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Improvement of seed germination and early growth of Caucasian whortleberry (Vaccinium arctostaphylos L.) by plant growth promoting rhizobacteria and cold stratification

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Abstract

Vaccinium arctostaphylos is a threatened species in Caspian forests of Iran that its seeds germinate in a long time due to internal dormancy. The purpose of this research was to investigate the effects of plant growth-promoting rhizobacteria and the cold stratification periods on seed germination of *Vaccinium arctostaphylos.* The seeds were inoculated with growth-promoting bacteria including *Bacillus subtilis, Enterobacter cloacae, and Pseudomonas putida* and combination (co–inoculation) of all strains. Then they were subjected to cold stratification in a refrigerator at 4 ± 1°C for 0, 1, 2, 3, 4 and 5 months. At the end of periods, seeds were sown in polyethylene bags (15 cm×8 cm) containing cocopeat, perlite, and sand (1:1:2) and were placed in greenhouse with temperature of 22 and 25°C, and relative humidity of 60% and 70%, respectively. After 40 days, germination percentage, germination speed, and seed vigor index (SVI) were calculated. Results showed that, bacteria inoculation and their interactions affected germination traits. Germination percentage ranged from 0 to 58.50%. Both inoculation and CS had positive effect on Germination percentage. The highest percentages of seed germination (57.50–58.50%) and speed of germination (2.26 n/d) belonged to co-inoculated seeds by the combination of all bacterial along with 4 and 5 months of cold stratification periods, respectively. The maximum shoot length (23. 25 mm), root length (17.98 mm), and seed vigor index (24.12) were recorded for co-inoculated seeds by the combination of all bacterial inoculants and five months of cold stratification. The results confirmed to overcome seed dormancy, increase of seed germination components, and early seedling growth of *V. arctostaphylos*, Plant growth promoting rhizobacteria is better to be applied in combination with cold stratification.

Introduction

The genus *Vaccinium* is one of the important genera of *Ericaceae*, which is distributed in most of the forest areas of Asia, the Caucasus, Siberia, North America, Europe, and especially in the forest areas of Turkey and Iran (Fischer et al. 2007). Caucasian whortleberry (*Vaccinium arctostaphylos* L.) belongs to the genus *Vaccinium* and family *Ericacea*. It is the only existing species of the genus *Vaccinium* in Iran (Mozaffarian 2005). The most important Caucasian whortleberry natural stands in Iran are located in the Caspian forests. This is typically a multi-stemmed shrub attaining a height of 2.5–3.5 m, with elongated oval or egg-shaped leaves. The flowering period is early to late June. the bisexual flowers are red placed axillary or subterminal in the branches. The fruit is spherical and red to purple-black with a diameter of 8 to 10 mm (Sabeti 1976). The fruit appears in July and matures in September.

V. arctostaphylos is one of the extremely exploited (harvested) species for its fruits as its high potential in the pharmaceutical and spice industries. The fruit of this species has strong antioxidant and medicinal properties due to the presence of phenolic compounds, especially anthocyanin and procyanidin (Karcheva-Bahchevanska et al. 2017). In traditional Iranian medicine, the fruit is used to treat various diseases such as diabetes, high blood pressure, and blood fat (Limaei and Amiri 2024). As a result, it has been considered as an important non-timber forest producing species having the potential income for the rural communities. However, the natural habitats of the species are under high pressure due to

human activities such as deforestation (felling for agricultural expansion and road construction), habitat loss, and irregular harvesting to meet the rising demands of fruits for medicines (Hasanloo et al. 2019).

In addition to overexploitation, there are a number of several factors such as low natural regeneration and limited distribution in forest stands which are responsible for its low population. On the other hand, long dormancy and poor germination rate of the seeds are major limitations to cultivation development and restoration of its natural habitats. Therefore, it is necessary to find effective dormancy-breaking treatments for the mass propagation of this medicinally important species.Different treatments have been reported to break seed dormancy and increase the germination percentage of *Vaccinium* seeds with stratification, as the the most important method (Baskin and Baskin 1998). Cold stratification is used as a treatment to break seed dormancy in *Vaccinium* species. Stratification is a standard and effective method at the temperature of 0 to 10°C to overcome the physiological dormancy of seeds (Baskin et al. 2000). Few studies have been to brake the seed dormancy of Caucasian whortleberry. Seed germination testing showed that chilling seeds for 15 to 90 days could remove *V. arctostaphylos* seed dormancy, even though the highest percentage of germination (11%) was obtained in the treatment of 90 days of chilling (Shahram 2007). The effect of different periods of cold stratification on the percentage of seed germination of *Vaccinium membranaceum* showed that the highest germination (70%) was obtained in the treatment of four weeks (28 days) of cold stratification. (Barney, Shafii, and Price 2001). In another study, the highest germination of blueberry (*Vaccinium myrtillus* L.) was obtained after 15 days of cold stratification (Bilgin and Alım 2021).

