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Effect of Plant Growth Promoting Rhizobacteria (PGPR) and Cold Stratification on Seed Germination and Early Growth of *Corylus avellana* L

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Keywords: *Pseudomonas putida*, hazelnut, seed dormancy, seed vigor index, shoot length

Abstract

In this study, three putative plant growth promoting rhizobacteria (PGPR) including *Pseudomonas putida* strain 168, *Bacillus subtilis* strain FzB24 and *Enterobacter cloacae* strain 12, were applied as single and combination of three strains for their ability to improve seed germination traits and early growth in three *Corylus avellana ecotypes*. After inclusion, seeds were sown in 45 cm×20 cm×10 cm plastic containers filled with wet sterilized river sand and subjected to cold stratification (CS) in a refrigerator at (5±1°C) for 0, 3 and 4 months. The experiment was done based on factorial in a completely randomized design with 3 replicates of 20 seeds each. The numbers of germinated seeds were recorded daily for each ecotype. At the end of the experiment, germination percentage (GP), germination speed (GS), seed vigor index (SVI) of the

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seeds were calculated. Results showed that, CS, ecotypes, bacteria inoculation and interactions of these factors affected germination traits. Over all treatments, GP ranged from 1.66% to 65.40% among the ecotypes. Both CS and inoculation had positive effect on GP. The greatest GP (61.35-65.40%) and onset of germination occurred in seeds co-inoculated by the combination of 3 bacterial inoculants followed by 4 months CS. Among ecotypes, the highest SVI (160.77) belonged to seeds of Makesh ecotype inoculated by the combination of 3 bacterial inoculants full in some points owed by 4 months CS. In case of application of bacterial as individual, the highest germination traits and early growth were obtained in seeds inoculated with *Pseudomonas putida*. The findings confirm that PGPR is better to be used in combination with cold stratification to overcome seed dormancy, improvement of seed germination treats and early growth of *C. avellana*.

Introduction

Hazelnut species belongs to the genus *Corylus* and family *Corylaceae* (Davis, 1988; Mozaffarian, 2005). Hazelnut is typically a multi-stemmed shrub reaching 3–5 m tall. The leaves are ovate to broadly ovate, 5–12 ×6-13 cm long and across, softly hairy on both surfaces, and with a double-serrate margin (Davis, 1988). The fruit is a nut and is roughly spherical to oval, 15–20 mm long and 12–20 mm (Mozaffarian, 2005). It is widely distributed throughout Europe, from Britain and Scandinavia eastwards to the Ural Mountains in Russia, and as far south as Spain, Italy and Greece. It also occurs in Morocco, Algeria, Turkey, Iran and the Caucasus region (Davis, 1988; Mozaffarian, 2005). The most important hazel natural stands in Iran are located in three main areas (Guilan, Ardebil and Arasbaran).

Seed dormancy has been described as a least understood phenomena in seed biology (Finch-Savage & Leubner-Metzger, 2006) that is an adaptation to delay germination after the seed has been dispersed from the tree until the suitable time for germination (Bewley, 1997). Seed dormancy affects both seed germination and subsequent growth of seedlings (Finch-Savage & Leubner-Metzger, 2006). Today, one of the methods used to break dormancy in seeds and increase germination percentage is inoculation of seeds with plant growth promoting rhizobacteria (PGPR). Term of "PGPR" was defined by Kloepper & Schroth in 1978. Many microorganisms are plant growth promoting rhizobacteria and include typical species of the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Azospirillum* and *Acinetobacter*, (Benizri et al., 2001; Cakmakci et al., 2006; Ahemad & Kibret, 2014) which can help increasing seed germination and seedling vigor (El-Mohamedy et al., 2006).

Experiments on the application of PGPR in forest tree are less widespread than in agricultural application. Although, there are several researches demonstrating the positive effects of PGPR on tree species. Ahmad et al. (2008) reported that PGPR could

