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*Full Length Article*

# A Hidden Plant Growth Promoting Bacterium Isolated from *In Vitro* Cultures of Fraser Photinia (*Photinia* × *fraseri*)

**Mine Gül Şeker[1], Irmak Şah[1,2], Emrah Kırdök[1], Hamit Ekinci[1], Yelda Özden Çiftçi[1] and Özlem Akkaya[1*]**
[1]*Gebze Technical University, Faculty of Basic Sciences, Department of Molecular Biology and Genetics, 41400, Kocaeli, Turkey*
[2]*Gebze Technical University, Institute of Nanotechnology, 41400 Gebze, Kocaeli, Turkey*
[*]For correspondence: ozlem@gtu.edu.tr

## Abstract

Microbial contamination in the plant tissue culture systems considered as *in vitro* pathogens and should be eliminated to prevent culture loss. However, some of these contaminants could have beneficial influence on plant growth with supplying different additives and could have potential to use as bio-inoculants. Thus, isolation and characterization of these potential *in vitro* contaminants for production of biological compounds from plant cultures is becoming attractive for scientists. With this approach, the objective of this study was to isolate and identify putatively endophytic bacterium that was detected in the long-term *in vitro* cultured microshoots of fraser photonia (*Photinia* × *fraseri* Dress) and to screen its plant growth-promoting characteristics (PGPC). The strain was screened according to their phenotypical and biochemical properties together with PGPC abilities including phytostimulation, biofertilization and hydrolytic activities. Our results showed that this strain exhibited nitrogen-fixing ability as well as gibberellic acid ($GA_3$) and indole-3-acetic acid (IAA) producing capability. Gram negative putative endophytic bacterium showed similarity to uncultured bacteria and Rhizobiales according to 16S rRNA sequencing. When the proliferation medium was supported with cytokinin, maximum multiple shoot formation together with the highest microshoot length were obtained in fraser photinia *in vitro* cultures with the presence of bacteria. Thus, the isolated bacterial strain with various PGPC can be considered as a beneficial microbe for the facilitation of fraser photinia growth. © 2017 Friends Science Publishers

**Keywords:** Endophytic bacteria; $GA_3$; IAA; Nitrogen fixation; PGPC

## Introduction

*In vitro* plant cultures are generally considered as aseptic since surface decontamination of plant explants were always carried out prior to their transfer to *in vitro* conditions. Likewise, ornamental plants like fraser photinia (*Photinia* × *fraseri* Dress) were surface decontaminated and then micropropagated by not only research institutes but also commercial laboratories in order to provide large amounts of plants to the market. However, persistent bacterial and fungal contaminations are often reported by these laboratories (Boxus and Terzi, 1988; Kunneman and Faaij-Groenen, 1988; Cassells, 1990). These contaminations can be epiphytic, endophytic or accidental contaminants (Stead *et al*., 1998). Among them, endophytic infections especially cause problems, as they may not be appeared at the culture establishment stage but emerge weeks later and persist through subsequent transfers (Thorpe and Harry, 1990). The presence of endophytic contaminants such as *Paenibacillus* spp., *Methylobacteria* spp., *Bacillus* spp., *Pseudomonas* spp., *Mycobacteria* spp. *Rhodopseudomonas* spp., and *Microbacterium* spp. has been highlighted in different woody plant species (i.e., hazelnut, sour cherry, Scots pine, poplar, and cherry, respectively) by several researches (Kamoun *et al*., 1998; Reed *et al*., 1998; Laukkanen *et al*., 2000; Pirttilä *et al*., 2000; Van Aken *et al*., 2004; Ulrich *et al*., 2008; Quambusch *et al*., 2014; Quambusch *et al*., 2016). Among them, one strain of *Paenibacillus* spp., which was isolated from poplar, had a growth promoting effect on seedling (Ulrich *et al*., 2008).

Despite the possible detrimental effects of pathogenic endophytic bacteria including reduced plant growth and rooting rates (Leifert and Cassells, 2001) and interfered with the somaclonal variation (Moreno-Vázquez *et al*., 2014), improvement of plantlet stress tolerance can also be achieved with either co-cultivation of plantlets with *Pseudomonas* sp. (Nowak *et al*., 1998) or occurrence of those bacteria in later subcultures (Ulrich *et al*., 2008) without inoculation. Moreover, it is well-known that sometimes these contaminants could have plant growth-promoting characteristics (PGPC) such as supplemention of nitrogen by atmospheric nitrogen fixation, production of siderophores for iron provision to the plant, solubilization of phosphorous, synthesis of plant growth

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regulators, antibiotics or enzymes (Davison, 1988; Patten and Glick, 1996).

