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The Effect of Plant Growth-Promoting Rhizobacteria on Growth and Physiological Characteristics of *Corylus avellana* Seedlings

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ABSTRACT The effects of plant growth promoting rhizobacteria (PGPR) on growth and physiological characteristics of seedlings *Corylus avellana* were determined in a forest nursery. For this purpose, in a completely randomized design (CRD) and four replicates, three putative bacteria, including *Pseudomonas putida*, *Bacillus subtilis* and *Enterobacter cloacae*, as alone and mixed, were examined. The greatest height (26.88 cm), collar diameter (7.11 mm), leaf area (23.87 cm^2)net photosynthesis (17.23µmol CO_2 $m^{-2}s^{-1}$), transpiration rate (3.22 mmol H_2O $m^{-2}s^{-1}$), stomatal conductance (0.189 mol CO_2 $m^{-2}s^{-1}$), water use efficiency (5.33 µmol CO_2 $mmol^{-1}$ H_2O) and chlorophyll content (26.16 SPAD) allocated to seedlings inoculated with a combination of three bacteria. The greatest root dry weight (3.98 g), root volume (10.87 cm^3), total plant dry weight (9.91g) was detected in seedlings inoculated with *P. putida*. In total, for the beneficial effects of PGPR on growth and physiological traits of *C. avellana* seedlings, all three bacteria either as individual or as mixed were found suitable for seedling inoculation. In fact, it is affirmed that inoculation of root with PGPR can be a proper approach to produce healthy and strong seedlings in nursery.

Key words: *Chlorophyll content, Hazelnut, Gas exchange, Growth, Pseudomonas putida*

1 INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots, produce phytohormones, asymbiotic nitrogen, siderophores, antibiotics, enzymes and fungicidal compounds and increase the rate of plant growth (Ahmad *et al*., 2008). The direct promotion of plant growth by PGPR and generally inoculation of nursery planting stock with selected PGPR can affect morphological and physiological traits of the seedlings (Domínguez-Núñez*et al*., 2013). *Pseudomonas putida, Bacillus subtilis and Enterobacter cloacae* are putative PGPR; they are able to colonize new ecological niches, especially in the rhizosphere and to play crucial roles in plant growth (Ahemad and Kilbert, 2014).

Hazelnuts belong to the genus *Corylus* in family Corylaceae. *C. avellana* occurs in

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Morocco, Algeria, Turkey, Iran and the Caucasus region (Mozaffarian, 2005; Falistocco *et al*., 2012). In Iran the most important hazelnut stands are located in the northern provinces of Gulilan, Mazandaran and as far as the northwest of Arasbaran and Fandoghlou forests where the rainfall and atmosphere humidity is high (Mozaffarian, 2005). Hazelnut plays an important role in human nutrition and health because of its specific composition in fat (mainly oleic acid), protein, carbohydrate, dietary fiber, vitamins, minerals and antioxidant phenolics (Alasalvar *et al*., 2003). The habitats of hazelnut, like most other forest habitats, have been suffering from severe destruction and deterioration by livestock grazing, land use change, fire and over-logging (Ansari *et al*., 2008). Thus, attention should be paid to restoration of the hazelnut through production of suitable seedlings in forest nurseries.

Using standard and healthy seedling is fundamental for the success of any plantation program (Espahbodi, 2015; Heydari *et al*., 2015). On the other hand, the generous fertilizer and pesticide used in nurseries to promote growth can develop seedlings root system from the beneficial symbiotic bacterial and mycorrhizal fungi. The produced seedlings of *C. avellana* in the nursery do not reach the size for reforestation purposes within the first year, which increases the cost of seedling production. This is while the biological growth properties of the root and stem are very important during the first year in forest nursery (Cerovic *et al*., 2007). Although application of bio-fertilizers as well as plant growth promoting rhizobacteria (PGPR) has been found to increase the growth of seedlings (Salimi and Hoseinova, 2012), no report is available on *C. avellana* seedlings simultaneously inoculated with PGPR.

