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ORIGINAL ARTICLE

The association of fraser photinia and its beneficial bacterium (PGB_invit) provided in vitro storage without subculture

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Abstract

Endophytes play crucial roles due to their beneficial influence on plant development, growth, fitness, and diversification. Due to these important capabilities, they have received attention from the scientific community and many papers have been published recently about their beneficial role in in vivo and in vitro plant propagation. However, up to now, there is no research on utilization of these microbial endophytes in prolongation of in vitro storage. Thus, the aim of this study is to assess the influence of fraser photinia associated and putatively endophytic bacterium (Plant Growth Bacteria_ in vitro; PGB_invit) on in vitro storage of its host. When pure strain of the bacterium was inoculated, it enabled the storage of microshoots up to 16 months at 25 °C without requiring periodic subculture while control (unincubated with PGB_invit.) microshoots died after 2 months of storage without subculture as in vitro plant cultures definitely need periodic subcultures (once in every 4–6 weeks) in order to renew media and gaseous atmosphere. Moreover, while the presence of virulence (*vir D1*), auxin (*aux1*), and cytokinin (*ipt)* production genes was confirmed in plasmid DNA of the bacterium, nitrogen fixing gene (*nifH*) was detected by the PCR analysis using bacterial culture. Overall results demonstrated that with these capabilities PGB_invit could be useful for in vitro conservation of fraser photinia.

Key message

The novelty is the supplementation of in vitro plant growth without either periodic renewal of the media or decreasing the culture temperature by means of a beneficial plant-bacterium interaction.

Keywords *Aux1* · Endophytic · *Ipt* · *NifH* · Plant growth promoting bacterium

Introduction

Microbial endophytes (bacterial, archaeal, fungal, and protistic taxa), date back more than 400 million years (Remy et al. 1994), are considered as extremely important plant partners (Hallman et al. 1997) as they live intercellularly and/or intracellularly in host without causing any apparent disease (Wilson 1995; Nair and Padmavathy 2014; Hardoim et al. 2015). Besides, it may affect the growth of their host plants positively, (i) by producing some plant growth promoting (PGP) regulators such as auxin and cytokinin (Ryan et al. 2008; Hardoim et al. 2008; Jimtha et al. 2014), (ii) promoting plant disease resistance against many potential plant pathogen by not only increasing expression of defense-related genes in plants (Benhamou et al. 1996; Gond et al. 2015; Cabanas et al. 2014), but also synthesis and modulation of bioactive compounds that have potential to be used in medicine, agriculture or industry (Jasim et al. 2015; Singh et al. 2017; Patle et al. 2018), (iii) supplying tolerance to abiotic stress (Vigani et al. 2018). Moreover, endophytes have also been shown to increase nutrients uptake such as nitrogen or phosphorous into plants (Boddey et al. 1991; James 2000; Iniguez et al. 2004; Malboobi et al. 2009). Above all, they also have positive effect on in vitro proliferation of different plants (i.e., Pirtilla et al. 2000; Dias et al.

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2009; Quambusch et al. 2014). Those beneficial influences of endophytic bacteria together with their potential role in agro-ecosytems have also recently been reviewed by many authors (i.e., Mercado-Blanco 2015; Card et al. 2016; Santoyo et al. 2016; Patle et al. 2018; Akkaya et al. 2019) and seemed to be very promising hot topics of the plant microbiome studies (Azevedo et al. 2000; Schulz et al. 2002; Aly et al. 2010).

In accordance, a putatively endophytic beneficial bacterium, which will be nominated as PGB_invit, was isolated and characterized in in vitro grown microshoots of fraser photinia that has ability to fix nitrogen and produce some PGP regulators such as indoleacetic acid (IAA) and Gibberellic Acid (GA_3) in our previous study (Gul Şeker et al. 2017). More interestingly, it seemed to promote in vitro storage and proliferation of fraser photinia without routine subculture. Thus, this study was conducted to reveal out the influences of this PGB_invit on not only shoot storage and culture growth without subculturing up to 16 months but also rooting and acclimatization of in vitro proliferated shoots. Moreover, the presence of PGP genes including auxin (*aux 1*), cytokinin (*ipt*) and nitrogen fixation (*nif H*) together with virulence gene (*virD1*) was also assessed to understand the molecular interactions between photinia and PGB_invit. Although the beneficial influences of endophytic bacterium have been studied for many decades, to our knowledge, this is the first paper that demonstrates the potential role of endophytic bacteria on providing in vitro storage in 25 °C without the need of renewal of macro and microelements, carbon source and PGP regulators in economic and eco-friendly manner.

Materials and methods

In vitro culture conditions

Shoots of fraser photinia (*Photinia* × *fraseri* Dress.) were subcultured monthly to fresh MS medium containing 4.4 µM 6-benzyladenine (BA) according to Akdemir et al. (2010) until contamination of PGB_invit was visually detected in the medium.