In our laboratory, fraser photinia was 'aseptically' *in vitro* micropropagated on 4.4 µM 6-benzyladenine (BA) containing QL medium as previously described (Akdemir *et al*., 2010). The *in vitro* plants were cultured at 25 ± 2°C under irradiance of 36 µmol $m^{-2}$ $s^{-1}$ for 16 h photoperiod and subcultured every 4 weeks. Presence of putatively endophytic bacterium was detected visually in *in vitro*-grown microshoots approximately after 4 years of subculturing without any detrimental effects in plant health and vigor. Thus, the aims of this work concerned the isolation and characterization of the putatively endophytic bacterium with using standard biochemical and morphological tests together with evaluation of its possible plant growth promoting capabilities.

## Materials and Methods

### Isolation of Putatively Endopyhtic Bacterium Associated with Fraser Photinia

Putative endophytic bacterial strain was isolated from segments of visibly contaminated *in vitro*-grown plantlets collected directly from plant tissue culture. It was inoculated into Nutrient Broth (NB) and examined for bacterial growth at 30°C. After incubation of 7 days, visible bacterial growth emerged from the culture in NB medium. Then, the serial dilutions from the obtained bacterial culture were inoculated onto Mineral-Pepton-Yeast-Extract (MPYE) (Davidson *et al*., 1989), NB, Luria-Bertani (LB), Triptic Soy Broth (TSB), Azotobacter medium, Rhizobium medium (Atlas and Parks, 1997), MS (Murashige and Skoog, 1962) and QL (Quoirin and Lepoivre, 1977) agar plates. After 4 days, distinct morphotypes of bacterium were screened on the basis of colony color, shape and size. Each morphotype was purified by restreaking on fresh MPYE agar plate, which supplemented bacterial growth more than other tested media. Colonies were repeatedly restreaked on MPYE media until the colony morphology of bacterium reached homogenous. The purified isolate was stored at -80°C in 50% glycerol.

### Culture Conditions

Putative endopyhtic bacterium was grown in individual 250 mL flasks containing 100 mL MPYE medium at 30°C with or without shaking for 10–15 days until exponential growth phase. An aliquot was taken from each pure culture for evaluation of its phytostimulation, biofertilization, and hydrolytic activities together with biochemical properties.

### Identification of Endophytic Bacterial Strain

**Strain morphology, physiological characteristics, and biochemical tests:** Morphological and cultural characterization were performed on the basis of colony size, shape, color, margin, opacity, consistency, elevation, motility and gram staining, endospore, capsule staining. Catalase activity, carbon utilization from different carbon sources (fructose, sucrose, mannitol, trehalose, lactose, mannose, xylose, arabinose, cellobiose, and sorbitol), nitrate reduction, oxidation/fermentation (OF) and urease tests were determined by using the previously described methods (Smibert and Krieg, 1994). To determine the optimal temperature and pH during 15 days of incubation period, the bacterial isolate was inoculated into MPYE medium and continuously grown at various temperature scales (22, 30, 37, and 40°C) and pH (4.0, 4.5, 5.0, 5.5, 5.8, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10.0). After incubation, bacterial growth was estimated by OD measurement.

**TEM analysis:** Transmission Electron Microscopy (TEM) was used to investigate the photosynthetic membrane to identify if the bacterium was photosynthetic or not. In this procedure, the bacterial culture was incubated at 30ºC on MPYE liquid medium and then cell suspension was centrifuged at 7000 g for 10 min. After pellets were washed with liquid MPYE medium, cell suspension was pre-fixed with 0.1 M phosphate buffer [4% (v/v) formaldehyde, 2.5% (v/v) glutaraldehyde on equal volume] and incubated overnight at room temperature. Pre-fixed cells were washed with the same buffer for 15 min thrice. The fixed cell pellets were embedded in agar beads (3%), post-fixed with osmium tetroxide ($OsO_4$) in phosphate buffer for 1h at room temperature, rinsed with the same buffer for several times and dehydrated gradually by using different ethanol concentration. The thin sections on 200 meshes coated with Form Var grids were embedded in Araldite then stained with uranyl acetate and lead citrate and were photographed with TEM (LEO 906 E TEM, Germany).