Investigations on the application of PGPR in forest seedlings are less widespread than in agricultural application. However, several studies have indicated the positive effects of various PGPR on tree species (e.g. Rincon *et al*., 2008; Mafia *et al*., 2009; Hasani *et al*., 2012; Yu *et al*., 2013; Bahmani *et al*., 2014; Karlicic *et al*., 2015; Liu *et al*., 2015).

Due to the limited studies on the effects of PGPR inoculation on growth and physiological parameters in the slow-growing seedlings of *C. avellana*, this research is aimed to assess the effect of PGPRs (*Pseudomonas putida*, *Bacilus subtilis* and *Entrobacter cloacae*) on growth characteristics and physiological parameters of this species in the nursery conditions.

2 MATERIALS AND METHODS

2.1 Plant material and experimental conditions

This investigation was carried out from Mar. to Oct. 2015 in the Fandoghlou Forest Nursery, Northwest Iran (48°36´E, 38°19´N, 1380 m altitude). The annual average precipitation is 487 mm; the annual mean min and max temperature are 3.7°C and 14.7°C, respectively.

Mature seeds of hazelnut were collected from healthy trees with similar diameter and height in Fandoghlou Forest. The seeds were sown in plastic pots containing 4 kg of sterilized nursery soil with silty-loam texture (Table 1). The pots were kept under natural photoperiod in the forest nursery of Fandoghlou. Three putative bacterial strains, *Pseudomonas putida* DSM291, *Bacillus subtilis* strainFzB24 and *Enterobacter cloacae* with 10^9 colony forming unit (cfu) ml^{-1} were obtained from the microbial collection of the soil microbiology department of Soil and Water Research Institute (SWRI), Iran. The PGPR species used in this experiment were selected for the following reasons: (1) all three bacteria are phosphate solubilizing, nitrogen fixing, and they have the ability to produce auxin, siderophores, HCN and ammonia (Ahmad *et al*., 2008), (2) all have the synergistic effect on each other, (3) they are found in various

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environments (especially in woodlands) (Ahemad and Kilbert, 2014).

Based on SWRI guideline, one month after the beginning of the experiment (Mid-April), all bacteria and their combination were applied through a syringe; 20 ml^{-1} of bacterial suspension was inoculated into the middle of seedling roots (5 cm depth) and the control plants received 20 ml of distilled water (Yu *et al*., 2014). The treatments of experiment comprised: (1) Control; (2) *P. putida*; (3) *B. subtilis*; (4) *E. cloacae*; (5) *P. putida* + *B. subtilis* + *E. cloacae*, 1:1:1 in volume. The seedlings were irrigated regularly to fulfill the plant needs, depending on climate and to ensure that the water was not the limiting factor during the experiment. The experiment was done based completely randomized design with four replicates.

Table 1 Some physical and chemical properties of the soil used for the experiment

pH	EC ($ds\ m^{-1}$)	Clay (%)	Silt (%)	Sand (%)	OC (%)	N (%)	P (ppm)	K (ppm)
6.34	0.386	23	48	29	1.21	0.13	9.44	174

2.2 Plant growth parameters

At the end of the experiment, three plants were randomly selected per replicate for measurements of the height (cm), collar diameter (mm), leaf surface (cm^2) and specific leaf area (cm^2g^{-1}). The same seedlings were destructively harvested and soil adhering to root system gently cleaned and the above-ground parts and roots separated at the root collar. The roots were washed and root volume measured. Root, shoot and leaf biomass were determined after oven drying at 70°C for 90 h.