Currently, one of the methods used to break seeds dormancy and improve germination traits is the inoculation of seeds with plant growth promoting rhizobacteria (PGPR). Various microorganisms have been identified as PGPR include strains of the genera *Bacillus*, *Azospirillum Enterobacter*, *cinetobacter*, and *Pseudomonas* (Çakmakçi et al. 2006, Ahemad and Kibret 2014) which can help increasing seed germination and seedling vigor. Experiments on the application of PGPR in forest trees are less widespread than in crops. Although, several researches are demonstrating the positive effects of PGPR on tree species. The inoculation of *Bacillus licheniformis* or *Sinorhizobium kostiense* alone and as the co-inoculants supported the maximum growth of the *Acacia senegal* seeds. It is attributed to the fact that *B. licheniformis* produces gibberellins which has a positive effect on seed germination and seedling traits in *Acacia senegal* and *Prosopis cineraria* species (Singh et al. 2011). the application of PGPRs (*Azotobacter chroococcum*, *Azospirillum lipoferum*, *Pseudomonas fluorescens* and *Bacillus subtilis*) on seed germination of *Crataegus pseudoheterophylla* showed 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 (Fatemeh et al. 2014).

Considering that Bacterial inoculation along with with cold stratification may have different mechanisms to breaking dormancy and improving seed germination, this study aimed to evaluate the effect of individual or combined inoculation of different PGPR strains on germination and Early Growth stage of *V. arctostaphylos*.

Methods

Source of seeds and seeds collection

Fresh mature berries were collected from 5 randomly shrubs having mean collar diameter (4.90 ± 0.18 cm); mean height (2.40 ± 0.23 m) in August 2020 from Soha vilage in Namin, Ardabil province, Iran (N 38°16´ 24.21", E 41°16´ 35.33", 1670 m a.s.l.). The seeds were separated by shaking and beating the fruits in the laboratory. To measure the quantitative and qualitative traits of seeds and fruits, 100 seeds each in replicates fruits were selected. Then, their weight was measured with a digital scale with an accuracy of 0.001 g. The seeds length and width were measured with a caliper with an accuracy of 0.1 mm. The seed viability was calculated by tetrazolium test (ISTA 2003) (Table 1)

Table 1
Quantitative and qualitative characteristics of Caucasian Whortleberry fruit and seeds

Fruit width (g)	Fruit length (g)	Fruit weight (g)	Seed width (mm)	Seed length (mm)	1000 Seeds weight (g)	Viability %
9.83 ± 1.23	10.24 ± 1.3	0.29 ± 0.05	1.02 ± 0.12	2.51 ± 0.35	3.12 ± 0.19	80.9 ± 7.55

Inoculation of seeds with PGPR strains and cold stratification

The PGPRs strains, *Bacillus subtilis strain FzB24* (B), *Enterobacter cloacae* (E), and *Pseudomonas putida* 169 (P) were obtained from the soil microbiology department of Soil and Water Research Institute (SWRI), Iran. The healthy seeds were selected by flotation from the damaged and empty ones. Then, the surface disinfection of seeds was done using 0.5% sodium hypochlorite for 10 min and washed withstrile distilled water four times. The seeds 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) *Bacillus subtilis strain FzB24*, (2) *Enterobacter cloacae* strain 12 (E), (3) *Pseudomonas putida* strain 169 (P), (4) combination of three bacteria (B + E + P), and control (non inoculation). Inoculated seeds were storedin plastic bags containing sterilized perlite and subjected to cold stratification in a refrigerator at 4 ± 1°C for 0, 1, 2, 3 ,4 and 5 months.

Seed germination

At the end of cold stratification (CS) periods, the seeds were sown in polyethylene bags (5cm diameter, 10 cm height) containing a mixture of cocopeat, perlite, and sand (1:1:2). The physical ans chemical properties of this medium have given in Table 2. The planted seeds were placed in the greenhouse with temperature of 20 and 24°C, and relative humid of 60% and 70%. The Seeds were irrigated three times a week.

Table 2
physical and chemical Characteristics of planting substrate cocopeat: perlite: sand (1:1:2).

