudomonas and Bacillus. These results confirmed the results of phenotypic traits.

According to the results of this study, the isolate of *Pseudomonas fluorescens* A33 was found to drastically promote growth indexes (shoot heights, root lengths and dry biomass levels and fresh biomass Levels) of bean (*P. vulgaris*). Yanti *et al.* (2018) reported that *Bacillus pseudomycoides* NBRC 101232 was able to increase height and number of leaves in tomato plants. Safdarpour (2017) reported that *Pseudomonas mosselli*, *P. fuorescence*, increased seed germination and growth parameters of tomato seedlings. It also reduced the disease and improved the growth parameters of the plants in challenging with *V. dahlia* in greenhouse. The results of the present study are consistent with the findings of Yanti *et al.* 2018 and Safdarpour (2017). Siddiqui and Shaukat (2002) reported that Plant growth-promoting rhizobacteria (PGPR) strains CHAO (*Pseudomonas fluorescens*), IE-6 S^+ (*Pseudomonas aeruginosa* increased shoot and root length of tomato plants. The result of the current study is in accordance with the results of Siddiqui and Shaukat (2002). Growth promotion activity and health benefits of endophytes have been indicated by several reports (Sturz *et al.* 2000).

According to the results of this study, two isolates of *B. simplex* Z51, and *B. pumilus* Z73 were found to dramatically promote growth indexes (shoot heights, root lengths and dry biomass levels and fresh biomass levels) of bean. Kalam *et al.* (2020) reported that seven plant growth promoting *Bacillus* strains promoted the root length shoot length and dry weight of tomato seedlings under *in vitro* and *in vivo* conditions. Greenhouse experiments with these strains indicated an overall increase in the growth of tomato plants, over 60 days. The result of the present study is in accordance with those observed by Kalam *et al.* (2020). Wang *et al.* (2018) reported that *Bacillus amyloliquefaciens* subsp. *plantarum* XH-9 significantly increased the wheat plant shoot heights, root lengths, dry biomass levels and fresh biomass levels compared to the un-inoculated plants. They reported that antagonistic mechanisms and PGP characteristics were revealed in terms of nitrogen fixation, phosphate and potassium solubilization, and production of growth hormones, ACC deaminase, diffusible and volatile antibiotics, siderophores, cellulase, glucanase, protease, and chitinase. The result of this study confirmed those obtained by Wang *et al.* (2018). Akinrinlola *et al.* (2018) reported that four strains *Bacillus megaterium* R181, *B. safensis* R173, *B. simplex* R180, and *Paenibacillus graminis* R200 increased the shoot height, shoot fresh weight and root fresh weight of corn, wheat and soybean plants. The results in the present study are consistent with the results of Akinrinlola *et al.*

(2018). The mechanisms by which plant growth is promoted by endophytes may be similar to the mechanisms exerted by rhizosphere microorganisms and include phytohormone production, promotion by enhanced accessibility of nutrients, production of antibiotics, reduction of ethylene level, induced systemic resistance and competition with pathogens (Krishnan *et al.* 2015). Kalam *et al.* (2020) reported that seven *Bacillus* strains that promoted the root length, shoot length and dry weight of tomato seedlings, had phosphate and zinc solubilization, production of indole acetic acid (IAA), siderophore, hydrogen cyanide (HCN), as well as phytase and 1-aminocyclopropane-1- carboxylate (ACC) deaminase activities. According to results of this study, three isolates *P. fluorescens* A33, *B. simplex* Z51, and *B. pumilus* Z73 had also significant inhibition effect on *X. axonopodis* pv. *phaseoli* on bean. A similar report by Krishnan *et al.* (2015) stated that *Bacillus subtilis* var. *amyloliquefacieons* (FZB24) effectively inhibited the growth of *Xanthomonas oryzae* pv. *oryzae*, *Pyricularia grisea* and *Rhizoctonia solani*, *in vitro*. The antagonistic effect of *B. amyloliquefaciens* against *Alternaria alternata*, *Colletotrichum crassipes* and *Fusarium oxysporum* under greenhouse conditions was reported (Li *et al.* 2015). Liu *et al.* (2018) in their new report declared that strains of *Bacillus altitudinid* (AP69), *B. velezensis* (AP197- AP199- AP298) had broad-spectrum biocontrol activity via antagonism in growth chamber against *X. axonopodis* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*. This growth inhibitory effect may be due to producing growth inhibitors i.e. antibiotics, bacteriocins, siderophores and lytic enzymes by PGPB (Tariq *et al.* 2017) or triggering induced systemic resistance (ISR) in bean plants through the salicylic acid-dependent SAR pathway, or require jasmonic acid and ethylene perception from the plant for ISR (Beneduzi *et al.* 2012). Chowdhury *et al.* (2015) reported that plant growth-promoting activity is linked with the ability to suppress soil-borne plant pathogens.

The potentials of endophytes as biocontrol agents or plant growth enhancers have been previously reported in different plant-endophyte-pathogen systems in Iran (Arshadi *et al.* 2019) However, to our knowledge, this is the first report of endophytic bacterial communities of wild legumes in Zagros forests in the country. The use of beneficial microorganisms is considered one of the most promising methods for safe crop management practices. The results of this study indicated that ability of some endophytes in biological control of Xap and growth promotion of bean plants; they are also appropriate candidates for biocontrol of other plant bacterial pathogens. Although, further experiments are needed to determine the effectiveness of our isolates under field conditions, we have confidence that this isolates can be developed as biocontrol agents for improving bean crop productivity in traditional and organic production systems.

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