2.3 Plant physiological measurements

Before the end of growing period, the net photosynthetic rate (*A*, in $\mu molCO_2\ m^{-2}s^{-1}$), transpiration rate (*E*, in mmol $H_2O\ m^{-2}s^{-1}$) and stomatal conductance (*gs*, in mol $m^{-2}s^{-1}$) of the seedlings were measured with a portable photosynthesis system (ADC Bio Scientific Ltd., UK). Instantaneous water use efficiency, (WUE_i =A/E) was calculated by dividing photosynthetic rate by transpiration (Zhang *et al*., 2005). The parameters were measured on the uppermost, fully expanded leaves of each plant from 09:00 AM to 11:00 AM under bright sunlight in a clear, cloudless day (Yu *et al*., 2014). Three leaves were selected for each seedling at each time. Leaf chlorophyll content was measured using a chlorophyll meter model (Minolta Co., Osko, Japan, SPAD 502) (Marcelo and Bruce, 2010).

2.4 Statistical analyses

Normality and homogeneity was confirmed using Kolmogorov-Smirnov and Levene tests, respectively. The data were analyzed using one-way ANOVA. Differences among means were analyzed by Least Significant Difference (LSD) test at $P \leq 0.05$. Statistical analyses were performed with the SAS statistical software version 9.3 (SAS Institute, Inc., Cary, NC, USA).

3 RESULTS

3.1 Growth parameters

Data analysis showed that the bacterial inoculations of seedling significantly affected the collar diameter, height, leaf area, specific leaf area, aerial dry weight, the total dry weight ($P \leq 0.05$), and root volume and root dry weight ($P \leq 0.01$) (Table 2).

All bacterial treatments significantly affected all growth parameters of seedlings (Table 3). In treatments (*P. putida*, *B. subtilis*, *E. cloacae* and *E. c+B. s+P. p*), collar diameter

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increased by 31.40%, 27.02%, 19.12%, and 49.48%, respectively and height by 25.43%, 18.01%, 8.83%, and 56.02%, respectively. The leaf area (28.87 cm^2) was greatest when the seedlings were inoculated with the combination of the three bacteria. The highest specific leaf area (123.21 $cm^2g^{-1}$and 120.89 cm^2g^{-1}) was observed in seedlings inoculated with *P. putida* and *B. subtilis*, respectively (Table 3).

Root volume (9.87 cm^3), root dry weight (4.08 and 3.98 g), and the aerial dry weight (6.01 g) were observed in seedlings inoculated with *P. putida* (Table 3). In each of the fourth-fold inoculation treatments (*P. putida, B. subtilis, E. cloacae and E. c+ B. s +P. p*), compared to control, total dry weight increased by 52.32%, 33.48%, 10.90%, and 29.18%, respectively (Figure 1).

Table 2 Analysis of variance (ANOVA) for effect of inoculation of PGPR on growth and physiological parameters of *C. avellana* seedling

Parameters	F	P- values
Collar diameter	2.96	0.030*
Height	4.13	0.029*
Leaf area	2.61	0.041*
Specific leaf area	6.68	0.022*
Root volume	19.78	0.000**
Root dry weight	8.23	0.000**
Arial dry weight	5.33	0.027*
Total dry weight	5.10	0.020*
Net photosynthetic	2.32	0.031*
Transpiration rate	2.76	0.042*
Stomatal conductance	2.26	0.044*
water use efficiency	10.04	0.000**
Chlorophyll content	7.43	0.016*

*, * Differences are significant at 1 and 5% level of probability, respectively.

Table 3 Effect of inoculation with PGPR on growth parameters of *C. avellana* seedling

Treatment	**Collar diameter (mm)**	**Height (cm)**	**Leaf area (cm^2)**	**Specific leaf area (cm^2g^{-1})**	**Root dry weight (g)**	**Aerial dry weight (g)**	**Root volume (cm^3)**
Control	4.81±0.42^{c}	17.10±1.30^{d}	16.15±1.03^{c}	100.87±11.72 ^{c}	2.22±0.64^{c}	4.27±0.23^{c}	6.44±1.01^{d}
P. putida (*P. p*)	6.32±0.09^{ab}	21.45±2.32^{b}	25.44±2.32^{ab}	123.21±17.32^{a}	3.98±0.72^{a}	6.01±0.78^{a}	9.87±1.33^{a}
B. subtilis (*B.s*)	6.11±1.22^{ab}	20.18±2.14^{b}	23.13±2.14^{b}	120.89±12.88 ^{a}	3.63±0.61^{ab}	5.11±1.02^{ab}	7.63±1.67^{c}
E. cloacae (*E .c*)	5.73±1.13^{b}	18.61±0.65^{c}	23.29±0.65^{b}	115.11±14.65^{ab}	3.03±0.15^{b}	4.69±1.22^{b}	6.11±1.23^{d}
P. p+B.s+E. c	7.19±0.77 ^{a}	26.88±1.43^{a}	28.87±2.62^{a}	108.42±13.34^{bc}	3.11±0.33^{b}	5.28±1.11^{ab}	9.08±1.42^{b}