**FAME analysis:** Bacterial isolate was cultivated at 30ºC on TSA for 10 days and obtained colonies used for fatty acids and its methyl esters (FAME) analysis according to the method described in the manufacturer's manual (Sherlock Microbial Identification System, version 6.0, MIDI, Inc., Newark, DE, USA). Gas chromatography (GC, HP6890, Hewlett Packard, USA) analysis with a fused-silica capillary column (25 m x 0.2 mm) was utilized to separate FAMEs. The operating parameters controlled automatically by computer program. TSBA 60 database with the MIS software package was used to identify FAME profile of the bacterium.

**Antibiotic susceptibility test:** Antibiotic resistance of the putatively endophytic bacterium was determined according to the agar diffusion method described by Bauer *et al.* (1966) by using MPYE agar. 20 different classes of antibiotics, i.e., Gentamycin (10 µg), Meropenem (10 µg), Cefepime (30 µg), Ciprofloxacin (5 µg), Ampicillin (10 µg), Aztreonam (30 µg), Erythromycin (15 µg), Ampicillin-sulbactam (20 µg), Rifamycin (30 µg), Levofloxacin (5 µg), Nitrofurantoin (300 µg), Chloramphenicol (30 µg), Polymixin B (300 U), Nalidixic Acid (30 µg), Meticillin (5 µg), Vancomycin (30 µg), Co-trimoxazole (25 µg),

Streptomycin (10 µg), Spectinomycin (100 µg), and Tetracycline (30 µg) were used. In order to verify the antibacterial effect of the antimicrobial test discs, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as control strains. All discs were obtained from Bioanalysis (USA) and the sensitivity of putatively endophytic bacterium was determined according to the manufacturer's recommendation.

**Screening for Biofertilization Activities**

Biofertilisers are known to fix nitrogen, solubilize phosphate and/or produce siderophore as those microbes increase the supplementation of these nutrients to plant utilization (Fuentes-Ramirez and Caballero-Melado, 2006). Therefore, in the present study we investigated the phosphate solubilization activity, nitrogen fixation property and siderophore production of the putative endophytic bacterium. All experiments were performed in triplicate.

**Nitrogen fixation:** Nitrogen fixation was analyzed by acetylene reduction assay (ARA) (Postgate, 1978). Nitrogenase activity of bacterial strain was examined by inoculating 8 x $10^6$ cfu $mL^{-1}$ bacteria into 15 mL vials containing 9 mL liquid nitrogen free broth (NFB). A rubber stopper was used to seal the vials and 1 mL headspace gas sample was quantified by GC (Perkin Elmer Clarus 580) for ethylene ($C_2H_4$) production after 10 days of incubation at 30°C. Then, 1 mL of acetylene gas was injected to the headspace air (10 mL). The vials were incubated for 1 day at 30°C. Reduction of acetylene to ethylene was measured with GC. *Azospirillum brasilense* SP7 was utilized as positive control.

**Phosphate solubilization:** Phosphate solubilization was measured by the methods of Sylvester-Bradley *et al*. (1982). For this purpose, putatively endophytic bacterium was cultivated in Glucose-Yeast (GY) medium. After 10 days of cultivation at 30°C, the solubilization of phosphate was determined with occurrence of halos surrounding the colonies. Both colony and phosphate solubilization diameters were measured and the colony diameter was subtracted from the total halo solubilization diameter to calculate solubilization halo. Positive control was made with *P. aureginosa* strain in similar culture conditions.

**Siderophore production:** Siderophore secretion by this strain was detected by the Chrome Azurol S (CAS) assay according to the Schwyn and Neilands (1987). Orange halos around the colonies on blue agar were indicative of siderophore excretion. Positive control samples were made with *P. aureginosa* and *E. coli* in similar culture conditions.

**Screening for hydrolytic activity:** Starch hydrolysis on starch plates and protein hydrolysis on skim milk were tested according to Claus (1988), while gelatin degradation of the bacterium was carried out by using standard protocol (Biling, 1970). Lipid hydrolysis was analyzed by the method of Leboffe and Pierce (2005). Experiments were performed in triplicate. Bacterial cultures were streaked on the medium and incubated at 30°C for 10–15 days. A clearing zone in the medium indicated positive enzyme activity.

**Production of Plant Growth Regulator for Phytostimulation**

**IAA production:** IAA production was measured according to Sheng *et al*. (2008). Briefly, isolated bacterial strain was grown in DYGS medium supplemented with L-tryptophan as a precursor. After 10 days of incubation, a 1 mL cell suspension was mixed with 2 mL Salkowski's reagent and was allowed at room temperature for 30 min. The cells were pelleted by centrifugation at 10.000 g for 5 min. The supernatant was collected and absorbance was measured at 540 nm by UV-Visible spectrophotometer. Experiments were performed in triplicate. The IAA concentration was determined using a calibration curve of pure IAA as a standard following the linear regression analysis.