produce phytohormones, asymbiotic N2, siderophores, antibiotics, enzymes and fungicidal compounds, which might increase the rate of seed germination. Mafia et al. (2009) showed *Pseudomonas* sp., was the best rhizobacteria for seed germination and growth promotion of *Eucalyptus cloeziana* and *E. grandis*. *Bacillus subtilis* was the most effective for *E. globulus*, and *Pseudomonas fulva, P. Putida, Stenotrophomonas maltophilia* as well as *B. subtilis* were the most promising for the *E. urophylla*. Kazaz et al. (2010) demonstrated that microbial inoculation (particularly *Rhodopseudomonas palustris*) with *Rosa damascene* seeds and a stratification period of 150 days released the seeds dormancy and highly improved the seed germination percentage. Singh et al. (2011) reported that inoculation of *Bacillus licheniformis* or S. ko-stiense alone and as the co-inoculants supported the maximum growth of the seeds and were significantly superior to other strains. It is attributed to the fact that *B. licheniformis* produces gibberellins and that has a positive effect on seed germination and seedling traits in *Acacia senegal* and *Prosopis cineraria* species. Ahmadloo et al. (2014) with application of PGPRs (*Azotobacter chroococcum* 12, *Azospirillum* lipoferum, *Pseudomonas fluorescens* 169, *Bacillus subtilis* FzB24) on seed germination of *Crataegus pseudoheterophylla* found that higher percentages of seed germination (18.33%) and germination speed (4.82 number/day) were recorded for co-inoculated seeds by the combination of all bacterial inoculants and the alternate temperature stratification regime.

The present study was carried out to breaking dormancy, improvement of seed germination traits and early growth of *Corylus avellana* inoculated with three different PGPR strains and different periods of cold stratification.

Material and Methods

Source of seeds and seeds collection: Mature seeds were collected from healthy trees with the same characteristics in terms of diameter and height from habitats of three hazel ecotypes (Makesh, Fandoglou and Makidi in areas of Guilan, Ardebil and Arasbaran). Characteristics of collection areas is shown in Table 1.

Determine of quantity and quality characteristics of seeds: Before seed germination experiments, 100 seeds were randomly selected from each ecotype and nut length (cm), nut width (cm), nut weight (g) and kernel weight (g) were measured. For measurement of moisture content of seed nuts, 100 seeds are weighted and placed in an oven at 103 ± 2°C for 16 hours (Normah et al., 1994). Kernel moisture content was determined by drying crushed kernels at 100±5 °C (2.0 g per sample) until they reached a constant weight (AOAC, 1990). For conducting the tetrazolium test, the nuts were manually cracked to remove the pericarp (to facilitate the quick penetration of tetrazolium) and soaked in water overnight at room temperature. The softened seeds were cut longitudinally. The seeds following the preparation were soaked in

1% tetrazolium solution and kept in dark at about 30°C for 4 hr. Then the seeds were rinsed three times with distilled water and then evaluated (Agrawal & Dadlani, 1992). Four replicates of 25 seeds each were subjected to moisture content determination, and tetrazolium test.

Seed preparation for inoculation with PGPR and cold Stratification: The bacterial strains, *Pseudomonas putida* DSM291 (P), *Bacillus subtilis* strain FzB24 (B), and *Enterobacter cloacae* (E) were obtained from the microbial collection of the soil microbiology department of Soil and Water Research Institute (SWRI), Iran.

First, the nuts were manually cracked to remove the pericarp and were selected only seeds with healthy cotyledon for experiment. Then seeds were soaked in a solution of hypochlorite sodium to surface-sterilized for 5 min and rinsed three times with distilled water prior to applying treatment in the following, for ulation seeds of three hazel ecotypes were coated with 20% gum arabic as an adhesive and rolled into the suspension of bacteria (10^8 cfu ml^{-1}). Four levels of bacterial species consisting of (1) *Pseudomonas putida* strain 168, (2) *Bacillus subtilis* strain FzB24, (3) *Enterobacter cloacae* strain (4) combination of three bacterial species and control (without use of bacterial). For treatments applied, a single and three strains, inoculum amounts, 300 and 100 ml were used for each bacteria, respectively. After seeds incubated at 28 ± 1° C for three days (Jahanian et al., 2012). Then, treatment application, seeds were sown in 45cm×20cm×10 cm plastic containers filled with wet sterilized river sand and subjected to cold stratification in a refrigerator at 5±1° C for 0, 3 and 4 months. During cold stratification (CS) periods, river sand moisture was checked periodically and distilled water added whenever necessary to keep its moist. Observations were recorded daily regarding germinated/non-germinated seeds up to 40 days for all ecotypes. At the end of (CS) periods, plastic containers were placed in room with temperature of 22 and 25°C, and relative humid of 65% and 75%. The containers were watered three times a week. The seeds were considered as germinated when the radicle length was more than 2 mm (ISTA, 1999).

