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Improving Seed Germination and Early Growth of Caucasian Whortleberry (*Vaccinium arctostaphylos* L.) by Plant Growth Promoting Rhizobacteria (PGPR) and Cold Stratification

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ABSTRACT

Aims: 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.*
Material & Methods: The seeds were inoculated with growth-promoting bacteria, including Bacillus subtilis, Enterobacter cloacae, and Pseudomonas putida, as well as a 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 each period, seeds were sown in polyethylene bags (15 cm × 8 cm) containing cocopeat, perlite, and sand (1:1:2) and placed in a greenhouse with temperatures of 22°C 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.
Findings: Results indicated that bacterial inoculation and their interactions influenced germination traits. Germination percentage ranged from 0 to 58.50%. Both inoculation and CS positively affected germination percentage. The highest seed germination rates (57.50-58.50%) and germination speed (2.26 n. d^{-1}) were observed in co-inoculated seeds with all bacteria combined and 4 to 5 months of cold stratification, 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 with all bacterial inoculants and 5 months of cold stratification.
Conclusion: The results confirmed overcoming seed dormancy, increased seed germination components, and early seedling growth in *V. arctostaphylos*. Plant growth-promoting rhizobacteria are more effective when combined with cold stratification.

Keywords: Caucasian Whortleberry; Pseudomonas Putida; Seed Dormancy; Vigor Index.

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Introduction

The genus *Vaccinium* is one of the important genera of *Ericaceae*, distributed across most forest areas of Asia, the Caucasus, Siberia, North America, and Europe, and especially in the forest regions of Turkey and Iran [1]. Caucasian whortleberry (*Vaccinium arctostaphylos* L.) belongs to the genus *Vaccinium* and family *Ericacea*e. It is the only species of the genus *Vaccinium* known to occur in Iran [2]. The most important Caucasian whortleberry natural stands in Iran are located in the Caspian forests and Ardabil Province. This is typically a multi-stemmed shrub, reaching 2.5-3.5 m in height, with elongated, oval, or egg-shaped leaves. The flowering period is early to late June. The bisexual flowers are red and are axillary or subterminal on the branches. The fruit is spherical and red to purple-black, with a diameter of 8-10 mm [3]. The fruit appears in July and matures in September.

V. arctostaphylos is one of the extremely exploited (harvested) species for its fruits due to 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 [4]. In traditional Iranian medicine, the fruit is used to treat various diseases such as diabetes, high blood pressure, and high blood fat [5]. As a result, it has been considered an important non-timber forest product species with the potential to generate income for rural communities. However, the natural habitats of the species are under high pressure from human activities, including deforestation (felling for agricultural expansion and road construction), habitat loss, and irregular harvesting to meet the rising demand for fruits for medicinal purposes [6].

In addition to overexploitation, several factors, such as low natural regeneration and limited distribution within forest stands, contribute to its low population. On the other hand, long dormancy and low germination rates of the seeds are major limitations to cultivation development and the restoration of their natural habitats. Therefore, it is necessary to find effective dormancy-breaking treatments for the mass propagation of this medicinally important species. Various treatments have been reported to break seed dormancy and increase the germination percentage of *Vaccinium* seeds, with stratification being the most effective [7]. Cold stratification is used as a treatment to break seed dormancy in *Vaccinium* species. Stratification is a standard and effective method for overcoming seed physiological dormancy at 0-10 °C [8]. Few studies have been conducted to break the seed dormancy of the Caucasian whortleberry. Seed germination testing showed that chilling seeds for 15 to 90 days could remove *V. arctostaphylos* seed dormancy, even though the highest germination percentage (11 %) was obtained with 90 days of chilling [9]. 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 with 4 weeks (28 days) of cold stratification [10]. In another study, the highest germination of Blueberry (*Vaccinium myrtillus* L.) was obtained after 15 days of cold stratification [11]. Currently, one method used to break seed dormancy and improve germination traits is inoculating seeds with plant growth-promoting rhizobacteria (PGPR). Various microorganisms have been identified as PGPR, including strains of the genera *Bacillus*, *Azospirillum, Enterobacter*, *Cinetobacter,* and *Pseudomonas* [12-14], which can help