Influences of PGB_invit on in vitro storage of fraser photinia microshoots

As no detrimental effect of bacterium was determined on 4 weeks PGB-invit incubated in vitro fraser photinia cultures (Gul Şeker et al. 2017), visibly contaminated microshoots were maintained (without periodic subculturing on fresh medium) in QL medium (Quoirin and Lepoivre 1977) containing 4.4 µM BA for different storage periods (6, 9, 12, 15, 16 months) at 25 ± 2 °C with 16 h photoperiod under 36 µmol m^{-2} s^{-1} photosynthetic photon flux provided by cool-white fluorescent lamps. Control cultures (without bacteria) were also maintained in the same conditions without subculture. The percentage of green shoots, average length of the microshoots, presence–absence of roots, average number of roots per microshoots, average length of the roots, dry-fresh weight was assessed together with the number of both green and abscised leaves per microplant in order to reveal the plant quality during storage. Dry weight of the shoots was determined by drying a batch of microshoots (a minimum of 20) in an oven at 80 °C, weighting the shoots every 4 h until two successive weights gave the same value. Moreover, leaf senescence index was also calculated per microplant according to $\sqrt{x/\sqrt{y+0.5}}$ formula in which "x" represents the number of green leaves per microplant whereas "y" represents senescence plus abscised leaves per microplant (Sarkar et al. 1999). In addition, visual preference scale from 0 to 3 was also scored based on plant appearance: 0-dead plant; 1-microshoots were brown, in some places green; 2-microshoots were green–brown; 3-microshoots with bright green leaves and stems (modified from Sarkar and Naik 1998).

Influences of PGB_invit on shoot retrieval after conservation

Shoot apices were excised from in vitro conserved microshoots, which were contaminated, and transferred to fresh 4.4 µM BA containing QL medium in order to assess shoot retrieval after conservation. The percentage of shoot apex that regenerated at least one elongated shoot, the average number of shoots proliferated per explant and the average length of the shoots were evaluated after 4 weeks in culture. In addition, the shoot forming capacity (SFC) index (Lambardi et al. 1993) was also calculated based on the formula with using the average number of shoots per proliferating explant × percentage of proliferating explants/100.

Influences of PGB_invit on rooting and acclimatization

A pool of elongated microshoots (at least 1–1.5 cm long) from control and contaminated were transferred to semi-solid QL medium supplemented with various concentrations (0.49, 2.46, 4.92 µM) of indole butyric acid (IBA) for rooting. After 7-days of culture, half of the shoots were transferred to PGR-free QL medium. Microshoots that had at least 0.2 cm root were considered as rooted. The date of the first root emergence in each experiment was recorded in order to calculate average days of rooting time. Rooting time was calculated according to formula $\sum (N_x T_x)/n°$ of

rooted shoots where N_x is the n° of rooted shoots within consecutive intervals of time; T_x is the n° of days between the beginning of the test and the end of the specific interval of time. Moreover, average number of roots per shoot and length of the root/microshoot were determined after 30 days of culture.

In order to acclimatize to in vivo conditions, rooted shoots from both control and contaminated cultures, that were stored for 12 months at 25 °C, were rinsed with tap water and transferred to pots containing peat and perlite (1:1). Pots were covered with a polyethylene bag to maintain high relative humidity and placed in a culture room for 4 or 5 weeks. Three holes (less than 1 mm) were opened after 2 days and doubled each day. After 4–5 weeks, the plants were transferred to bigger pots in greenhouse conditions for their further growth.

Genomic and plasmid DNA isolation of PGB_invit

PGB_invit was inoculated in MPYE liquid medium and incubated at 30 °C for 10 days. Genomic DNA extraction was performed by using Promega WizardR Genomic DNA Purification Kit (Madison USA). Plasmid isolation was carried out with Macherey–Nagel Nucleospin Plasmid Kit. The isolated DNAs were visualized on 1.5% agarose gel electrophoresis along with 1 kb + 100 bp DNA ladder (Invitrogen Cat. No. 10787-018) as size marker and quantified by UV spectrometer on 260 nm wavelength (Shimazu Biotech, BiospecNano Spectrometer).