**$GA_3$ production:** To quantify the production of $GA_3$ by the putatively endophytic bacterium, the isolate was grown in MPYE liquid medium in exponential growth phase as defined in "Culture Conditions". After 10 days of incubation, amount of $GA_3$ present in the supernatant of the culture was determined by a modified method of Tien *et al*. (1979). $GA_3$ in the ethyl acetate phase was measured by UV spectrophotometer at 254 nm. The amount of $GA_3$ was calculated from the standard curve.

**16 S rRNA Gene Amplification, Sequencing and Phylogenetic Analysis**

Putatively endophytic bacterium was inoculated in MPYE liquid medium and incubated at 30ºC for 10 days. Genomic DNA extraction was performed by using Promega Wizard® genomic DNA purification kit (Madison, USA). The genomic DNA was visualized on agarose gel and quantified by UV spectrometer on 260 nm wavelength (Shimadzu Biotech, BiospecNano spectrometer). 16S rRNA PCR was carried out by using universal primer pairs [27F (5′ - AGAGTTTGATCCTGGCTCAGA - 3′ and 1491R 5′ ACGGCTACCTTGTTACGACTT - 3′)]. The PCR mixture was prepared with 1X Taq buffer, 3 mM $MgCl_2$, 0.5 µg template DNA, 200 µM dNTP's mix, 0.5 µM F - R primers and 1.25 u/µL Go Taq Flexi polymerase (Promega) in a final volume of 50 µL. PCR (MJ Research, PT-20, USA) programme was designed as 95ºC for 5 min and 30 cycles of 95ºC for 1 min, 58ºC for 1 min, 72ºC for 1,5 min and final extension at 72ºC for 10 min. The PCR amplification products were analyzed on agarose gel electrophoresis and purified by using Macherey-Nagel Nucleo Spin Extract II kit. DNA fragments sequenced with ABI 3130XL Genetic Analyzer by using Bigdye Cycle Sequencing kit v.3.1. Similarity of partial 16S rRNA gene nucleotide sequences with both nucleotide (nr/nt) and reference RNA sequence (refseq_rna) databases in the NCBI gene bank determined with using BLASTN 2.2.28+ (Basic Local Alignment

Search Tool) (Zhang *et al.*, 2000). The phylogenetic tree showing the similarity results of the bacterium and related strains was constructed by MEGA7 software (Kumar *et al.*, 2016) with neighborhood joining clustering parameter. In addition, overview phylogenetic tree was also constructed in order to predict the order of the isolated bacterium by using the same software. For this purpose, 16S rDNA sequences of different bacteria were taken from "Ribosomal Database Project". Moreover, similarity was also compared with the sequences existed in the same database (Cole *et al.*, 2009). The partial 16S rRNA gene sequence data generated in the present study has also been deposited with the NCBI Genebank under the accession number JQ715617.

### Influence of Bacterium on *in Vitro* Proliferation of Fraser Photinia

For uncontaminated controls, *in vitro* cultures were initiated from apical buds that were excised from fraser photinia plants located on fields of Gebze Technical University, Kocaeli, Turkey. 70% (v/v) ethanol (15 sec) was used for surface sterilization of excised apical buds followed by 1,5% (v/v) 'Domestos' (2% available chlorine, 20 min) treatment for disinfection. Then, buds were rinsed in sterile distilled water ($dH_2O$) at least 3 times and cultured on 15 mL of semi-solid QL medium containing 4.4 µM BA. Uncontaminated and contaminated cultures were kept at 25°C under a 16-h photoperiod of 36 µmol $m^{-2}$ $s^{-1}$ irradiation for 4 week as standard culture conditions. Following 4 weeks of culture, contamination frequency (%), shoot proliferation frequency (%), mean number of shoot proliferated per explant and mean shoot length were scored for controls. Then, apical buds were excised from both uncontaminated control and visibly contaminated with putative endophytic bacterium plantlets and transferred to plant growth regulator free QL medium (QL0) or 4.4 µM BA containing semi-solid QL medium in order to reveal influence of putative endophytic bacterium on *in vitro* fraser photinia proliferation.

### Data Collection and Statistical Analysis

At least 50 apical buds were used for each proliferation treatment and each experiment was repeated at least twice. Data were recorded 4 weeks after culture initiation and consisted of: i) the percentage of apical buds that visibly showed bacterial and/or fungal contamination, ii) the percentage of apical buds that proliferated at least one elongated microshoot, (iii) the average number of microshoots formed per proliferating explant and (iii) the average length of elongated microshoots.