BD (g/cm^3)	EC (dS /m)	pH	OC (%)	N (%)	P (mg/ L)	K (mg/ L)	Fe (mg/ L)	Zn (mg/ L)
0.72	0.91	6.5	3.21	0.17	91	484	18	12

BD: Bulk density, EC: Electrical conductivity, pH: Acidity, OC: Organic Carbon, N: Total nitrogen. K: Potassium, Fe: Iron, Zn: Zink

Seed germination

The seeds were considered as germinated when the radicle length was more than 2 mm (ISTA 2003). Observations were recorded daily regarding germinated/non-germinated seeds up to 40 days. Percentage of germination (PG), germination speed (GS), the mean germination time (MGT), and Seed Vigor index (SVI) were determined according to equals number 1 to 4, respectively (Panwar and Bhardwaj 2005). N is total number of sown seeds, n is number of germinated seeds on day, d is the number of days counted from the beginning of germination, SL and RL is shoot and root length, respectively. to determine the length of root and shoot in the studied treatments, 5 samples were randomly selected from each replicate, and then the shoot and root were separated and measusred with a caliper with an accuracy of 0.1 mm.

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

$GS=\sum (n /d)$ (2)

$MGT = \Sigma(dn)/\Sigma n$ (3)

SVI = PG × Mean (SL + RL) / 100 (4).

Data analysis

This experiment was carried on afactorial completely randomized design, with four replications of 50 seeds in each treatment. Homogeneity and normality of data were determined (checked) using Levene's and Shapiro -Wilk tests. Two-way analysis of variance (ANOVA) was used to determine the significant effect of treatments on germination traits (germination percentage, germination speed, Seed Vigor index, and shoot and root length). The means comparison was determined by Tukey's test at $p \leq 0.01$. Excel software (2016) was used to draw all the graphs.

Results and Discussion

The analysis of variance demonstrated that PGPRs inoculant, Cold stratification periods and their interaction effects on germination traits were significant (Table 3, $P < 0.05$). Seed inoculation

significantly increased seed germination, germination speed, mean germinayion time, seed vigor index, and root and shoot length of *Caucasian whortleberry*.

Table 3
Analysis of variance for PGPR inoculants and stratification on germination traits of Caucasian whortleberry seeds

Treatment	Source variance	DF	F	P
PGPRs inoculant	Germination percentage	4	511.2	0. 011**
	Germination speed	4	19.56	0.025**
	Mean germination time	4	8.56	0.012*
	Root length	4	3.10	0.042*
	Shoot length	4	18.96	0.020**
	Seed Vigor index	4	10.23	0.000***
Cold stratification	Germination percentage	5	833.78	0.000***
	Germination speed	5	26.90	0.004**
	Mean germination time	5	10.34	0.043*
	Root length	5	114.42	0.000***
	Shoot length	5	78.64	0.008**
	Seed Vigor index	5	2.11	0.045*
PGPRs inoculant * stratification	Germination percentage	20	264.44	0.015**
	Germination speed	20	44.56	0.029**
	Mean germination time	20	7.79	0.019*
	Root length	20	3.61	0.031*
	Shoot length	20	45.89	0.020**
	Seed Vigor index	20	128.90	< 0.044**
* P < 0.05; ** P < 0. 01 and *** P < 0.001				

Effect of bacterial inoculation and cold stratification on germination percentage (GP)

The percentage of seed germination was 9, 7. And 6.5 I the seeds which inoculated by *P. putida*, *E. cloacea* and *B. subtilis*, 9, 7 and 6.5%, respectivelyThe non inoculated seeds did not germinate (Fig. 1). *P. putida* increased the seed germination percentage more than *B. subtilis* and *E. cloacea*.

Many researches have shown that PGPR inoculations can increase the germination traits in many seeds of woody species such as *Acacia senegal* (Singh et al. 2011). *Crataegus pseudoheterophylla* (Fatemeh et al. 2014), *Abies religiosa* and *A. hickelii* (Zulueta-Rodríguez et al. 2015), *Corylus avellana* (Rostamikia et al. 2016), *Acasia mearnsii* (São José et al. 2019), and *Abies nordmanniana* (Garcia-Lemos et al. 2020).

The effect of different bacteria is various on seed germination and plant growth (Basu et al. 2021). In this context, it can be stated *pseudomonas* strains by affecting different parts of the seed, playing a role in the biosynthesis and secretion of pklant growth hormones and reducing the ratio of abscisic to gibberellin, which breaks embryo dormancy and stimulates germination (Miransari and Smith 2014). It is a well-known that inoculation of seeds with PGPB strains increases the uptake of water and nutrient and effective synthesis of auxins, gibberellins, and reduces inhibitors such as ABA in the seed endocarp (de Andrade et al. 2023).