PCR analysis

In order to assess whether PGB_invit is producing auxin or cytokinin, *aux*1 (tryptophan monooxygenase) and *aux2* (indoleacetamide hydrolase) genes, responsible for auxin biosynthesis, and *ipt* (isopentenyl transferase) gene, responsible for cytokinin biosynthesis were amplified using the following primers: *aux1*-FW (5′-CTCCGATTCCTTTCC AACCG-3′) and *aux1*-RV (5′CGCACGTTATCCTCATAC CC-3′), *aux2*-FW (5′-CTGTCAACGGAGGCTGTTGGG-3′) and *aux2*-RV (5′ACCCTAGTCTCATCCCAGGG-3′) (Camilleri and Jouanin 1991) and *ipt*-FW (5′-GATCG(G/C) GTCCAATG(C/T)TGT-3′) and *ipt*-RV (5′-GATATCCAT CGATC(T/C)CTT-3′), respectively according to Haas et al. (1995). *Rhizobacterium rhizogenes* (*Agrobacterium rhizogenes*) ATCC 15834 for *aux* genes and *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) ATCC 15955 for *ipt* gene were used as positive controls in PCR. The reaction mixture contained 2.5 × PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 50 nanograms plasmid DNA, 1 U Taq DNA polymerase (i-Taq™, Intron) and 0.4 µM for both forward and reverse primers. PCR conditions for *aux* genes was 94 °C for 2 min pre-denaturation; 30 cycles of 94 °C for 15 s denaturation, 60 °C for 30 s annealing, 72 °C for 1 min extension and 72 °C for 10 min final extension steps. PCR program for *ipt* gene was 94 °C for 2 min predenaturation; 30 cycles of 94 °C for 15 s denaturation, 55 °C for 30 s annealing, 72 °C for 1 min extension and 72 °C for 10 min final extension steps. PCR products except were visualized on 1.5% agarose gel electrophoresis along with 1 kb DNA Ladder (Intron 24074).

To identify whether the bacterium has gene transfer capability like *Agrobacterium* Ti plasmid, we tried to amplify *virD1* gene region. The primer pairs were *virD1*-FW (5′-ATGTCGCAAGGCAGTAGGCCCACCT-3′) and *virD1*-RV (3′-CTACAAGGCGTCTTTCAGCAGCGAGC-5′) (Rogorowsky et al. 1990). The PCR mixture for *virD1* amplification contained 1 µl plasmid DNA as template, 5 µl Solis BioDyne 5x FIREPol Master Mix, 0.2 µM from each primer in a final volume of 25 µl. PCR conditions was 95 °C for 5 min pre-denaturation and 40 cycles of 95 °C for 1 min denaturation, 55 °C for 2 min annealing, 72 °C for 2 min extension and 72 °C for 10 min final extension steps. With biochemical tests, we observed that the PGB_invit has the ability to fix nitrogen (Gul Şeker et al. 2017). To verify this, the presence of *nifH* gene was assessed by using *nifH* forward (5′-TGCGAYCCSAARGCBGACTC-3′) and reverse (3′-ATSGCCATCATYTCRCCGGA-5′) degenerated primers. The PCR mixture contained 1 µl bacterial culture as template, 5 µl Solis BioDyne 5x FIREPol Master Mix, 0.2 µM from each primer in a final volume of 25 µl. PCR programme was 95 °C for 5 min predenaturation and 40 cycles of 95 °C for 1 min denaturation, 58 °C for 2 min annealing, 72 °C for 2 min extension and 72 °C for 10 min final extension steps. *virD1* and *nifH* PCR products were visualized on 1.5% agarose gel electrophoresis along with 1 kb and 100 bp DNA Ladders (Thermofisher).

Carbohydrate and alditols analysis

PGB_invit was grown in MPYE medium for 10 days. Then, bacterial culture was centrifuged at medium speed (8000 rpm) for 5 min at RT, and the supernatant was used for the determination of carbohydrate and alditols including mannitol, inositol, and sorbitol by HPLC method according to Agilent's protocol (https://www.agilent.com). The flow rate of the isocratic elution was 600 µl/min, the sample injection volume was 5 µl, the MetaCarb 87P Carbohydrate Column Pb + column was used at ambient temperature (80 °C) and sample run time was 60 min. Mobile phase composed of Milli Q water.

Experimental design and statistical analysis

Each experiment concerning the biochemical and molecular characterization of the bacterium was repeated at least twice

whereas experiments regarding shoot proliferation, the number of shoots proliferated per explant, plant shoot growth, shoot retrieval and rooting were carried out with using at least 50 explants/microshoots and repeated at least thrice.

Statistical analysis of the non-parametric data (frequencies) was carried out by the test for homogeneity of proportions and significant treatment differences selected by a non-parametric statistical test: Post Hoc Multiple Comparison (Marascuilo and McSweeney 1977). Discrete data were subjected to analysis of variance (ANOVA), followed by the least significant difference (LSD) test at $P \leq 0.05$ to compare means.