Evaluations for percentage of germination (PG), germination speed (SP) and Seed Vigor index (SVI) were calculated according to Eqs. (1 to 3), respectively (Panwar & Bhardwaj, 2005), where N is total number of sown seeds, n is number of germinated seeds on day d, d is the number of days counted from the beginning of germination, SL and RL is shoot and root length, respectively.

$PG = \sum n / N \times 100$ (1).

$SP = \sum (n / d)$ (2).

$SVI = GR \times Mean (SL+RL) / 100$ (3).

The experiment was conducted using three replications of 20 seeds per treatment. Term "seed" refers to the seed without pericarp.

Experimental design and statistical analysis: The experiment was done as factorial in a completely randomized design and 3 replicates of 20 seeds each. Normality and homogeneity were confirmed using Shapiro -Wilk and Levene's tests. Data of germination percentage and germination speed were transformed to arc-sine square root before analysis of ANOVA. Statistical analyses were performed with the SAS statistical software version 9.3 (SAS Institute, Inc., Cary, NC, USA). Differences among means were analyzed by Least Significant Difference (LSD) test at $p \leq 0.01$.

Results

Before seed germination experiments, quantitative and qualitative measurements of seeds were determined. Makesh ecotype had the highest nut length, nut width, nut weight and kernel weight with 1.88 cm, 1.45 cm, 2.42 g and 0.66 g, respectively, whereas Makidi seeds had the lowest with 1.48 cm, 142 cm, 1.42g and 0.38g, respectively. Makesh seeds retained high percent moisture (28.1±4.9), whereas Makidi seeds had low moisture content (20.8±3.60). Viability percentage of Makesh, Fandoglou and Makidi seeds was 90.80, 80.10 and 71.80, respectively (Table 2). Analysis of variance illustrated that triple interaction effects on traits of germination were significant (Table 3, $P \leq 0.05$). Seed inoculation significantly increased seed germination and seedling vigor of hazel ecotypes; however, the rate of enhancement varied with bacterial strains.

Germination percentage (GP):

Over all treatments, germination by ecotype ranged from 1.66% to 65.40% (Fig. 1). Both cold stratification periods and bacteria inoculation had positive effect on germination rate (Fig. 1), so that the highest germination percentage was observed in seeds co-inoculated by the combination of three bacterial followed by 4 months cold stratification ranged from 65.40±3.84% (Makesh), 61.67±7.40% (Fandoghlou) and 61.35±6.23% (Makidi). In each 3 and 4 months stratification, seeds inoculated with bacteria exhibited higher germination percentages than non-inoculants ($p<0.05$). In individual applications of bacterial in treatment without CS, higher percentages for seed germination were obtained with *P. putida*. There was an increase in the CS periods from 3 to 4 months in GP for all ecotype seeds. The lowest germination percentage among ecotypes in all treatments belonged to Makidi ecotype with 1.66%. Treatments co-inoculated by the combination of three bacterial plus four months cold stratification and control (without bacteria + 0 months CS) resulted in most rapid (low MGT) and slowest (high MGT) dormancy breaking in hazel ecotypes.

The beginning time of germination indicated that treatments co-inoculated by the combination of three bacterial plus four months cold stratification and control (without bacteria + 0 months CS) resulted in the most rapid (low MGT) and slowest (high MGT) dormancy breaking in the hazel ecotype assayed (Fig 2 to 4). Germination period in Makesh ecotype seeds had a faster process of other ecotypes and seeds germination started 9 days after seed sowing (Fig. 4).

Germination speed (GS): CS Period, seed source, bacteria inoculation and all interactions of these factors impacted germination speed. Germination occurred sooner when seeds were inoculated by individual and co-inoculated with combination of three bacterial stratified in longer period compared to control (Fig. 5). The highest germination speed was obtained in seeds inoculated with combination of all bacteria followed by four months cold stratification (3.63 seed per day).

Root and shoot length: Different strains of rhizobacteria had variable effects on root and shoot length in various ecotype assays. Among three bacteria used in assay, *P. putida* and *B. subtilis* were the best ones on enhancement of root and shoot length in all CS periods. The highest root length was allocated to the seeds inoculated by combination of all bacteria followed by 4 months cold stratification (Fig. 6). Among ecotypes, the lowest and highest root length belonged to Makidi and Makesh with 12.48, 14.53 cm, respectively.