The results showed the mixed inoculion of bacteria had better effect compared to individual inoculation on germination traits. The application of cmixed inoculum (*B. subtilis, E. colacea and P. putida*) increased the germination percentage to 14 showing the synergic effect of bacteria. Similar to our findings, the co-inoculation seeds of C. *pseudoheterophylla* with *Azotobacter chroococcum*, *Azospirillum lipoferum*, *Pseudomonas fluorescens* and *Bacillus subtilis* increased seed germination (Fatemeh et al. 2014).

The germination was 6.5, 16, 18, 40 and 40.5% in seeds, which stratified for 1, 2, 3, 4, and 5 months, respectively while non-stratified seeds did not germinate in the control (Figure.1). a period of 1, 2 and 3 months of cold stratification was insufficient to induce percentage of germination in *V. arctostaphyos* seeds. While 4 and 5- month cold stratification resulted significant increase in seed germination percentage, which indicates the presence of physiological dormancy in the seed, which controls a significant part of the seed dormancy in this species. the highest germination (70%) of *V. membranaceum* seeds was obtained by 28 days stratification at 1–3°C (Barney, Shafii, and Price 2001) and the maximum germination of seeds of *V. myrtillus* and *V. vitis-idaea* were 62 and 100%, respectively, in light at 20:10°C and 25:15°C (Baskin et al. 2000). The chilling reduces the content of abscisic acid (ABA) by increasing the content of gibberellic acid. These changes are made simultaneously as creating a balance between two hormones (Bewley and Black 2013). It is possible that the cold factor, in addition to stimulating the endogenous gibberellin synthesis, activates other stimuli that increase the percentage of seed germination (Chen et al. 2009). Also, during Cold stratification, a significant increase in the level of phosphate pathway enzymes coincided with breaking seed dormancy.

The results of our study indicated the highest germination percentage was observed in seeds co-inoculated by three bacteria followed by 5 -months cold stratification period with 58.5% that did not differ significantly ($p > 0.05$) from the GP (57.5%) of the coi-noculation of three bacteria followed by 4-month CS period pretreatment (Fig. 1).

Efect of bacterial inoculation and cold stratification on germination speed (GS) and mean germination time (MGT)

In general, germination speed is considered one of the most important factors affecting plant establishment (Chen et al. 2007). The results showed that the inoculation of PGPRs, period of CS and the interaction of these factors had a significant effect on the germination speed (Table 1). when the seeds were inoculated with PGPR individually and mixed, the germination rate increased. So that, the highest germination speed (2.26 n/d) was observed in seeds co-inoculated by three bacteria followed by 5-months cold stratification (Fig. 2). Earlier studies have indicated that bacterial inoculums are able to increase the germination speed. In this context, the highest germination speed (4.82 n/d) was obtained in *Crataegus pseudoheterophylla* seeds using the combination of all bacterial of *Azotobacter chroococcum*, *A. lipoferum*, *Pseudomonas fluorescens* and *Bacillus subtilis* (Fatemeh et al. 2014). these findings may be due to the increased synthesis of hormones like gibberellins and better synthesis of auxins, which have a positive effect on germination speed (Ahemad and Kibret 2014).

The results of our study showed, the minimum of mean germination time (MGT) was observed in seeds co-inoculated followed by 5-month CS period with 13.84 day. The mean germination time (MGT) was 25.50, 28.26 and 32.92 days when the seeds were inoculated by *P. putida*, *E. colacea* and *B. subtilis*, respectively. Co-inoculation decreased the mean germination time (MGT) compared in individual inoculation (Fig. 3). *P. putida* and *B. subtilis* are among the most important phosphate solubilizes (Kalayu 2019).

In CS treatments (without inoculation), the increase in CS period (from 1 to 5 months) led to a gradual decrease in MGT. These results confirmed the importance of the cold stratification in accelerating the growth of the embryo, increasing the germination speed and reducing the germination period.