Results

Influences of PGB_invit on in vitro storage of fraser photinia microshoots

It was possible to conserve fraser photinia microshoots at 25 °C with the bacterium up to 9 months without any decline in the percentage of green microshoots (Table 1) whereas control (unincubated with PGB_invit.) microshoots dies after 2 months of storage without subculture. However, with the prolongation of the conservation time, a significant decline in the green microshoot percentage was observed [95.5% for 12 months (Fig. 1a), 80.4% for 15 and 79.8%

Table 1 The influence of the PGB_invit on fraser photinia microshoot growth and rooting after storage

Parameters	Storage time at 25 °C (months)				
	6	9	12	15	16
Green microshoot (%)[ab]	100a	100a	95.5b	80.4c	79.8c
Microshoot lenght (mm)[bc]	11.5 ± 0.07b	12.9 ± 0.05a	8.84 ± 0.04c	11.5 ± 0.19b	10.0 ± 0.03c
Root formation (%)[ab]	58.3a	35.5b	32.2b	36.7b	14.7d
Root/microshoot[bc]	1.86 ± 0.23b	3.50 ± 0.87a	1.28 ± 0.10c	3.00 ± 1.08a	1.21 ± 0.11c
Root length (mm)[bc]	74.5 ± 1.08b	54.8 ± 0.78c	104.7 ± 2.32a	85.9 ± 1.22b	64.7 ± 1.03d
Fresh weight (g)[bc]	3.69 ± 0.09b	5.23 ± 0.57a	5.34 ± 0.28a	4.67 ± 0.32a	5.93 ± 0.94a
Dry weight (g)[bc]	0.79 ± 0.09b	1.00 ± 0.12a	1.00 ± 0.10a	1.02 ± 0.09a	1.04 ± 0.02a
Visual preference scale[d]	3	3	2	2	2

The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least 2 times

[a]The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$)

[b]The same letters following the means show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The mean difference was analyzed horizontally)

[c]Mean ± standard error

[d]The microplant growth was scored on a 0–3 visual preference scale in which: 0-dead plant; 1-microshoots were brown, in some places green; 2-microshoots were green–brown; 3-microshoots with bright green leaves and stems (modified from Sarkar and Naik 1998)

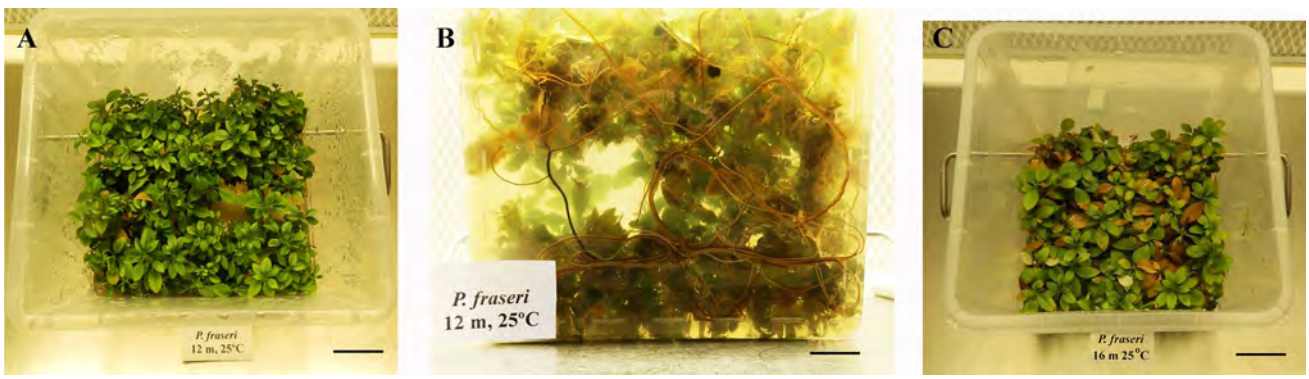

Fig. 1 Post-storage status of microshoots of fraser photinia containing PGB_invit stored under proliferation conditions (25 °C) together with PCR analysis of genes related with beneficial influence of bacterium. **a** The status of microshoots after storage for 12 months (bar = 1.2 cm). **b** Root formation in fraser photinia microshoots stored for 12 months (bar = 1.2 cm). **c** Healthy microshoots obtained after 16 months of storage (bar = 1.2 cm)

for 16 month]. There is a possibility to obtain about 80% green shoot after storage for 16 months at 25 °C without transferring the plants to fresh medium and not applying low temperature to reduce plant metabolism. After 6 months of conservation in vitro, root development was also occurred on microshoots. The rooted microshoots that were stored for 12 months (were shown in Fig. 1b) and rooted plantlets still could be observed in 16 month-stored microshoots although with a relatively lower percentage (14.7%).

No significant difference was observed in fresh and dry weight of the microshoots that were conserved up to 16 months. However, relatively lower fresh and dry weights (3.69 and 0.79, respectively) were measured in 6 months of conservation. As regard visual preference scale, although relatively lower amount of microshoots with bright green leaves and stems were observed with prolongation of storage, they seemed to be healthy with green–brown color (Table 1; Fig. 1a, c).