The same trend was observed in shoot elongation. On the other hands, the highest shoot length was observed when the seeds inoculated with combination of all bacteria followed by cold stratification. In individual applications of bacterial, higher shoot length *P. putida* and *E. cloacae* had the least effect on shoot elongation (Fig. 7). The lowest and highest shoot length values in treatment of mixture bacteria and 4 months stratified belonged to Makidi and Fandoglou with 9.48, 12.81 cm, respectively.

Seed vigor index (SVI): The inoculation of different bacterial strains and their mixtures significantly influenced seed vigor index. The results of effect of different bacterial treatments on SVI in three ecotypes of *C. avellana* are shown in (Fig. 8). Treatment of Makesh, Fandoglou and Makidi seeds inoculated with mixture of three bacteria, gave the highest vigor index of 160.77, 151.18 and 134.77, respectively. Among inoculated bacteria in state on individual applications, *P. putida* had the highest effect on SVI. Seed vigor index was increased with increasing CS periods in both PGPRs inoculation and un-inoculation.

Discussion

Hazelnut is a most important species in northern Iran but the knowledge of pre-sowing treatment of its seed has been so far limited. Prerequisites for its high germination capacity and emergence rate are good quality of seed as well as choice of appropriate pre-sowing treatment (Stejskalová et al., 2015).

In the present study the positive influences of PGPR strains in various species were clearly determined when applied (Minaxi et al., 2012). The bacterial strains evaluated in this work were *P. putida*, *B. subtilis* and *E.cloacae*. Of course, in individual application of bacteria, the highest germination trait was obtained with *P. putida* in all treatmants. The most important characteristics of *Pseudomonas* spp. is production of IAA, siderophores, HCN, ammonia, exo-polysaccharides and phosphate solubilization (Ahemad & Khan, 2012; Ahemad & Khan, 2011). Mafia et al. (2009) reported the highest evaluation for seed germination percentage (GP) in tests on *Eucalyptus* grandis as a result of seed inoculation with *Pseudomonas fulva*, which produced an increase compared to the control. *Bacillus subtilis* was the most effective treatment for E. globulus, promoting a significant increase in seed germination. *B. licheniformis*, *Sinorhizobium saheli*, *S. kostiense* inoculation both individually or as a co-inoculant had a positive effect on germination traits (Singh et al., 2011). Previous studies have shown that bacterial inoculants were able to improve germination rate (Mafia et al., 2009; Kazaz et al., 2010; Ahmadloo et al., 2014), plant growth, responses to external stress factors and protect plants from disease. The improvement in seed germination by PGPR was also found in *Crataegus pseudoheterophylla* (Ahmadloo et al., 2014), *Acacia senegal* (Singh et al., 2011), *Rosa damascene* (Kazaz et al., 2010) and *Abies* spp. (Zulueta-Rodríguez et al., 2015), whereas PGPRs induced increased seed emergence in some cases up to 100% greater than control (Maifa et al., 2009). These findings may be due to the increased synthesis of hormones like gibberellins and better synthesis of auxins (Ahmad & Khan, 2012) which would have triggered the activity of specific enzymes that promoted early germination, such as amylase, which have brought an increase in availability of starch assimilation (Bharathi et al., 2004).

Patten & Glick (2002) found that the PGPR bacteria may enhance the growth the radicle of seedlings by inducing of phytohormones production such as auxins (usually IAA). In addition, significant promotion in root and shoot vigor would have occurred by better synthesis of auxins. In our investigation in the case of application of bacterial as individual, the highest germination speed was obtained in inoculated seeds with *P. putida* and *B. subtilis*. Emergence was happened faster in inoculated seeds with *P. putida* and integrated application. In these conditions the mean germination time (MGT) decreased. This result is similar with the findings of Ahmadloo et al. (2014) who assessed the inoculation effect of PGPRs *Azospirillum lipoferum*, *Pseudomonas fluorescens, Bacillus subtilis* on germination of *Crataegus pseudoheterophylla*. We observed that inoculated seeds resulted in better germination, and also enhanced in germination speed compared to control in all ecotypes. Morpeth & Hall (2000) reported that

microorganisms accelerated seed germination by macerating the hard-coated seed pericarp.