Mean ±SE followed by same letter in column are not significantly different, according to LSD test (p = 0.05)

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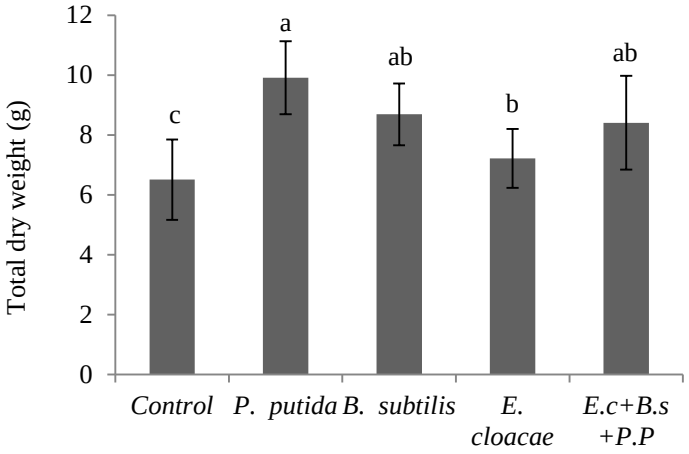

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Figure 1 Effect of inoculation with PGPR on total dry weight of *C. avellana* seedling
Same letters on columns are not significantly different, according to LSD test (p = 0.05)

3.2 Physiological parameters

Data analysis showed that the bacterial inoculations significantly affected the photosynthetic rate (P_n), transpiration rate (E), stomatal conductance (g_s), chlorophyll content ($P \leq 0.05$), and water use efficiency (WEF) ($P \leq 0.01$) (Table 2). In four treatments (*P. putida*, *B. subtilis*, *E. cloacae* and *E. c+ B.s+ P. P*), net photosynthesis (P_n) increased by 32.84%, 26.50%, 10.58%, and70.42%, respectively (Table 4).

The highest transpiration rate (E) was observed with *P. putida* (3.05 mmol H_2O $m^{-2}s^{-1}$) and *E .c+B. s+P. p* (3.23 mmol H_2O $m^{-2}s^{-1}$) as compared with control (2.85 mmol H_2O $m^{-2}s^{-1}$). The inoculation with combination of the three PGPR strains showed higher stomatal conductance (0.189 mol $m^{-2}s^{-1}$) compared to the control (0.156 mol $m^{-2}s^{-1}$). Water use efficiency (WUE) also increased significantly in all the PGPR strain treatments. In four treatments (*P. putida*, *B. subtilis*, *E. cloacae* and *E. c+B. s+P. p*), WUE increased by 34.92, 23.94%, 10.98%, and 50.14%, respectively, compared with controls (Table 4). The highest chlorophyll content (26.16 SPAD) was detected in seedlings inoculated with the combination of three bacteria (Figure 2).

Table 4 Effect of inoculation with PGPR on the net photosynthetic rate, transpiration rate and stomatal conductance and water use efficiency of *C. avellana* seedling.