Influences of PGB_invit on number of green/abscised leaves per microshoot and leaf senescence

There was no significant influence of bacterium on number of green leaves obtained per microshoot (5.13 and 6.32) that conserved up to 16 months (Table 2). However, significantly higher number of abscised leaves and lower leaf senescence index were obtained with conservation of microshoots up to 15 and 16 months.

Influences of PGB_invit on shoot retrieval after in vitro storage

Although the proliferation of shoot apices excised from contaminated microshoots started to decline after conservation up to 12 months, more than 93% proliferation was obtained after 16 months (Table 3). Moreover, relatively higher number of microshoots was obtained with the prolongation of the storage time as maximum multiple shoot formation was scored with the tested longest storage period. So that the highest SFC index was obtained from shoot apices from microshoots that stored for 9 or 15 months. With increasing the storage time up to 16 months, lower SFC index (4.4) was

Table 2 The influences of PGB_invit on number of green/abscised leaves per microshoot and leaf senescence after storage of fraser photinia microshoots

Parameters	Storage periods (months) at 25 °C					
	6	9	12	14	15	16
Green leaves/microshoot[a]	6.32a	5.81a	5.13a	6.08a	5.15a	5.44a
Abscised leaves/microshoot[a]	0.58b	1.52ab	0.65b	0.90b	2.33a	2.19a
Leaf senescence index[b]	2.46a	2.02ab	2.18a	2.26a	1.74b	1.82b

Each treatment consisted of at least 50 explants and repeated at least twice

[a]Means followed by the same letter are not significantly different at $P \leq 0.05$ by the ANOVA, followed by the LSD test (i.e., mean significativity is per horizontal lines)

[b]Leaf senescence index of the microshoots were calculated according to $\sqrt{x}/\sqrt{(y+0.5)}$ formula

Table 3 The influence of PGB_invit on proliferation of shoot apices excised from in vitro-stored fraser photinia microshoots

Parameters	Storage time (months)				
	6	9	12	15	16
Proliferation (%)[a]	100a	98.9a	95.6b	98.8ab	93.3b
Microshoot/explant[bc]	4.40 ± 0.17a	3.85 ± 0.18b	3.51 ± 0.14b	4.42 ± 0.18a	4.44 ± 0.13a
Microshoot length (cm)[bc]	3.46 ± 0.14a	3.70 ± 0.15a	3.56 ± 0.01a	4.73 ± 0.14a	4.41 ± 0.14a
SFC index[d]	4.1	3.8	3.8	4.1	4.1

The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least 2 times

[a]The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$)

[b]The same letters following the averages show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The average difference was analyzed horizontally)

[c]Mean ± standard error

[d]SFC index of microshoots; It is calculated by multiplication the proliferation of the explant by the percentage of proliferating explants and divided by 100

obtained due to significantly lower proliferation percentage obtained in that storage period. Also, relatively longer shoots were obtained after 15 and 16 months (4.7 and 4.8 mm, respectively).

Influences of PGB_invit on rooting and acclimatization

Significant differences were obtained on root induction responses between control and contaminated microshoots cultured on different IBA concentration (Table 4). Maximum root formation (70%) was obtained with the tested highest IBA concentration (4.92 µM) in control microshoots whereas 66.6% of rooting was scored with 2.46 µM IBA enriched QL media in contaminated microshoots. Doubling the IBA concentration resulted in decline as 43.3% of rooting in contaminated microshoots. Although no significant differences were obtained in number of roots formed per control microshoot in response to different auxin concentrations, the lowest adventitious root formation per contaminated microshoots (1.2) was obtained with the tested lowest IBA concentration (0.49 µM). On the contrary, the longest roots were obtained in contaminated microshoots cultured on this medium.

Root formation was occurred on control microshoots in between 22 and 27 days while contaminated shoots were rooted in between 27 and 32 days. Except of the roots formed in the QL medium supplemented with the lowest concentration of IBA, no difference was obtained as regard rooting time between contaminated and control microshoots.

Although spontaneous rooting was observed with the presence of PGB_invit during storage at 25 °C, they were inadequate to support plantlet survival during acclimatization (Fig. 2). With the inclusion of IBA to the medium, the adventitiously rooted microshoots showed survival during acclimatization to in vivo conditions. Moreover, no statistical difference was observed in acclimatization success between control and 12 month in vitro stored contaminated microshoots except the ones that were rooted with the presence of the lowest IBA concentration.