Effect of bacterial inoculation and cold stratification on root and shoot length and seed vigor index

Different strains of rhizobacteria had variable effects on root and shoot length in various ecotype assays. Individual inuculation of bacteria, the maximum root and shoot length were observed in *P. putida* with 8.74 and 12.30 mm, respectively (Fig. 4). Similary, Rostamikia et al. (2016) reported the increase in root and shoot of hazelnut (*Corylus avellana* L.) inoculated with *Pseudomonas putida.* Chaín et al. (2020) also observed significant root and stem growth in seedlings of *Eucalyptus grandis* inoculated with two *Pseudomonas* strains after 50 days. The most important characteristics of *Pseudomonas* spp. is production of auxines, siderophores, hydrogen cyanide (HCN), ammonia (NH3), exo-polysaccharides, and also solubilization of phosphate (Costa-Gutierrez et al. 2022). Plant hormones such as auxins, gibberellins, and cytokinins produced by rhizobacteria play an important role in cell division, and root and shoot elongation (Chaín et al. 2020).

In each bacterial treatment, the increase in CS period led to a gradual elongation in root and shoot length. tThe highest root length was allocated to the seeds co-inoculated by bacteria followed by 5-month cold stratification with 17.98 mm (Fig. 4). The same trend was observed in shoot length. The maximum shoot length was observed when the seeds co-inoculated by bacteria followed by 5 months CS with 23.25 mm (Fig. 5).

Seed vigor is the most important indicator for determining seed quality, the potential for fast and uniform emergence of seedlings (Wen et al. 2018). In this study, seed germination index was significantly affected by individual and combined inoculation of different bacterial strains. The results of variation bacterial treatments of *V. arctotaphylos* are presented in Fig. 6. the highest value of seed germination index was obtained in the treatment of seeds inoculated with the combination of three bacteria. Among inoculated bacteria in state on individual applications, *P. putida* had with 18.30 the highest effect on SVI. Seed vigor index was improved with increasing CS periods in both PGPRs inoculation and un-inoculation. So that, the highest value of seed germination index with 24.12 belonged to the appliction of combined inoculation three bacteria (*B. subtilis, E. colacea and P. putida*) with 5 months CS treatment (Fig. 6). The reason for the increase in the seed vigor index can be related to the movement of nutrient reserves, the activity and resynthesis of some enzymes, the start of the resynthesis of RNA and DNA, and the rapid growth of the embryo after the elimination of the barriers to germination

Conclusion

In this study, the effects of PGPRs strain inoculation and cold stratifications periods on seed germination behavior of *V. arctotaphylos* were evaluated. The results showed that all treatments increased the components of germination of *V. arctotaphylos* seeds. Non-stratified seeds did not germinate in the control treatment confirming the existence of physiological dormancy in *V. arctotaphylos* seeds. Overall, PGPRs strain when applied as individual or mixed inocloum, have considerable outcomes on seed germination and early growth. it can be claimed that the effect of bacteria on seedling growth is much greater in compared to cold stratification alone or combined with cold stratification. Although the cold stratification can be considered as one of the most important treatments for stimulating germination, but inoculation with bacteria activities a set of processes leading to germination over timeIn conclusion, bacteria provide germination stimuli and removiephysiological barriers increasing the growth of root and shoot and helping establishment and subsequent growth, a process that cold stratification cannot do in alone. Finally, the co-inoculation of bacteria (*B. subtilis, E. colacea and P. putida*) along with 5months CS treatment can be recommended as a suitable treatment to break dormancy and increase of germination of *V. arctotaphylos* seeds.

Declarations

Author Contribution

Younes Rostamikia (Author 1): Data curation, Formal analysis, Project administration, Software, Writing – original draft.Maryam Teimouri (Author 2): Conceptualization, Funding acquisition, Methodology, Validation, Writing – review & editing.Farnoosh Jafari (Author 3): Investigation, Supervision, Resources, Visualization.

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Mexican fir tree species in danger of extinction. Forests 6(9):3109–3122Enrique Troyo-Diéguez, and Miguel V Córdoba-Matson