Among the treatments, the highest root and shoot length and seed vigor index was observed in integrated applications of *P. putida*, *B. subtilis* and *E. cloacea* followed by 4 months cold stratification in all ecotypes. It may be stated that integrated application of P solubilizing and N fixing bacteria as well as their ability produce growth-promoting substances (such as synergistic effect of IAA) to increase the root and shoot length and seed vigor index. Similarly, improving effect of inoculation with the mixture of the microorganism (synergistic effect of bacteria) was reported on legumes (Zaidi et al., 2003) and *Crataegus pseudoheterophylla* (Ahmadloo et al., 2014).

El-Refaey & El-Dengawy (2005) reported that cold stratification affected phosphate metabolism in seeds. Cold stratification increases levels of organic phosphates such as fructose 2, 6-biphosphate, ATP, nucleotides and differential changes in enzyme concentrations (Bewley & Black, 1994; Garcia-Gusano et al., 2004). Also, during cold stratification, a significant increase in the level of phosphate pathway enzymes coincided with breaking seed dormancy (Gosling & Ross, 1980; Han et al., 2010). For this reason, in the present study, phosphate-solubilizing bacteria (*Pseudomonas* and *Bacillus*) had a greater effect than *E. cloacea* on seeds germination. Zimmer & Bothe (1989) reported that the promoting effect of *Pseudomonas* is due to more production of plant growth regulators such as auxin and gibberellin. In our study, the increase of cold stratification period from 0 to 4 months, without any other treatment, caused an analogous significant increase in germination percentages of *C. avellana* seeds.

We found differences in germination percentage, speed, root and shoot length and seed vigor index among ecotypes. Variability in seed germination of ecotypes may be due to local genetic adaptation, climatic conditions or the environment conditions during flowering and seed maturation (Baskin & Baskin, 2008). Likewise, it can be stated that one of the factors influencing the level of seed dormancy is the environment conditions under which the seeds are developed on the parent plant (Fenner & Tompson, 2005).

Conclusion

The role of Plant Growth Promoting Rhizobacteria (PGPR) and cold stratification on germination traits and early growth of three hazelnut ecotypes were determined in our finding. Overall, results indicated that PGPR inoculant and cold stratification treatment and their interaction had a positive effect on seed germination traits and early growth. In addition, it is confirmed that PGPR can be used in combination with cold stratification to overcome seed dormancy. Makesh and Fandoglou ecotypes had the highest seed germination and seed vigor index. Finally, PGPR inoculation followed

by 4 months CS can be recommended as a good treatment to break seed dormancy, improvement of seed germination treats and early growth of *Corylus avellana*.

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Appendix

Table 1. Geographic and climatic details of the selected seed sources of Corylus avellana

Region	Elevation (m)	Latitude (°S)	Longitude (°E)	Aspect	Climate type*
Guilan	1550-1620	37° 43′ 02"	47° 53′ 10"	Southern	Cold Humid with 2 months dry season
Ardebil	1430-1470	38° 19′ 16"	48° 36′ 28"	Southwest	Cold Semi-humid with 3 months dry season
Arasbaran	1470- 520	38° 51′ 15"	48° 39′ 17"	Southern	Hyper-cold Semi-humid with 3 months dry season

*Climate zones were calculated based on the Demarton formula

Table 2. Quantitative and qualitative measurements of seeds on ecotypes studied.

Ecotype	Nut length (cm)	Nut width (cm)	Nut weight(g)	Kernel weight (g)	Non-kernel seed (%)	Kernel moisture content (%)	Viability (%)
Makesh	1.88±0.11	1.45±0.12	2.42±0.29	0.66±0.29	8.1±0.92	7.81±4.9	90.80±10.9
Fandoglou	1.61±0.09	1.55±0.14	1.77±0.21	0.57±0.21	17.6±2.35	5.13±3.35	80.10±9.80
Makidi	1.48±0.15	1.42±0.23	1.42±0.41	0.38±0.19	31.2±3.40	4.86±3.60	71.80±12.60

Data presented in the table are Mean± S.D.

Table 3. Two ways ANOVA with PGPR inoculants and stratification on germination traits of seeds

S.O.V	df	Germination percentage	Germination speed	Root length	Shoot length	Vigor index
Ecotype (E)	2	11.18**	70.22**	11.57**	34.94*	17.61**
Cold stratification (CS)	2	609/16**	634.87**	12.08**	82.80**	412.22**
Bacteri (B)	4	24.630**	56.67**	58.17**	66.80**	26.19**
E × B	4	151.58**	19.30*	1.30 ns	0.32 ns	33.32*
E × CS	4	44. 3*	9.72 *	22.34*	27.90**	13.31**
CS × B	8	56.30*	34.13**	6.30**	58.38*	13.31**
E × B × CS	8	32. 2**	12.10*	16.55**	12.26**	44.40**
CV		13.40	9.09	15.19	15.31	17.45

ns: P > 0.05; * P < 0.05; ** P < 0.001.