Table 4 Influences of PGB_invit on root induction

Parameters	IBA concentrations (µM)					
	0.49	2.46 Control	4.92	0.49	2.46 Contaminated	4.92
Root formation (%)[a]	40.0b	50.0b	70.0a	40.0b	65.0a	43.0b
Root/microshoot[bc]	2.0 ± 0.6a	1.8 ± 0.2a	1.8 ± 0.3a	1.2 ± 0.2b	1.6 ± 0.1a	2.5 ± 0.5a
Root length (cm)[bc]	1.9 ± 0.6b	1.7 ± 0.4b	1.6 ± 0.4b	3.2 ± 0.6a	2.2 ± 0.3b	2.3 ± 0.3ab
Rooting time(day)[d]	22.3	26.2	27.6	32.0	27.6	26.5

The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least twice

[a]The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$)

[b]The same letters following the averages show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The average difference was analyzed horizontally)

[c]Mean ± standard error

[d]Rooting time of microshoots was calculated according to $\Sigma (N_x T_x)$/rooting microshoot number. N_x is rooted shoot number; T_x is the number of days between the beginning of the test and the ending of test

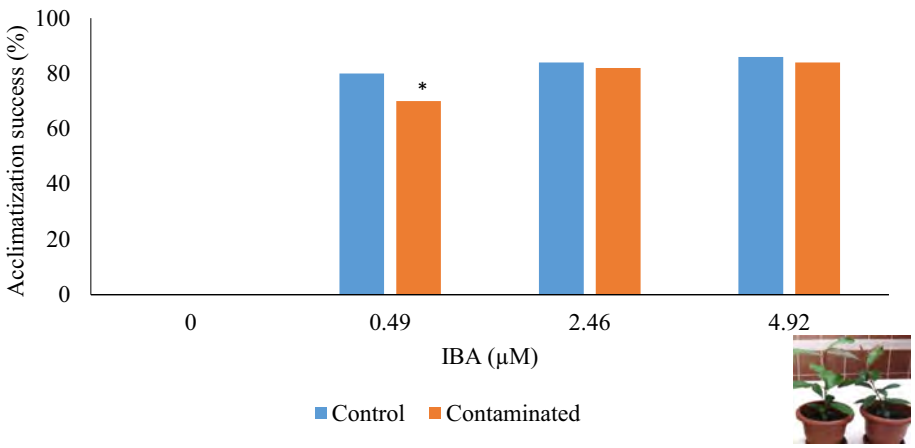

Fig. 2 Acclimatization results of control and microshoots proliferated from shoot apices excised from 12 month-stored contaminated fraser photinia in vitro cultures on different concentrations of IBA containing medium

Auxin and cytokinin synthesis

When PCR was performed using *aux1* and *ipt* primers and genomic DNA as a template, no band was obtained. PCR amplification with *aux*1-FW and aux1-RV primers a single band of ~ 950 bp for the plasmid DNA of PGB_invit and a single band of 791 bp for the plasmid DNA of *R. rhizogenes* ATCC 15834 (Camilleri and Jouanin 1991); with *ipt*-FW and *ipt*-RV primers a single band of ~ 800 bp with plasmid DNA of PGB_invit and 427 bp (Haas et al. 1995) with plasmid DNA of *R. radiobacter* ATCC 15955 were obtained (Fig. 3a). The obtained PCR results show that *aux1* and *ipt* genes in PGB_invit were present in different sizes the reference strains. The presence of *aux2* genes could not be verified due to the several numbers of non-specific bands obtained from the plasmid DNA as template.

Virulence and nitrogen fixing ability

When PCR with *virD*1 primers was performed using plasmid DNA of PGB_invit as template, ~ 550 bp single band (data not shown) was detected. Moreover, expected ~ 750 bp band was obtained with amplification of *nifH* primers by using bacterial culture (Fig. 3b).

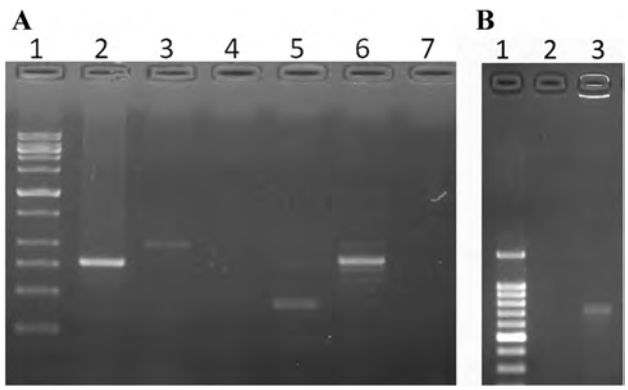

Fig. 3 PCR analysis of *aux1, ipt* and *nifH* genes. **a** Agarose gel electrophoresis of PCR amplification products obtained with *aux*1 and *ipt* gene primers by using plasmid DNA as template. (1) Size marker, 1 kb DNA ladder (Intron 24074), (2) PCR amplification of *Rhizobacterium rhizogenes* ATCC 15834 plasmid DNA (pRi) with *aux*1 primers; (3) PCR amplification of endophytic bacterium plasmid DNA with *aux*1 primers; (4) negative control, (5) PCR amplification of *Rhizobacterium radiobacter* ATCC 15955 plasmid DNA (pTi) with *ipt* primers; (6) PCR amplification of endophytic bacterium plasmid DNA with *ipt* primers; and (7) PCR amplification with *ipt* primers as negative control. **b** Agarose gel electrophoresis of the *nifH* gene by using endophytic bacterial culture (1) size Marker, 1 kb and 100 bp DNA Ladders (Thermofisher), (2) PCR amplification of bacterial culture with *nifH* primers

Carbohydrate and alditols production

According to HPLC analysis, no significant difference was obtained between control (MPYE medium) and bacterium, indication that PGB_invit do not secrete tested carbohydrate or alditols into the medium (data not shown).