increase seed germination and seedling vigor. Experiments on the application of PGPR in forest trees are less widespread than in crops. However, several studies 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 have a positive effect on seed germination and seedling traits in *Acacia senegal* and *Prosopis cineraria* species [15]. 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 $n.d^{-1}$) were recorded for co-inoculated seeds by the combination of all bacterial inoculants and the alternate temperature stratification regime [16].

Physiological dormancy markedly constrains the germination of *Vaccinium arctostaphylos* seed under natural conditions, thereby representing a substantial impediment to the efficient utilization of this species beyond its native range [7]. Considering that Bacterial inoculation, along with cold stratification, may act through different mechanisms to break dormancy and improve seed germination, this study aimed to evaluate the effects of individual or combined inoculation with different PGPR strains on germination and early growth of *V. arctostaphylos*.

Materials & Methods

Source of Seeds and Seed Collection

Fresh mature berries were collected from 5 randomly selected shrubs having a mean collar diameter (4.90± 0.18 cm); mean height (2.40±0.23 m) in August 2020 from Soha Village in Namin, Ardabil Province, Iran (N 38° 16’ 24.21” E 41° 16’ 35.33“, 1670 m above sea level). 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 per replicate were selected. Then, their weight was measured with a digital scale accurate to 0.001 g. The seeds’ length and width were measured with a caliper with an accuracy of 0.1 mm. Seed viability was determined by the tetrazolium test (Table 1).

Inoculation of Seeds with PGPR Strains and Cold Stratification

The PGPR strains, *Bacillus subtilis FzB24* (B), *Enterobacter cloacae* (E), and *Pseudomonas putida* 169 (P), were obtained from the Soil Microbiology Department of the Soil and Water Research Institute (SWRI), Iran. The healthy seeds were selected by flotation from the damaged and empty ones. Then, the seeds were surface-disinfected with 0.5% sodium hypochlorite for 10 min and washed 4 times with sterile distilled water. 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 *Bacillus subtilis* strain FzB24, *Enterobacter cloacae*

Table 1) Quantitative and qualitative characteristics of Caucasian Whortleberry seeds collected in Soha village, Namin County, Ardabil Province, Iran.

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

strain 12 (E), *Pseudomonas putida* strain 169 (P), and a combination of three bacteria (B+E+P), and a control (non-inoculation). Inoculated seeds were stored in plastic bags containing sterilized perlite and subjected to cold stratification at 4±1°C in a refrigerator 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 and chemical traits of this medium are given in Table 2.

The planted seeds were placed in the greenhouse at 20 and 24°C and relative humidities of 60% and 70%, respectively. The Seeds were irrigated three times a week. The seeds were considered to have germinated when the radicle length was more than 2 mm. Observations were recorded daily regarding germinated/non-germinated seeds up to 40 days. Percentage of germination (PG), germination speed (GS), mean germination time (MGT), and the Seed Vigor Index (SVI) were determined using Eqs. (1) to (4), respectively [17]. where N is the total number of sown seeds, n is the number of seeds that were germinated on day d, d is the number of days counted from the beginning of germination, and SL and RL are the shoot and root length. 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 measured with a caliper with an accuracy of 0.1 mm.

$$PG = \frac{\sum_{i=1}^{n} n}{N} \times 100 \quad \text{Eq. (1)}$$

$$GS = \sum_{i=1}^{n} \left(\frac{n}{d}\right) \quad \text{Eq. (2)}$$

$$MGT = \frac{\left(\sum_{i=1}^{n} d.n\right)}{\sum_{i=1}^{n} n} \quad \text{Eq. (3)}$$

$$SVI = PG \times Mean\left(\frac{SL + RL}{100}\right) \quad \text{Eq. (4)}$$

Experimental Design and Statistical Analysis

This experiment was conducted in a factorial completely randomized design, with 4 replications of 50 seeds per 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 were compared using Tukey's test at $p \leq 0.01$. Excel software (2016) was used to draw all the graphs.