Treatment	Net photosynthetic (µmol $CO_2m^{-2}s^{-1}$)	transpiration rate (mmol H_2O $m^{-2}s^{-}$)	Stomatal conductance (mol $CO_2m^{-2}s^{-1}$)	water use efficiency (µmol $CO_2mmol^{-1}H_2O$)
Control	10.11±1.34 d	2.85±0.11 b	0.156±0.007 d	3.55±0.41 d
P. putida (*P. p*)	13.43±1.22 b	3.05±0.43 ab	0.181±0.009 ab	4.79±1.76 b
B. subtilis (*B. s*)	12.79±1.03 bc	2.92±0.67 b	0.177±0.014 b	4.40±0.42 bc
E. cloacae (*E. c*)	11.18±0.98 c	2.84±0.51 b	0.161±0.013 c	3.93±0.97 c
P. p+B. s+E. c	17.23±1.47 a	3.23±0.45 a	0.189±0.006 a	5.33±1.01 a

Mean ±SE followed by same letter in column are not significantly different, according to LSD test ($p = 0.05$).

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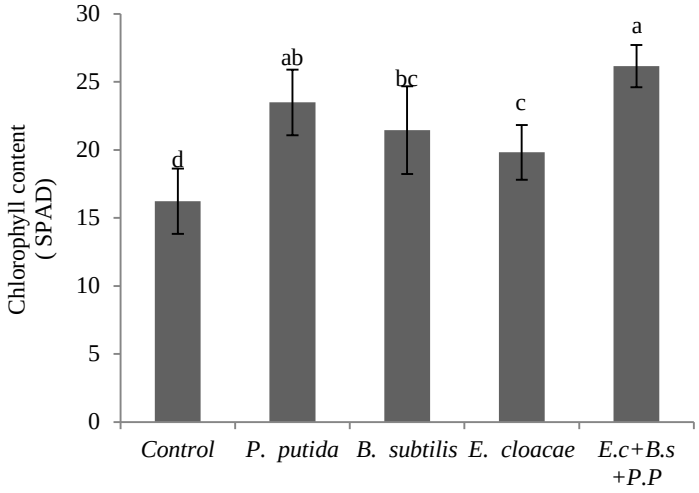

Figure 2 Effect of inoculation with PGPR on leaf chlorophyll content of *C. avellana* seedling. Same letters on columns are not significantly different, according to LSD test (p = 0.05)

4 DISCUSSION

In the present study, three bacterial strains, including *P. putida*, *B. subtilis* and *E. cloacae* were evaluated for improving growth and physiological parameters of *C. avellana* seedlings under nursery conditions. The results showed both single and mixed rhizobacteria applications increased growth parameters (Table 3). The combinations of three rhizobacteria resulted in significant increase in collar diameter (49.48%), height (57.19%) and leaf area (78.76%). Karlicic *et al*. (2015) also reported significant increase (11-24%) in the growth characteristics of *Pinus sylvestris* and *Robinia pseudoacacia* seedlings following inoculation with mixed rhizobacteria (*B. licheniformis*, *Aeromonas hydrophila* and *P. putida*). In this respect, it can be stated that there is a positive synergism effect between *P. putida*, *B. subtilis* and *E. cloacae* to improve the seedlings growth of hazelnut (Yang *et al*., 2010).

In current research, among the microbial inoculants tested as individual, *P. putida* showed significantly higher growth followed by *B. subtilis* and *E. cloacae*. The most important characteristics of *Pseudomonas* sp. is production of indoleacetic acid (IAA), siderophores, HCN, ammonia, exo-polysaccharides and phosphate solubilization (Mafia *et al*., 2009; Rincon *et al*., 2009; Ahemad and Khan, 2012). In line with Hasani *et al*. (2012) on *Pistacia vera*, in our study single inoculation of hazelnut seedlings with *P. putida* resulted in significant increase in specific leaf area, root dry weight, root volume (Table 3) and total dry weight (Fig. 1). These effects might be explained by the capability of the *Pseudomonas* strains to synthesize IAA that was resulted in development and root proliferation (Zhu *et al*., 2010), plant mineral uptake and indirect stimulation of plant growth (Spaepen *et al*., 2007).