Discussion

Some endophytic bacteria that are beneficial for host plant can be present naturally in soil and may penetrate the plant and translocate to the above ground organs and, upon colonization. Endophytic bacteria can affect the plant growth, health, and productivity positively by enhancing the plant's capacity for nutrient obtaining, better water management, and/or resistance to abiotic and biotic stresses (some of them may be antagonistic to pathogens) via regulation of hormones and increase expression of defense related genes in plants (Kim et al. 2012). Organisms identified as endophytes are usually fungi (Yuan et al. 2016) and bacteria (Fahey et al. 1991; Wilson 1995). Presence of a wide range of common gram positive and negative bacteria including *Enterobacter, Pseudomonas, Staphylococcus, Xanthomonas, Agrobacterium, Methylobacterium* spp. have been reported previously in tissue culture of different plant species (Leifert and Cassels 2001; Herman 2004; Kulkarni et al. 2004; Thomas 2004, b, 2007; Thomas et al. 2006). Additionally, Thomas et al. (2008) also identified some uncommon endophytic organisms such as *Ochrobactrum intermedium, Alcaligenes faecalis, Ralstonia mannitolilytica, Oceanobacillus picturae, Bacillus neonatiensis, Brachybacterium, Brevibacterium, Kocuria rosea, Tetrasphaera* spp. etc. Although the presence of bacteria in micropropagated plants is generally considered as microbial contamination that must be prevented and eliminated (George et al. 2008; Quambusch et al. 2016), the association of beneficial endophytic bacteria and micropropagated plants can have positive effects on micropropagation (Dias et al. 2009; Jimtha et al. 2014). For instance, endophytes in tissue cultures of several woody plants showed beneficial influence due to plant growth promotion (i.e., Pirttilä et al. 2000; Quambusch et al. 2014; Pham et al. 2017; Perez-Rosales et al. 2018). In accordance with this, PGB_invit exist in in vitro microshoots of fraser photinia did not result any decline in growth and vigor of the cultures.

The culture media used for the proliferation of in vitro plantlets contains minerals, a carbon source, vitamins and generally low concentration of growth regulators. However, in vitro grown plantlets exhausted the nutrients in 2–3 months and therefore they should be transferred frequently (once every 4–6 weeks depending on the species) to fresh media (Ozden-Tokatli et al. 2010). The lengthening of the

subculturing periods through growth rate reduction could be achieved by modification of media components with incorporation of the culture medium some growth retardants like abscisic acid (ABA) (Kovalchuk et al. 2009) or osmotica like mannitol (Negash et al. 2001; Divakaran et al. 2006) or sucrose (Kovalchuk et al. 2009) together with reduction of the culture temperature usually from 25 to 15 °C (Negash et al. 2001) or 4 °C (Negri et al. 2000; Kovalchuk et al. 2009). In accordance, microshoots of fraser photinia were in vitro-stored at 4 °C up to 15 months on sucrose and mannitol containing QL medium in both baby food jars and vitrovents without subculture (Akdemir et al. 2010). However, the presence of PGB_invit enabled the maintenance of the fraser photinia microshoots without any further incorporation of growth retardants to the culture medium or reduction in culture temperature. With the beneficial effect of the bacterium, microshoots could be stored at standard culture medium and conditions for up to 9 months without significantly losing any viability and vigor. Moreover, all shoot apices excised from 16 months conserved microshoots were capable of resuming or initiating new and organized growth following their transfer to fresh medium.