Findings

Results of Analysis of Variance

The analysis of variance demonstrated that PGPRs inoculant, cold stratification periods,

Table 2) Physical and chemical characteristics of planting substrate cocopeat: perlite: sand (1:1:2).

BD ($g.cm^{-3}$)	EC ($dS.m^{-1}$)	pH	OC (%)	N (%)	P ($mg.L^{-1}$)	K ($mg.L^{-1}$)	Fe ($mg.L^{-1}$)	Zn ($mg.L^{-1}$)
0.72	0.91	6.50	3.21	0.17	91.00	484.00	18.00	12.00

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

and their interaction effects on germination traits were significant (Table 3, $P<0.05$). Seed inoculation significantly increased seed germination, germination speed, mean germination time, seed vigor index, and root and shoot length of *Caucasian whortleberry*.

Effect of Bacterial Inoculation and Cold Stratification on Germination Percentage (GP)

The seed germination percentages were 9, 7, and 6.5% for seeds inoculated with *P. putida*, *E. cloacea*, and *B. subtilis,* respectively. The non-inoculatedseeds did not germinate (Figure 1). *P. putida* increased the seed germination percentage more than *B. subtilis* and *E. cloacea*. The results showed that mixed bacterial inoculation had a greater effect on germination traits than individual inoculation. The application of a mixed inoculum (*B. subtilis, E. coli, and P. putida*) increased the germination percentage to 14, demonstrating the synergistic effect of the bacteria. Germination was 6.5, 16, 18, 40, and 40.5% in seeds 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 the percentage of germination in *V. arctostaphyos* seeds. At the same time, 4- and 5-month cold stratification resulted in a significant increase in seed germination percentage, indicating the presence of physiological

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

Treatment	Source Variance	DF	F	P
PGPRs Inoculant	Germination Percentage	4	511.20	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**

Note: * P < 0.05; ** P < 0. 01 and *** P < 0.001

dormancy in the seed, which controls a significant part of the seed dormancy in this species.

The results of our study indicated the highest germination percentage was observed in seeds co-inoculated by three bacteria, followed by a 5-month cold stratification period with 58.5%

that did not differ significantly ($p>0.05$) from the GP (57.5%) of the co-inoculation of three bacteria, followed by a 4-month CS period pretreatment (Figure 1).

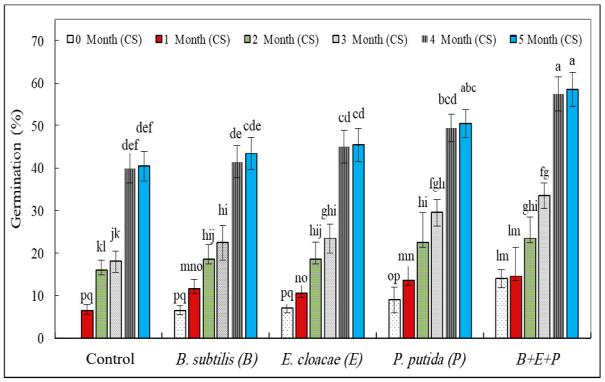

Figure 1) Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on seed germination of *Vaccinium arctostaphylos*. The means ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

Effect of Bacterial Inoculation and Cold Stratification (CS) on Germination Speed (GS) and Mean Germination Time (MGT)

The results showed that inoculation with PGPRs, the CS period, and their interaction significantly affected germination speed (Table 1). When the seeds were inoculated with PGPR either individually or in a mixture, the germination rate increased. So, the highest germination speed (2.26 n. d^{-1}) was observed in seeds co-inoculated with three bacteria after 5 months of cold stratification (Figure 2). Earlier studies have shown that bacterial inocula can increase germination speed. In this context, the highest germination rate (4.82 $n.d^{-1}$) was obtained with *Crataegus pseudoheterophylla* seeds using a combination of all bacteria: *Azotobacter chroococcum*, *A. lipoferum*, *Pseudomonas fluorescens,* and *Bacillus subtilis* [16].