Percentage data were subjected to the Post Hoc Multiple Comparisons Test (Marascuilo and McSweeney, 1977) while ANOVA followed by the least significant difference (LSD) test ($P \leq 0.05$) was used to compare means of discrete data for statistical analysis.

## Results

### Isolation and Characterization of Putatively Endophytic Bacterium

When the bacterial dilutions were cultured on different agar plates as mentioned in "Materials and Methods" section for 15 days at 30°C, bacterial growth was better in MPYE medium than the other tested media. Moreover, since bacterium reached its early exponential phase in MPYE broth after 3–5 days of incubation time, it can be regarded as a slow-growing one. The strain grew at 22–34°C. From multiple experiments, the average optimal temperature for growth was found to be 30°C. The strain grew between pH 5.8 and pH 10.0 with an optimum of pH 7.0.

The evaluation of biochemical characteristics of the isolated strain showed that it was positive for catalase production and nitrate reduction but negative for urease production, protein, starch, lipid and gelatin hydrolysis (Table 1). The strain was also able to poorly utilize carbonhydrates D-glucose and sucrose while mannose, lactose, trehalose, mannitol, sorbitol, fructose, arabinose, xylose and cellobiose were not utilized as energy sources. The isolated bacterium was found to be aerobic but can survive in the anaerobic conditions and grew well either with or without agitation. Therefore, this isolate was not strictly aerobe.

*In vitro* antibiotic susceptibility tests showed varying sensitives of bacterium to streptomycin (10 µg), tetracycline (30 µg), spectinomycin (100 µg), polimyxin B (300 U), erythromycin (15 µg) and nalidixic acid (30 µg). However, it was resistant to all other tested antibiotics.

### Phenotypical Identification and Microscopic Examination of the Bacterium

Microscopic examination of the bacterium revealed irregular rod-shaped cells with small opaque and circular colonies on MPYE agar plated after 10 days of incubation at 30°C (Fig. 1). Bacterial cells were stained as Gram-negative, non-motile and occurring singly or in pairs. No capsule or spore formation was observed but β-polyhydroxybutyrate production and intracellular polyphosphate accumulation occurred. Photosynthetic membrane was not visualized by TEM (Fig. 2).

### Chemotaxonomic Characterization

With extraction of whole-cell fatty acids and analysis of their methyl esters by GC revealed that $C_{18:1}w7c$ constituted the main fatty acid component (77.28% of the total content, Fig. 3). $C_{16:0}$ (6.61%) also constituted a significant proportion of the total. $C_{18:0}$ (4.61%), $C_{17:0\ iso}$ (3.68%), $C_{19:0}$ cyclo$w8c$ (3.57%), $C_{17:0}$ (2.62%), $C_{18:1}w9c$ (1.63%) constituted other minor fatty acids.

**Table 1:** Plant growth promotion (PGP) features of putatively endophytic bacterium

| PGP | Endophytic Bacterial Isolate |
|---|---|
| *Phytostimulation*[a] | |
| IAA | + |
| $GA_3$ | + |
| $C_2H_4$ | - |
| *Biofertilization*[b] | |
| $N_2$ fixation | + |
| $Ca_3PO_4$ | - |
| SID | - |
| *Hydrolytic activities*[c] | |
| GEL | - |
| LIP | - |
| AMY | - |
| PROT | - |

[a]IAA indole-3-acetic acid; $GA_3$ gibberellic acid; $C_2H_4$ ethylene production
[b]$Ca_3PO_4$ inorganic calcium phosphate solubilization; SID siderophore production
[c]GEL gelatinase; LIP lipase; AMY amylase; PROT protease activity