Alteration of plant growth and development with production of PGRs (i.e., cytokinins, auxins, etc.) was also reported with the presence of *Pseudomonas, Enterobacter, Staphylococcus, Azotobacter*, and *Azospirillum* (Arshad and Frankenberger 1991; Leifert et al. 1994; Bashan and Holguin 1997), which some strains of them could also be endophytic. The positive influences of them on plant growth have been attributed to its ability to co-synthesize compounds commonly known as plant products (Zabetakis 1997; Koutsompogeras et al. 2007) and PGRs (Ivanova et al. 2000, 2001; Koenig et al. 2002). Molecular genetic analysis of the PGB_invit reveal that it can produce cytokinin and auxin. Thus, the positive influence of endophytic bacteria obtained in in vitro cultures of fraser photinia on growth could also be due to the synthesis of PGRs. The spontaneous rooting observed in microshoots that were cultured on media without any auxin incorporation to medium could also support the presence of optimal endogenous levels of PGRs in the original tissues required for rooting (Divakaran et al. 2006). In collaboration, it is reported previously that auxin especially IAA synthesis by endophytic bacteria may have not only various regulatory effects in plant-bacterial interactions but also significant effect on plant growth promotion, i.e., root nodulation (Jasim et al. 2015). For instance, biotization of endophytic plant growth-promoting rhizobacterium (*A. brasilense* strain Cd) stimulated in vitro rooting of jojoba (Perez-Rosales et al. 2018). Moreover, Muromtsev et al. (1987) also reported that the ability of the colonized plants and explants to grow normally on the sucrose-free media and the bright green coloration of the plants infer that; by producing cytokinins, the methylobacteria promote chloroplast development and activity. Likewise, this similar positive influence of the isolated bacterium was also observed in contaminated fraser photinia in vitro cultures.

Besides, the beneficial influence of PGB_invit on storage of microshoots at 25 °C in in vitro conditions, it has no negative influence on proliferation of shoot apices excised from stored microshoots. Moreover, there is no statistical difference on rooting and acclimatization results of control and contaminated microshoots, possibly showing the continued beneficial influence of the bacterium. As there is no report on endophytic bacteria that enable to store microshoots in in vitro conditions without subculturing and renewal of the medium such a long-time, endophytes like PGB_invit seemed to be very original and have potential to be used for medium-term storage of plant germplasm.

It should also be noted that diverse species of bacteria such as *Agrobacterium tumefaciens, Rhizobium* sp., *Sinorhizobium meliloti* and *Mesorhizobium loti* could transfer genes to plants (Broothaerts et al. 2005). The presence of genes encoding *virD1* on the plasmid DNA isolated from PGB_invit may indicate the ability of this bacterium to transfer genes to its host plant as *virD1*, an endonuclease encoded by inducible locus of the virulence (vir) region of the *Agrobacterium tumefaciens* Ti plasmid, is required for site-specific nicking at T-DNA border sites (Wang et al. 1990).

In our previous study, we showed that the putatively endophytic bacterium may reduce NO_3 to NO_2 according to its biochemical assays. In this study, ability to fix nitrogen of PGB_invit was verified by the PCR amplification of *nifH* gene from this bacterium. Nitrogen is generally a limiting source of plant growth and development. *Nif* genes encode the enzymes, which are capable of fixing atmospheric nitrogen into a form available to plants. Plants only may take nitrogen as ammonia or nitrate forms. Therefore, nitrate reduction by a bacterium is important for the nitrogen availability of the plants (Mbai et al. 2013). In nature there are several nitrogen-fixing bacteria which may benefit the plants (Boddey et al. 1991; Triplett 1996; Malik et al. 1997; Reinhold-Hurek and Hurek 1998; Iniguez et al. 2004).

Although no carbohydrate or alditols production was evident in PGB_invit according to HPLC analysis, the presence of polyhydroxybutyrate (PHB), which was detected in transmission electron microscopy (TEM) analysis (Gul Şeker et al. 2017) could be used as carbon source as it is a carbon reserve of bacteria (Lemoigne 1926) that is synthesized when nutrient status is low (Borque et al. 1995; Khosravi-Darani et al. 2013). Thus, PHB accumulation detected in PGB_invit could ensure energy to the bacteria and enabled its survival under metabolic stress. Moreover, it should also be noted that some metabolites (i.e., furanoids and pavettamine) might also be produced by plant bacteria

association (Brader et al. 2014) and this possibility should also be investigated with further analysis.

In conclusion, the presence of PGB_invit in the culture medium and its synergistic effect with its host resulted in a significant improvement in microshoots growth during prolonged maintenance of fraser photinia shoot cultures in vitro at 25 °C. The positive influence of this bacterium is due to its ability to provide cytokinin and auxin together with its capability to nitrogen fixation. Moreover, the presence of especially *virD1* gene is also promising as it may have ability to transfer genes to plant and could be used for future genetic transformation studies. Hence, the isolated bacterium is useful for in vitro conservation of fraser photinia germplasm as frequent subculturing can enhance not only the risk of occurrence of somaclonal variation but also the cost of personnel, energy and materials. It should be noted that PGB_invit will also be inoculated with other plants especially model species (i.e., *Arabidopsis thaliana*) not only to reveal out its host specificity, but also the molecular mechanism underlying its beneficial influence on plant in our future studies.

Author contributions YÖÇ and ÖA designed the research project; HA and EK carried out the plant storage analyses; IS and ÖA carried out the molecular analysis; IS, ÖA and YÖÇ wrote the paper.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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