Figures

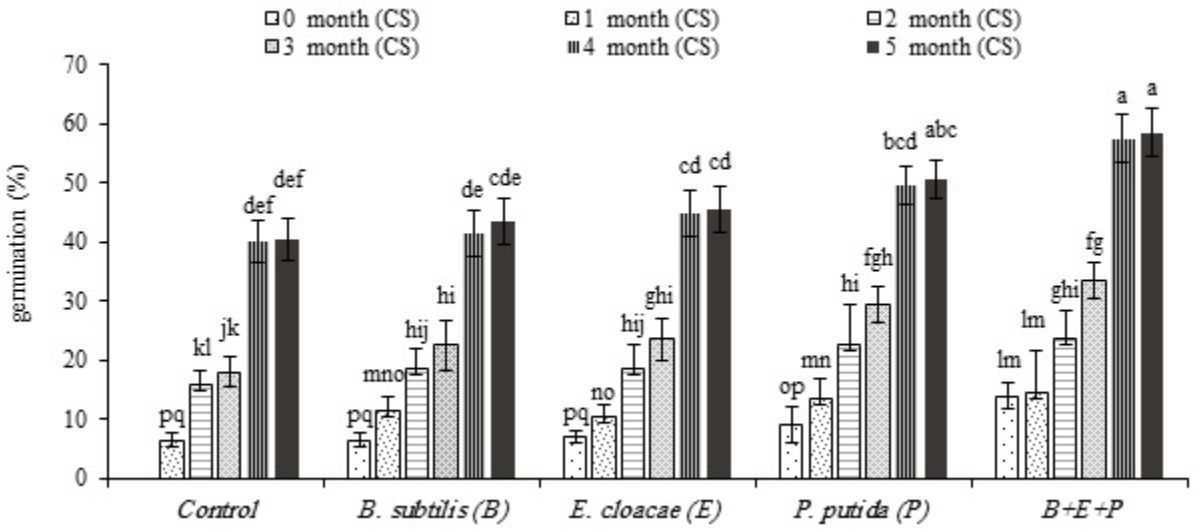


Figure 1

Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on seed germination of *Vaccinium arctostaphylos*. The mean ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

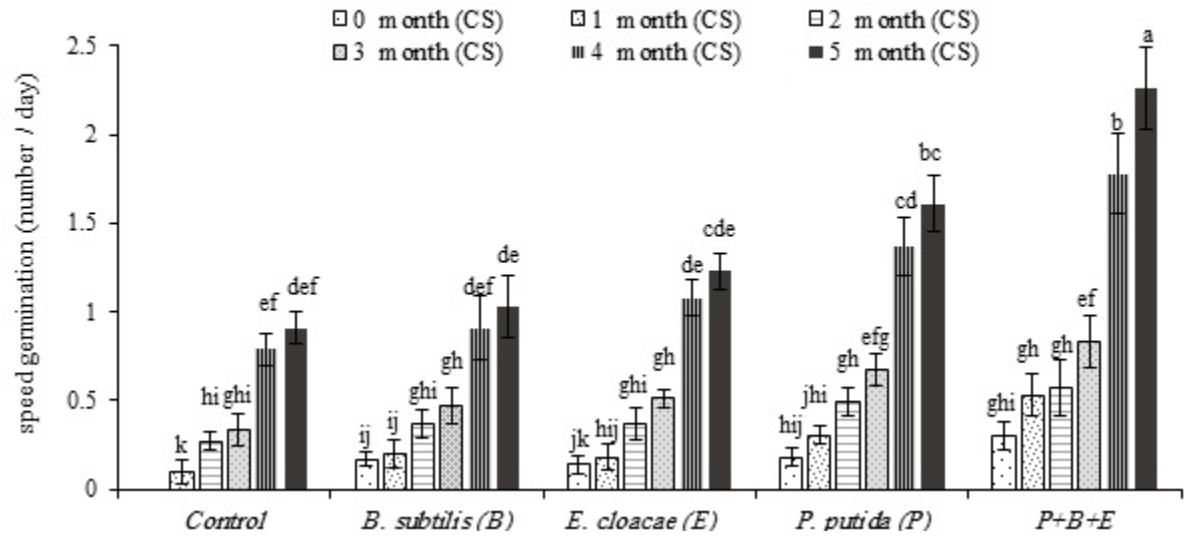


Figure 2

Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on germination speed of *Vaccinium arctostaphylos* seeds. The mean ±SE to Column followed by the same letter are not

significantly different, according to Tukey's test at (p = 0.05).