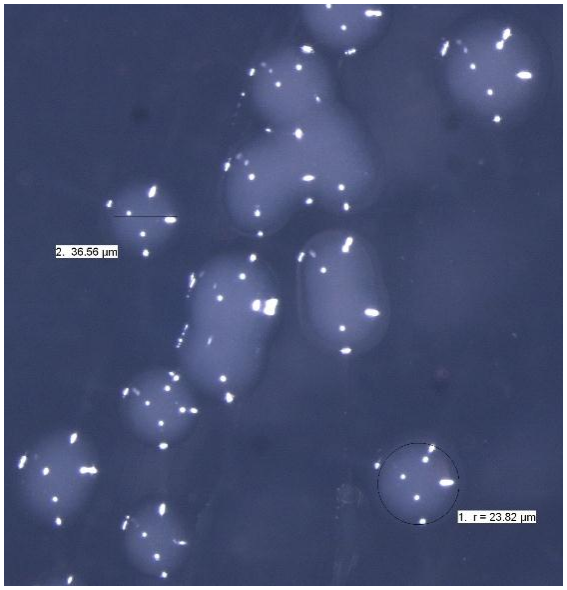


**Fig. 1:** Representative microscopic visualization of the plant growth promoting bacterium

With this FAME profile, the bacterium showed the highest similarity to *Ochrobactrum anthropi* (Achromobacter Vd, CDC group Vd) and *Phyllobacteria myrsinacearum* with 0,642 and 0,417 similarity rate, respectively according to TSBA 60 database. However, those similarity rates were not high enough to classify endophytic bacterium under these genera.

**Characterization of Potential Plant-beneficial Traits**

The isolate was analyzed for its plant growth promoting activities such as phosphate solubilization, IAA, $GA_3$ and siderophore production. The evaluation of PGPC of the isolated strain revealed that it produced IAA (10 µg $mL^{-1}$) and $GA_3$ (29 µg $mL^{-1}$) (Table 1); however, it neither

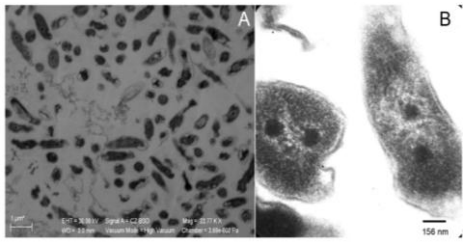


**Fig. 2:** TEM analysis of the putatively endophytic bacterium. (A) Zeiss SEM/TEM microscopy (22700X); (B) LEO 906 E (77500X)

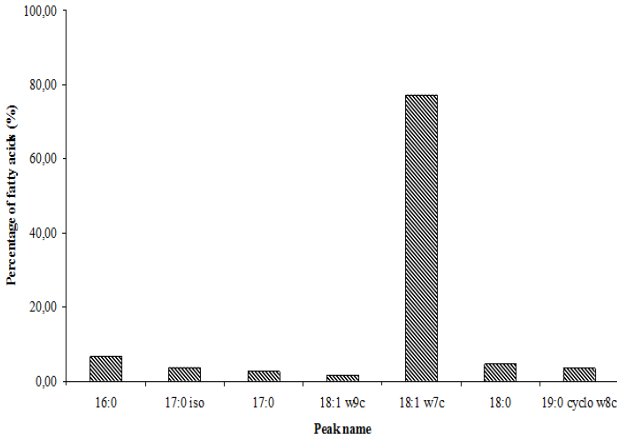


**Fig. 3:** FAME analysis of the bacterium

solubilized phosphorous nor produced siderophores. Moreover, the bacterium grew in NFB media and had nitrogenase activity that was detectable with ARA, which confirmed that it had ability to fix nitrogen. Thus, the bacterial strain isolated from the tissue culture of fraser photinia has a multitrait PGPC that exhibited nitrogen fixation and production of IAA and $GA_3$.

**Molecular Identification of Bacterium**

936 bases were sequenced and the most similar one was found to be *Pseudorhodoplanes sinuspersici* strain C16-RIPI 110 (JX500274.3) isolated from oil contaminated soils according to the phylogenetic tree, which was constructed with neighborhood joining parameter (Fig. 4). The majority of the other similar ones belonged to uncultured bacteria (KF494612.1, DQ123778.1, KF494607.1, EF219026.1, AF407716.1, JN217184.1 and LN565571.1 according to NCBI accession numbers). There were three hits that belonged to Proteobacteria and Rhizobiales (DQ123778.1, DQ123619.1, and EF219026.1). Also, three of them were related to permafrost environments (KF494612.1, KF494607.1, and AB630417.1).
In addition, isolated bacterium showed 96% similarity to *Rhodoplanes* sp UBH9 according to reference database hits. Moreover, the partial 16S rRNA sequence