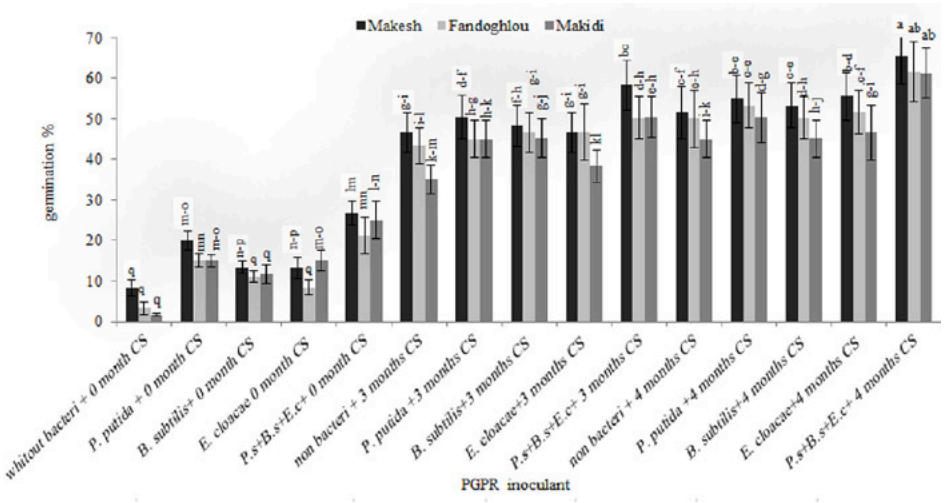

Fig. 1. Effects of bacteria inoculation and cold stratification from 0 to 4 months on germination percentage in ecotypes of Corylus avellana seeds. Mean ±SE to Bars followed by the same letter are not significantly different according to LSD test at (p = 0.05).

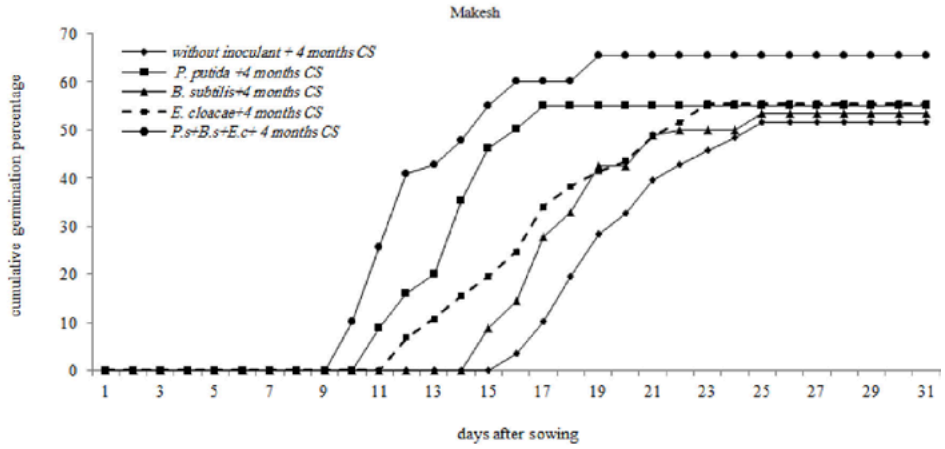

Fig. 2. Cumulative germination percentage in ecotype of Makesh under bacteria inoculation plus 4 months CS.

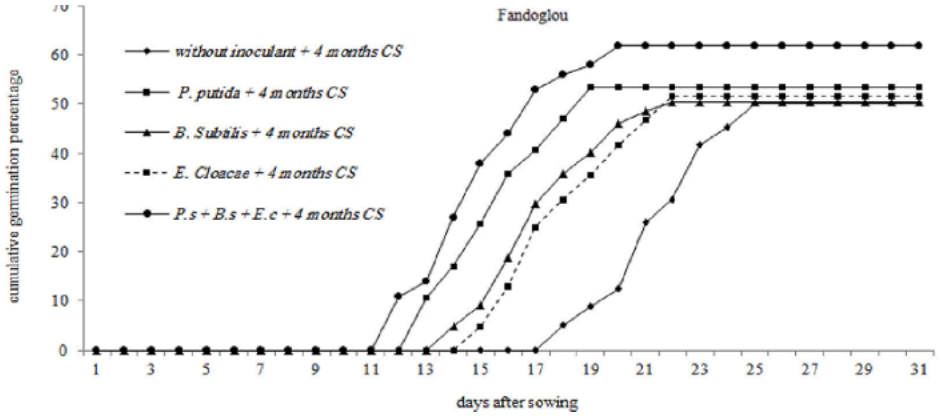

Fig. 3. Cumulative germination percentage in ecotype of Fandoglou under bacteria inoculation plus 4 months CS.