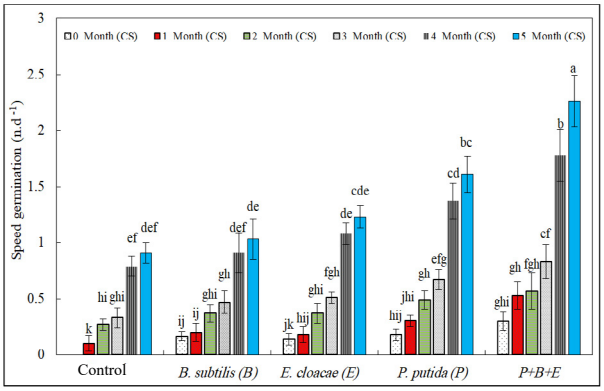

Figure 2) Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on germination speed of *Vaccinium arctostaphylos* seeds. The means ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

The results of our study showed that the minimum mean germination time (MGT) was observed in seeds co-inoculated, followed by a 5-month CS period, with a MGT of 13.84 days. 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 to individual inoculation (Figure 3). *P. putida* and *B. subtilis* are among the most important phosphate solubilizers. In CS treatments (without inoculation), the increase in the CS period (from 1 to 5 months) led to a gradual decrease in MGT. These results confirmed the importance of cold stratification in accelerating embryo growth, increasing germination speed, and reducing the germination period.

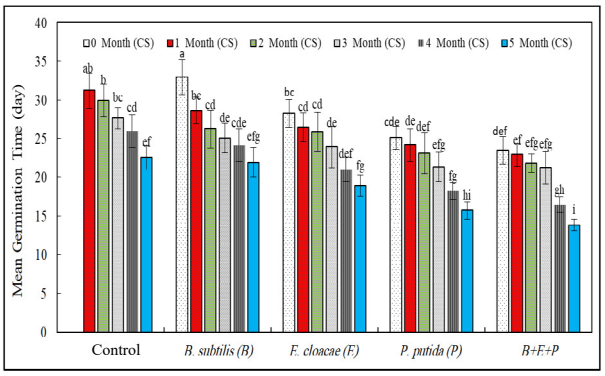

Figure 3) Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on mean germination time *of Vaccinium arctostaphylos* seeds. The means ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

Effect of Bacterial Inoculation and Cold Stratification (CS) on Root Length (RL), Shoot Length (SL), and Seed Vigor Index (SVI)

Different strains of rhizobacteria had variable effects on root and shoot length in various ecotype assays. In individual inoculation experiments, the maximum root and shoot lengths were observed in P. putida at 8.74 and 12.30 mm, respectively (Figure 4).

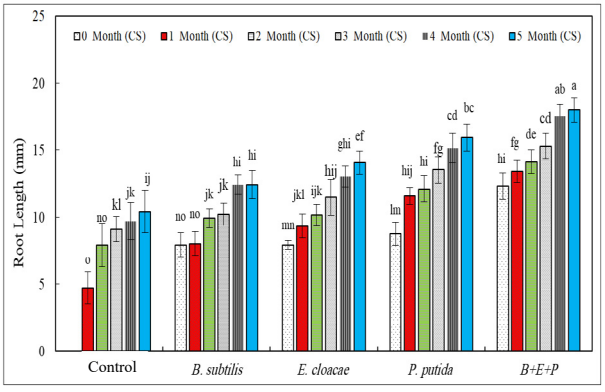

Figure 4) Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on root length of *Vaccinium arctostaphylos*. The means ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