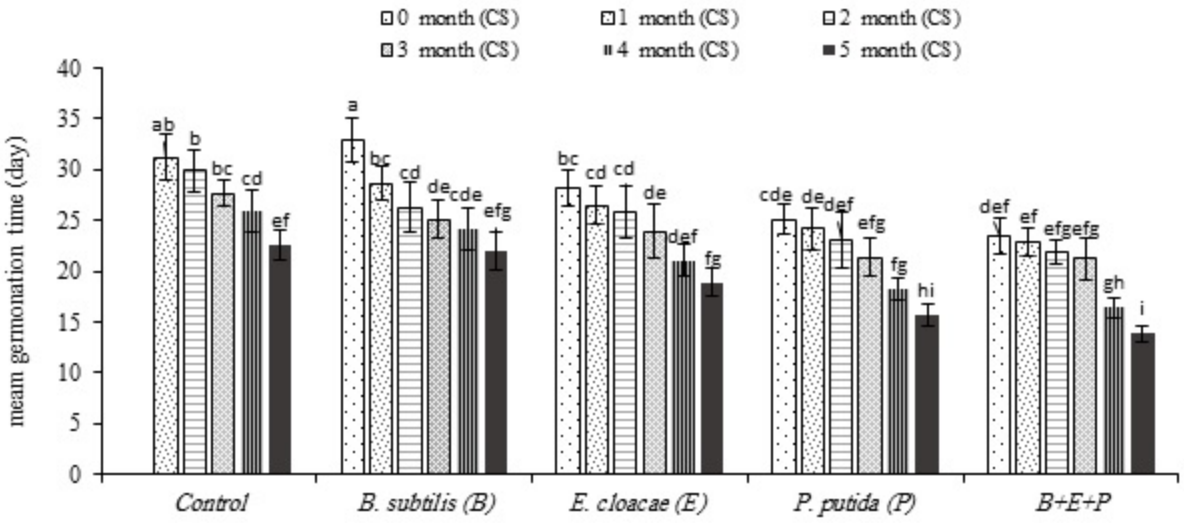


Figure 3

Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on mean germination time *Vaccinium arctostaphylos* seeds. The mean ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

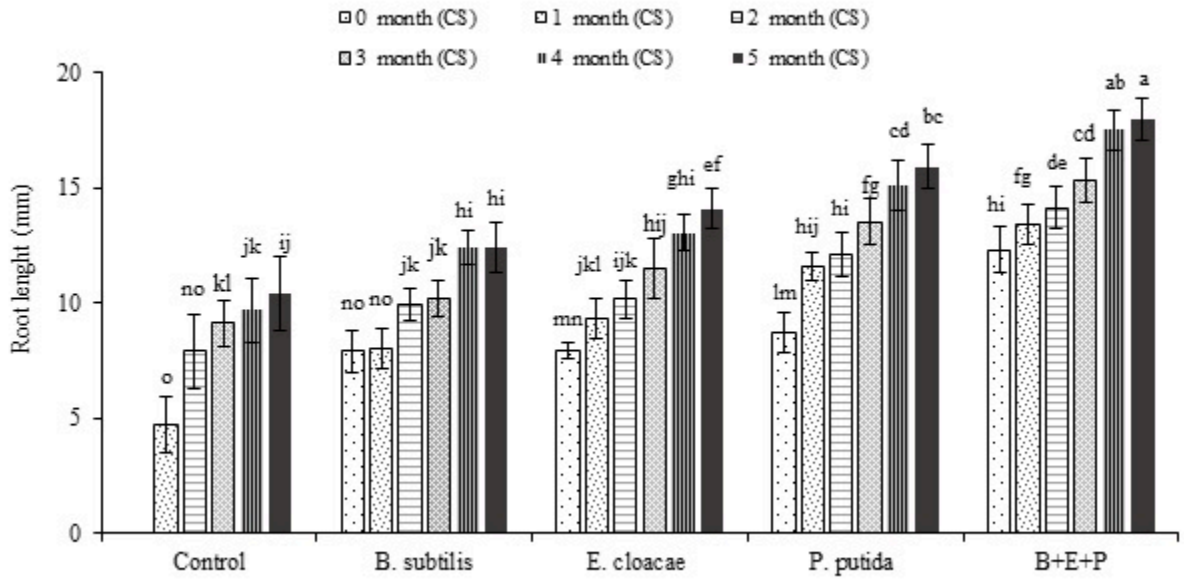


Figure 4

Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on root length of *Vaccinium arctostaphylos*. The mean ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