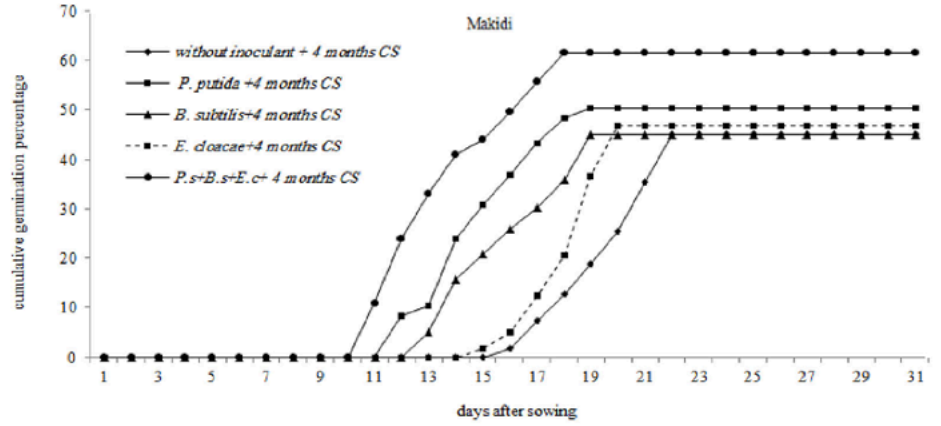

Fig. 4. Cumulative germination percentage in ecotype of Fandoglou under bacteria inoculation plus 4 months CS.

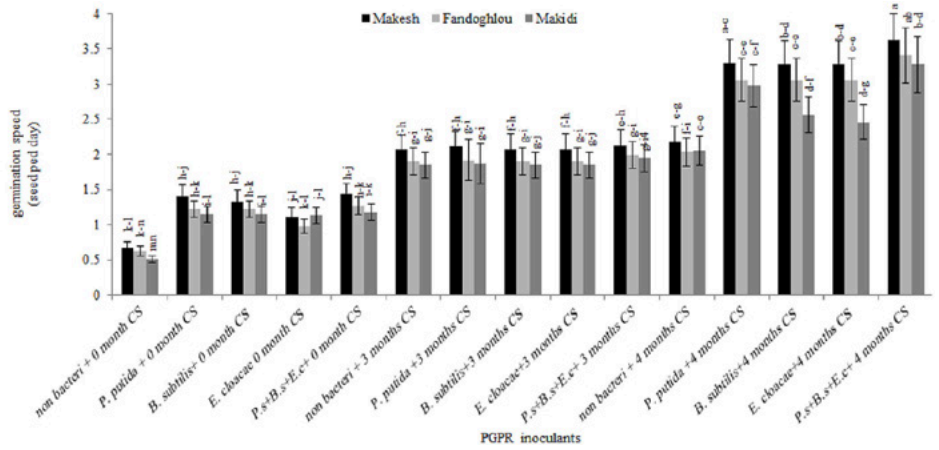

Fig. 5. Effects of bacteria inoculation and cold stratification from 0 to 4 months on germination speed in ecotypes of *C. avellana* seeds. Mean ±SE to Bars followed by the same letter are not significantly different, according to LSD test at (p = 0.05).