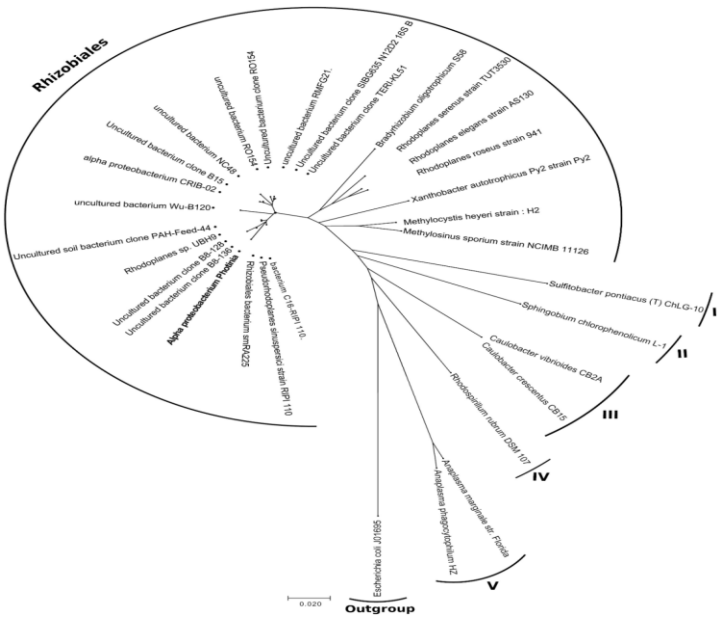


**Fig. 4:** Neighbor-joining phylogenetic tree based on bacterial 16S rRNA sequences of the isolate. *E. coli* was used as an outgroup. The scale bar shows the number of nucleotide substitutions per site. I: Rhodobacterales, II: Sphingominodales, III: Caulobacterales, IV: Rhodospiralles, V: Rickettsialles

**Table 2:** Influence of bacterium on *in vitro* proliferation of fraser photinia[a]

| *In vitro* cultures | Proliferation[b] (%) | Microshoot/explant[cd] | Microshoot length[cd] (mm) |
|---|---|---|---|
| *QL0* | | | |
| Control | 92ab | 1.00±0.00c | 4.00±0.51b |
| Contaminated | 80b | 1.00±0.00c | 3.27±0.53b |
| *QL + 4 µM BA* | | | |
| Control | 100a | 2.20±0.10b | 4.45±0.33b |
| Contaminated | 100a | 3.80±0.10a | 10.90±0.50a |

[a]Data were collected after 4 weeks of in vitro culture. Values represent the averages as determined from at least 50 explants and experiment was repeated at least twice

[b]Percentages followed by the same letter at each column are not significantly different at $P \leq 0.05$ by the Post Hoc Multiple Comparisons Test

[c]Mean± standard error

[d]Means followed by the same letter at each column are not significantly different at $P \leq 0.05$ by the ANOVA, followed by the LSD test

showed that the bacterium was also belong to Proteobacteria phylum under Alphaproteobacteria class under Rhizobiales order according to "Ribosomal Database Project" (Cole *et al*., 2009) (Fig. 4).

### Beneficial Influence of Bacterium on *in vitro* Proliferation of Fraser Photinia

40% of different bacterial and fungal contamination was detected from apical buds excised from *in vivo*-grown fraser photinia plants after 4 weeks of *in vitro* culture. Following the initiation of uncontaminated control *in vitro* cultures, apical buds were excised both from these microshoots and putatively endophytic bacterium contaminated ones and cultured on QL0 or 4.4 µM BA containing QL medium. After 4 weeks of *in vitro* incubation, 92% and 80% of proliferation were scored in QL0 medium in control and contaminated apical buds, respectively (Table 2). There was no significant difference in means of multiple microshoot formation or average microshoot length was recorded with the presence of bacterium. On the contrary, a beneficial influence with the presence of bacterium was evident in mean number of microshoots per explant and microshoot length as the maximum multiple shoot formation together with the highest microshoot length were obtained when the medium was supported with cytokinin.

## Discussion

There are a few studies on the determination of endophytic bacteria in *in vitro* plant tissue cultures (Almeida *et al*., 2009; Dias *et al*., 2009; Abreu-Tarazi *et al*., 2010; Moraes *et al*., 2012) as it is assumed that cultures without visible symptoms are bacteria free (Moreno-Vázquez *et al*., 2014). However, those cultures have great potential to isolate beneficial bacteria that resides within plant specific organs. Thus, it seems that an important proportion of plant micropropagation studies are carried out without knowledge of microbial presence (Abreu-Tarazi *et al*., 2010). Accordingly, this is the first report about the isolation and molecular characterization of a putatively endophytic bacterium that has potential to promote fraser photinia growth associated with its tissue culture. This isolated strain showed the highest similarity to *O. anthropi* and *P. myrsinacearum* (0.64 and 0.41,

respectively), according to major cellular fatty acid profiles. Although strains with a similarity index (SI) of 0.500 or greater are considered as a good match at the species level according to literature provided by MIDI (Sherlock Microbial Identification System, version 6.0, MIDI, Inc., Newark, DE, USA), 0.64 SI still was not high enough to classify putative endophytic bacterium under *Ochrobactrum* genus. Moreover, even though the results of some biochemical tests were common (i.e., Gram staining and catalase reaction), various biochemical characteristics (i.e., motility, oxidase, different sugar utilization as carbon sources) were strikingly different.