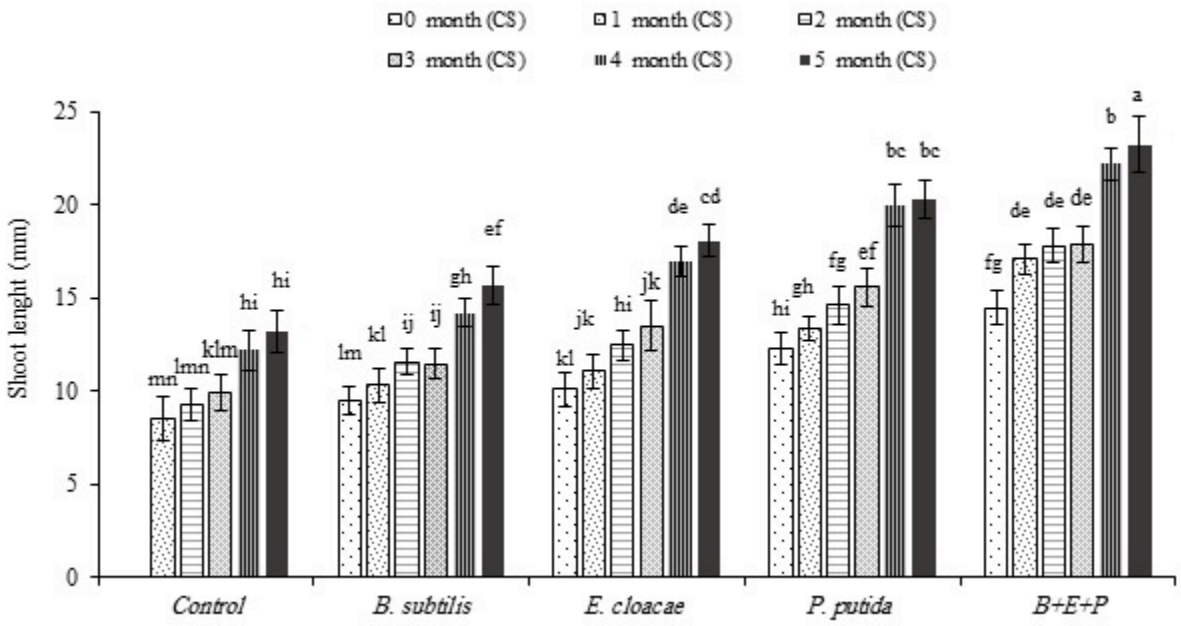


Figure 5

Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on shoot length of *Vaccinium arctostaphylos*. The mean ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

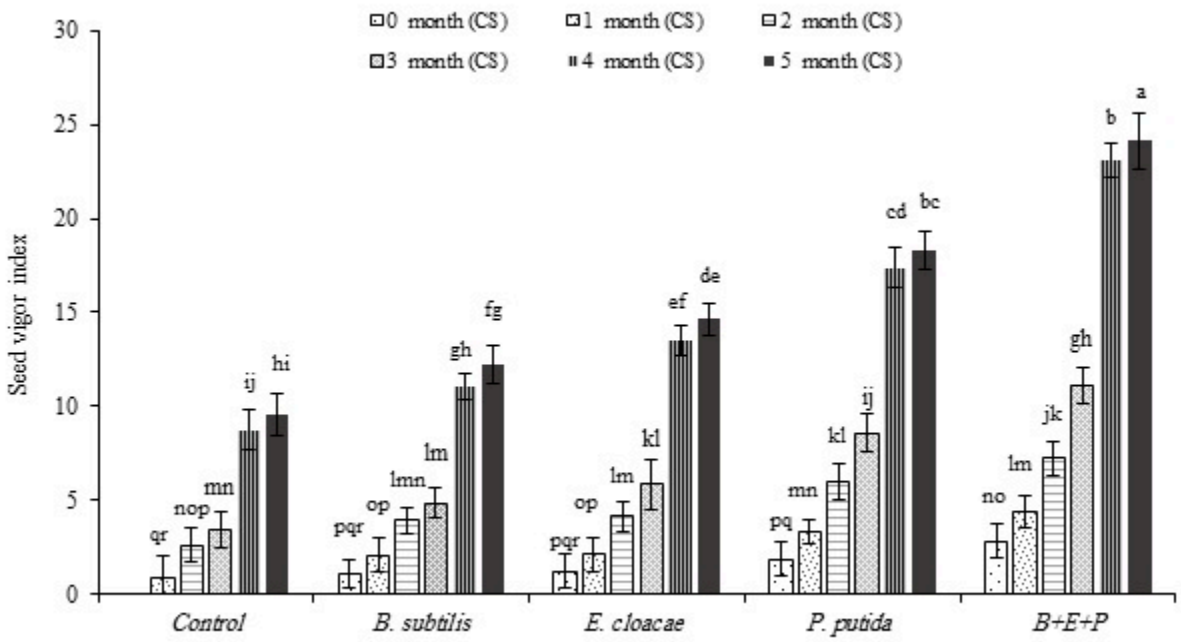


Figure 6

Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on seed vigor index (SVI) of *Vaccinium arctostaphylos*. The mean ±SE to Column followed by the same letter are not significantly

different, according to Tukey's test at (p = 0.05).