In our findings with application of *P. putida* as individual, net photosynthetic rate and stomatal conductance increased 32.8% and 16%, respectively (Table 4). Similar results were reported by Bahmani *et al*. (2014, on *Calotropis procera*) and Bisht *et al*. (2009, on *Dalbergiasisso*), following inoculation of seedlings with *Pseudomonas* strain. Such increases were also observed following inoculation of seedlings of *Pinus halpensis* and *Quercus coccifera* with *P. fluorescence* (Rincon *et al*., 2009). In present investigation, the net photosynthesis, transpiration rate and stomatal

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conductance in *C. avellana* seedlings as compared with individual inoculations indicated that the three strains could act synergistically during plant growth and development. This is similar with the findings of Yu *et al*. (2014), who with inoculation of *P. aurantiaca*, *P. fluorescens* and *B. cereus* on walnut (*Juglans siggillata* L.) seedlings founded that co-inoculation with the three strains increased net photosynthesis rate, transpiration rate. In our study, co-inoculation of the three strains improved chlorophyll content of the seedlings as compared with individual inoculation (Fig. 2). This may be attributed to increased photosynthetic activity which is a consequence of a higher N incorporation contributing the formation of chlorophyll content (Liu *et al*., 2013).

5 CONCLUSION

The present study clearly demonstrated the benefits of PGPR for enhancing growth and physiological parameters of *C. avellana* seedlings. In reality, it can be confirmed that inoculation of root with PGPR is a proper approach to produce high-quality seedlings in nursery.

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تاثیر باکتری‌های محرک رشد بر بهبود صفات رویشی و فیزیولوژیکی نهال‌های فندق (*Corylus avellana* L.)

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چکیده: تحقیق حاضر به‌منظور بررسی تاثیر باکتری‌های محرک رشد برای بهبود رشد و ویژگی‌های فیزیولوژیک نهال‌های فندق (*Corylus avellana* L.) در نهالستان انجام شد. برای این مقصود، به صورت طرح کاملا تصادفی در چهار تکرار، تاثیر سه نوع باکتری *Pseudomonas putida*، *Bacillus subtilis* و *Enterobacter cloacae* به‌صورت مجزا و ترکیبی به‌مدت ۷ ماه روی نهال‌های این گونه در نهالستان جنگلی فندقلو (شمال غرب ایران) بررسی شد. بیش‌ترین اندازه ارتفاع، قطریقه و سطح برگ به‌ترتیب با ۲۶/۸۸ سانتی‌متر، ۷/۱۱ میلی‌متر و ۲۳/۸۷ سانتی‌متر مربع، نرخ فتوسنتز با ۲۳/۱۷ میکرومول بر متر مربع بر ثانیه، هدایت روزنه‌ای با ۳/۲۲ میلی‌مول بر متر مربع بر ثانیه، نرخ تعرق با ۰/۱۸۹ مول بر متر مربع بر ثانیه، کارایی مصرف آب با ۵/۳۳ میکرومول بر متر مربع بر ثانیه و محتوی کلروفیل برگ با ۲۶/۱۶ در تلقیح ترکیبی سه باکتری مشاهده شد. وزن خشک ریشه با ۳/۹۸ گرم، حجم ریشه با ۱۰/۸۷سانتی‌متر مکعب و زی‌توده خشکِ کل نهال با ۹/۹۱ گرم با تلقیح مجزای باکتری *P. putida* به‌دست آمد. در حالت کلی، باتوجه به تأثیر مفید باکتری‌های محرک رشد در صفات رویشی و شاخص‌های فیزیولوژیکِ هر سه باکتری (چه مجزا یا ترکیبی)، برای تلقیح نهال فندق مناسب تشخیص داده شدند. در حقیقت، تایید می‌شود که تلقیح ریشهٔ نهال با باکتری‌های محرک رشد راه‌کاری مناسب برای تولید نهال سالم و قوی در نهالستان است.

کلمات کلیدی: تبادلات گازی، فندق، *Pseudomonas putida*، رویش طولی، محتوی کلروفیل

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