known as a predominant group of endophytic bacteria that have beneficial influences (i.e., growth promotion and plant pathogen antagonism) (Dias *et al*., 2009) on host plants. Moreover, the delayed detection of the bacterium in *in vitro* cultures of fraser photinia and the requirement Although genotypic identification by 16S rDNA sequencing had not showed a very high similarity to known bacterial species, the most similar cultures are belonging to Alphaproteobacteria or uncultured bacteria. Proteobacteria phylum is of relatively long period for optimum growth also supported and suggested the possibility of this isolate to be originally a viable but non-culturable (VBNC) bacterium. Even though the factor(s) that involved to the activation of VNBCs to cultivation have not been well-understood (Thomas *et al*., 2008), medium pH alterations, release of tissue breakdown products, and exposure of internal tissue during subculturing might have influences (Thomas *et al*., 2004 a,b; Thomas *et al*., 2006). Originally, unculturable endophytic bacteria were also isolated from *in vitro* cultures of papaya (Thomas, 2007), banana cv. Grand Naine (Thomas *et al*., 2008) and pineapple (Abreu-Tarazi *et al*., 2010) with prolonged culture. Thus, it appeared that an important proportion of *in vitro* studies are undertaken unawares of microbial presence (Thomas *et al*., 2008). Therefore, it is important to consider also the influence of endophytic bacteria on plant tissue cultures for development of better strategies on tissue culture associated issues. Moreover, this study also supported the phenomenon proposed by Thomas and co-workers (2006; 2008) that emphasize the influence of plant tissue culture system in obtaining some less common and/or novel endophytic organisms with generating the VBNC cells to cultivation.

The influence of this bacterium on fraser photinia microshoots could be associated with the possibility of supplying carbon to plant as *in vitro* plants are grown under exogenous supply of carbon source to medium (Pospóšilová *et al*., 1999). However, TEM visualization showed that this isolate was not a photosynthetic organism since it did not have internal photosynthetic membrane. Thus, other possible plant growth promoting factors including siderophore production, nitrogen fixation, phosphate solubilization and phytohormone production were investigated. Although siderophore production and phosphate solubilization were not detected, the bacterium was able to fix nitrogen and reduce nitrate. It is known that nitrogen-fixing endophytic bacteria that are present inside the plant tissues supply fixed nitrogen directly to the plant in comparison to its rhizospheric counterparts (Gupta *et al*., 2012).

In tissue culture medium, prolonging of subculture period results in decrease of pH of the medium and it leads the stimulation of nitrate uptake instead of ammonium, causing of pH to rise (George, 1993). Presence of the nitrate reduction ability of the bacterium may help plantlets to metabolize nitrate containing chemicals of the medium in the late periods of tissue culture. In addition to nitrogen-fixation and nitrate reduction ability, the bacterium may show beneficial influence through phytohormones. In accordance with this, our results showed that this isolate had the capacity to produce IAA (10 µg $mL^{-1}$) and $GA_3$ (29 µg $mL^{-1}$). This finding was compatible with literature as it was known that endophytes can produce phytohormones to promote plant growth (Mendes *et al*., 2007). Moreover, a beneficial influence of presence of this isolate on *in vitro* proliferation of fraser photinia was also detected in cytokinin enriched medium. These results clearly showed that putatively endophytic bacterium had no detrimental influence on micropropagation of fraser photinia. Besides, phytohormones produced by the bacterium and exogenously applied cytokinin had a synergistic beneficial influence on *in vitro* proliferation of this ornamental plant.

Last but not least, it was known that some bacterial endophytes may also contribute to their host plants by producing antimicrobial compounds that provide protection and increase survival rate of the plant (Yu *et al*., 2010). In accordance with this, the putative endophytic bacterium endowed with the intrinsic antibiotic resistance (IAR) could help its host plant to encounter with stress situations. Moreover, it was observed that the bacterial isolate was catalase positive which was important for not only bacterium to reproduce by avoiding cellular toxicity but also plant as it can help to struggle with oxidative stress caused by tissue culture conditions. Overall, plant growth promoting features of the bacterium and its positive influence on fraser photinia growth under *in vitro* conditions suggested that this strain is promosing to be developed as a bio-inoculant for agricultural applications.

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