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

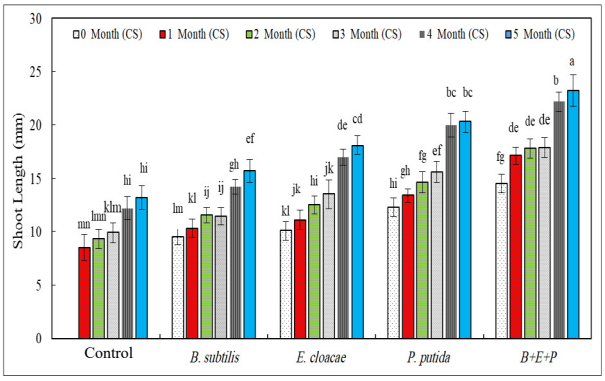

Figure 5) Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on shoot length of *Vaccinium arctostaphylos*. The means ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

In this study, the seed germination index was significantly affected by individual and combined inoculation of different bacterial strains. The results of the variation in bacterial treatments of *V. arctotaphylos* are presented in Figure 6. The highest seed germination index was obtained with seeds inoculated with the combination of three bacteria. Among inoculated bacteria in the individual application state, *P. putida* had the greatest effect on SVI, with 18.30. Seed vigor index improved with increasing CS periods in both PGPR-inoculated and uninoculated treatments. The highest seed germination index of 24.12 was observed with combined inoculation of three bacteria (*B. subtilis*, *E. coli*, and *P. putida*) and 5 months of CS treatment (Figure 6).

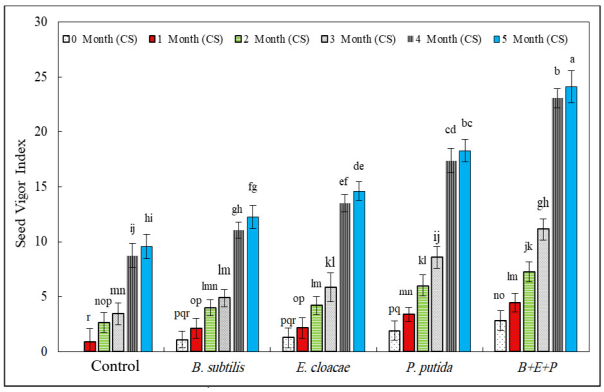

Figure 6) Effects of PGPRs inoculation and cold stratification periods (0 to 5 months) on seed vigor index (SVI) of *Vaccinium arctostaphylos*. The means ±SE to Column followed by the same letter are not significantly different, according to Tukey's test at (p = 0.05).

Discussion

In this study, the effects of PGPR strain inoculation and cold stratification duration on seed germination behavior in *V. arctotaphylos* were evaluated. The results showed that all treatments of bacteria increased the germination components of *V. arctotaphylos* seeds. Non-stratified seeds did not germinate in the control treatment, confirming the existence of physiological dormancy in *V. arctotaphylos* seeds. These findings may be due to increased synthesis of hormones such as gibberellins and auxins [16], which would have triggered the activity of specific enzymes that promote early germination, such as amylase, thereby increasing the availability of starch for assimilation [18].

Many studies have shown that PGPR inoculations can improve germination traits in various woody species, including *Acacia senegal* [15]. *Crataegus pseudoheterophylla* [16], *Abies religiosa* and *A. hickelii* [19], *Corylus avellana* [20], *Acasia mearnsii* [21] , and *Abies nordmanniana* [22]. In this study, *P. putida* increased the seed germination percentage more than *B. subtilis* and *E. cloacea*. The effects of different bacteria on seed germination and plant growth vary [18]. In this context, it can be stated that *Pseudomonas* strains, by affecting various parts of the seed, play a role in the biosynthesis and secretion of plant growth hormones and in reducing the abscisic-to-gibberellin ratio, thereby breaking embryo dormancy and stimulating germination [23]. It is well known that inoculating seeds with PGPB strains increases water and nutrient uptake, effectively synthesizes auxins and gibberellins, and reduces inhibitors such as ABA in the seed endocarp [24].