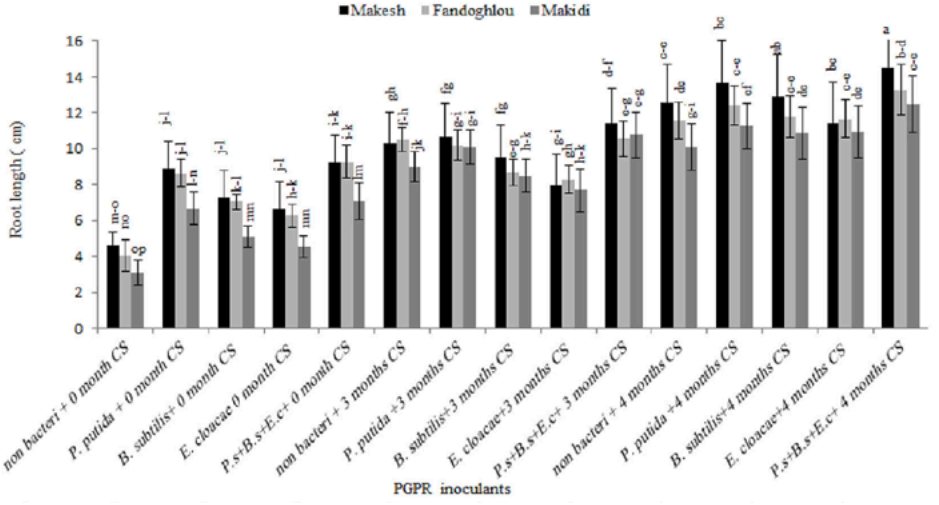

Fig. 6. Effects of bacteria inoculation and cold stratification from 0 to 4 months on root length in ecotypes of Corylus avellana seeds. Mean ±SE to Bars followed by the same letter are not significantly different, according to LSD test at (p = 0.05).

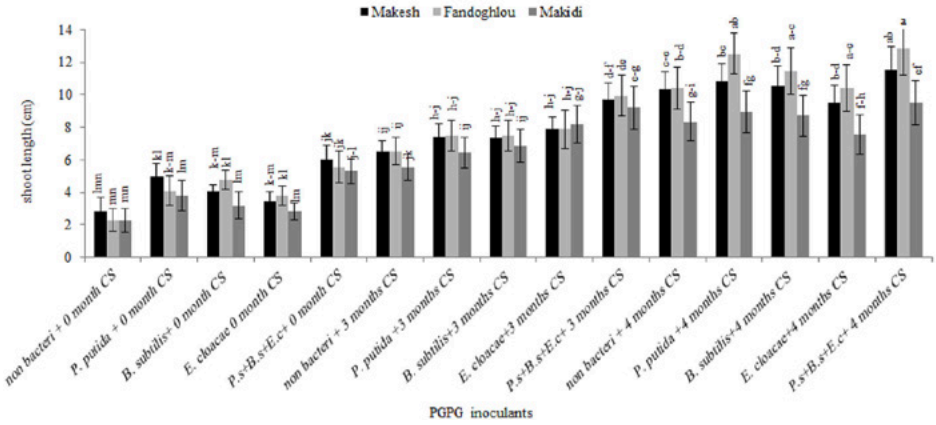

Fig. 7. Effects of bacteria inoculation and cold stratification from 0 to 4 months on shoot length in ecotypes of *Corylus avellana* seeds. Mean ±SE to Bars followed by the same letter are not significantly different, according to LSD test at (p = 0.05).

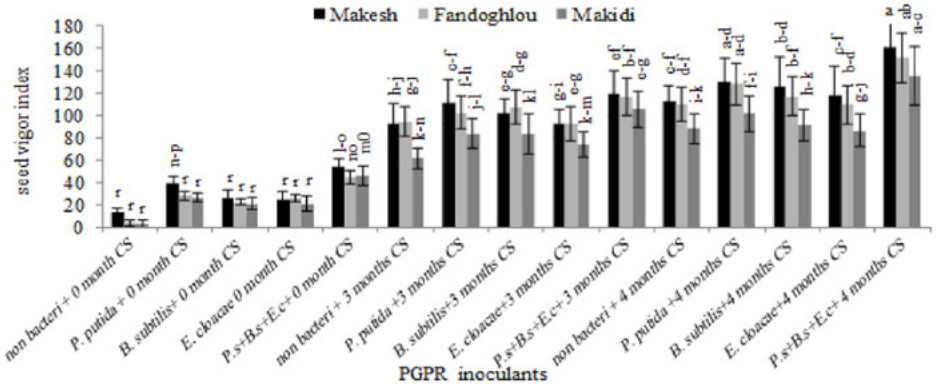

Fig. 8. Effects of bacteria inoculation and cold stratification from 0 to 4 months on seed vigor index in ecotypes of Corylus avellana seeds. Mean ±SE to Bars followed by the same letter are not significantly different, according to LSD test at (p = 0.05).