The results showed that mixed bacterial inoculation had a greater effect on germination traits than individual inoculation. The application of a mixed inoculum (*B. subtilis, E. colacea, and P. putida*) increased germination to 14%, demonstrating a synergistic effect of the bacteria. Similar to our findings, co-inoculation of C. *pseudoheterophylla* seeds with *Azotobacter chroococcum*, *Azospirillum lipoferum*, *Pseudomonas fluorescens,* and *Bacillus subtilis* increased seed germination [16]. A period of 1, 2, and 3 months of cold stratification was insufficient to induce the percentage of germination in *V. arctostaphyos* seeds. while 4- and 5-month cold stratification resulted in a significant increase in seed germination percentage, indicating the presence of physiological dormancy in the seed, which controls a significant part of seed dormancy in this species. [10] reported that the highest germination (70%) of *V. membranaceum* seeds was obtained by 28 days of stratification at 1-3°C, also 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 [8]. Chilling reduces abscisic acid (ABA) levels by increasing gibberellic acid levels. These changes are made simultaneously to balance

the two hormones [25]. 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 [26]. Also, during cold stratification, a significant increase in the level of phosphate pathway enzymes coincided with breaking seed dormancy. In our investigation of single-strain bacterial application, the highest germination rate was observed in *P. putida*-inoculated seeds. Earlier studies have shown that bacterial inocula can increase germination speed. In this context, the highest germination rate (4.82 $n.d^{-1}$) was obtained with *C. pseudoheterophylla* seeds using a combination of all bacteria of *Azotobacter chroococcum*, *A. lipoferum*, *Pseudomonas fluorescens,* and *Bacillus subtilis* [16]. These findings may be due to increased synthesis of hormones such as gibberellins and improved auxin synthesis, which have a positive effect on germination speed [12].

Different strains of rhizobacteria had varying effects on root and shoot length among the treatments. The maximum root and shoot length was observed in integrated applications of *P. putida*, *B. subtilis*, and *E. cloacea*, followed by 5 months of cold stratification. In individual inoculation experiments, the maximum root and shoot lengths were observed in *P. putida*. It may be stated that the combined use of P-solubilizing and N-fixing bacteria, as well as their ability produce growth-promoting substances (such as the synergistic effect of IAA), increases the root and shoot length. Similarly, [20] reported the increase in root and shoot of hazelnut (*Corylus avellana* L.) inoculated with *Pseudomonas putida* [27]. Also observed significant root and stem growth in Eucalyptus grandis seedlings inoculated with two *Pseudomonas* strains after 50 days. The most important features of *Pseudomonas* spp. are the production of auxins, siderophores, hydrogen cyanide (HCN), ammonia (NH3), exopolysaccharides, and the solubilization of phosphate. Plant hormones, such as auxins, gibberellins, and cytokinins, produced by rhizobacteria, play key roles in cell division and root and shoot elongation.

Seed vigor is the most important indicator of seed quality, reflecting the potential for rapid, uniform seedling emergence. In this study, the seed germination index was significantly influenced by both individual and combined inoculation of different bacterial strains. The increase in the seed vigor index can be attributed to the movement of nutrient reserves, the activity and resynthesis of certain enzymes, the beginning of RNA and DNA resynthesis, and the rapid growth of the embryo after barriers to germination are removed.

Conclusion

Overall, PGPR strains, whether applied as individual or mixed inoculants, have significant effects on seed germination and early growth. The impact of bacteria on seedling development is much greater than that of cold stratification alone or when combined with cold stratification. Although cold stratification is regarded as one of the most important treatments for stimulating germination, inoculation with bacteria triggers a series of processes that promote germination over time. In conclusion, bacteria provide germination stimuli, overcome physiological barriers, and enhance root and shoot growth, supporting establishment and subsequent development—effects that cold stratification alone cannot achieve. Finally,

co-inoculation of bacteria (*B. subtilis, E. colacea*, and *P. putida*) along with 5 months of CS treatment can be recommended as an effective method to break dormancy and increase germination of *V. arctotaphylos* seeds.

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