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Effect of plant growth promoting rhizobacteria (PGPRs) and stratification on germination traits of *Crataegus pseudoheterophylla* Pojark. seeds

Ahmadloo Fatemeh [a], Tabari Masoud [a,*], Azadi Pejman [b], Hamidi Aidin [c]

[a] Department of Forestry, Faculty of Natural Resources, Tarbiat Modares University, Noor Iran
[b] Agricultural Biotechnology Research institute of Iran, Karaj, Iran
[c] Seed & Plant Certification & Registration Institute, Karaj, Iran

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ABSTRACT

This study investigated seed germination of *Crataegus pseudoheterophylla* Pojark using different combinations of growth promoting rhizobacteria and stratification regimes. The PGPR treatments were *Azotobacter chroococcum* 12, *Azospirillum lipoferum* OF, *Pseudomonas fluorescens* 169, *Bacillus subtilis* FzB24 and combinations (co-inoculation) using two or all PGPR inoculants. The control was no inoculation followed by stratification. Temperature regulation in the stratification regime was 1 month at the constant temperature of 23 °C, then 3 months at 4 °C. The alternate temperature regime was 1 month at 4 °C, then 2 weeks at 23 °C followed by 1 month at 4 °C, then 2 weeks at 23 °C, then 1 month at 4 °C. The effects of these treatments were evaluated and results showed that treatments of PGPR inoculation stimulated germination in terms of percentage of seed germination, speed of germination and mean germination time, and effect was significant at ($p < 0.05$). Higher percentages of seed germination (18.33%) and speed of germination (4.82 number/day) were recorded for co-inoculated seeds by the combination of all bacterial inoculants and the alternate temperature stratification regime. Results showed a higher percentage of seed germination using alternate temperature stratification than for the constant temperature stratification regime. These results suggest that PGPRs in combination were effective in terms of promoting germination of *C. pseudoheterophylla* seeds.

1. Introduction

The genus hawthorn (*Crataegus*) belongs to the subfamily Maloideae in the family Rosaceae, it belongs to a group of complex genera that have interbreed freely (or hybridized) with the basal chromosome number of 17 (Mirek et al., 2002). The species is native to western Asia including countries such as Afghanistan, Iran, Turkey and the Central Asian republics. The fruit is spherical and about 10 mm in diameter, dark red and contains 3–5 nutlets, each with furrows (Mozaffarian, 2004).

Hawthorn species are grown worldwide as edible fruit and for medicinal and ornamental purposes as well as erosion control practice. Hawthorn has dense branches and thorns; it is an important plant for wildlife and is a good nesting site for birds. Characteristics of this genus include abilities to survive in poor soil, promote soil conservation, and adapt to drought (Nas et al., 2012). The genus is a valuable source of rootstock for peach, apple, and quince propagation (Qrunfleh, 1993).

Hawthorn seeds have orthodox storage behavior (Gosling, 2007) because they can be dried to maintain low moisture content (9–13%) without loss of viability. The seeds of many hawthorns exhibit double dormancy, such as embryo dormancy and endocarp dormancy and may not germinate for 2 to 3 years (Bujarska-Borkowska, 2006). A feature of all members of *Crataegus* is a stony endocarp, which may offer some resistance to germination (Bujarska-Borkowska, 2007). The presence of this hard seed coat acts to physically restrain enlargement of an embryo and prohibit radicle emergence (Baskin and Baskin, 2001). Degradation of the endocarp may lead to increased water uptake by an embryo and increased diffusion of oxygen that increases growth potential of the embryo (Bewley and Black, 1994). A study on physical restraint of the endocarp has demonstrated that its function is mechanical inhibition of germination. High concentrations of abscisic acid (ABA) in the pericarp and testa of *Crataegus* seeds also strongly inhibits germination (Hartmann et al., 2002).

* Corresponding author. Tel.: +98 9112246250/+98 9112246250; fax: +98 01226253499.
E-mail addresses: mahsa_ahmadloo@yahoo.com (A. Fatemeh), mtabari@modares.ac.ir (T. Masoud).

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One method used to break seed dormancy and promote germination is microbial inoculation of the seeds themselves or applied to the germination medium. Morpeth and Hall (2000) reported that microorganisms facilitate germination by macerating the hard-coated seed pericarp. Microorganism strains of *Azotobacter*, *Azospirillum*, *Pseudomonas*, and *Bacillus* can affect seed germination. Bacteria are required for cellulose biosynthesis in the seed coat. Reports have demonstrated that bacteria such as *Azotobacter chroococcum* (Singh et al., 1994), *Bacillus subtilis* (Rathnan et al., 2013), *Azospirillum* sp. (Mehdipour-Moghaddam et al., 2010) and *Pseudomonas fluorescens* (Dees et al., 1995) can break down cellulose or hemicelluloses in cell wall. The beneficial effect of these bacteria on seed germination is attributed to production of metabolites (siderophores, hydrocyanic acid), synthesis of antibiotics, enzymes and phytohormones (auxin, cytokinin, gibberellic acid) activity and other associated activities such as greater phosphate solubilization and competition in soil and root colonization (Kazaz et al., 2010). Tests were done on *Rosa damascena* Mill. seeds soaked in different microbial inocula such as Bioplin^{TM}, Phosfert^{TM}, Phosfert^{TM} + Bioplin^{TM} and EM•1® solutions and then subjected to 4 weeks of warm stratification at 25 °C, followed by 150 days of cold stratification at 4 °C. The highest evaluation for cumulative percentage of germination was obtained in EM•1® (100%), followed by Phosfert^{TM} and Phosfert^{TM} + Bioplin^{TM} (84%) and control (69.3%) (Kazaz et al., 2010). Beneficial effects such as those mentioned above have also been observed for *R. corymbifera* (Morpeth and Hall, 2000) and *Acacia senegal* (Singh et al., 2011).

Stratification is another method used to break dormancy (Hartmann et al., 1997). Several studies have tested pre-germination treatment of *Crataegus* seeds. Bujarska-Borkowska (2002) reported that *Crataegus monogyna* seeds were released from dormancy using warm stratification at 20 °C for 4–5 months followed by cold stratification at 3–5 °C for 4-5 months. Persson et al. (2006) reported a high rate of germination (90%) without warm stratification before cold stratification in *C. monogyna* seeds without endocarp. Seeds with endocarp required 112 days warm stratification followed by cold stratification to reach germination percentage of 75%. Mirzadeh Vaghefi et al. (2010) reported high percentages of germination for three species of hawthorn (*Crataegus aminii*, *C. persica*, *C. babakhanloui*) (32, 23.47, and 18.67%, respectively) by immersion in running water for 24 h, followed by 3 months of warm stratification, then 4.5 months of cold stratification.

Ahmad et al. (2008) reported that PGPR could produce phytohormones, asymbiotic N_2, siderophores, antibiotics, enzymes and fungicidal compounds, which might increase the rate of seed germination.

Although, research has reported dormancy mechanisms of *Crataegus* sp. seeds (Persson et al., 2006; Mirzadeh Vaghefi et al., 2010), no information is available on breaking seed dormancy of *Crataegus pseudoheterophylla* Pojark. To date research centers have not been able to germinate seeds of this species (Nasri et al., 2010). This study investigated effects of four different PGPR strains, stratification at constant and alternate temperature regimes and the interaction of these two factors on seed germination of *Crataegus pseudoheterophylla*. The goal was to develop an efficient protocol for propagation of this valuable plant.

2. Materials and methods

2.1. Seed collection

Fully ripe fruits of the *C. pseudoheterophylla* were collected from stock plants in the Dokhaharan village (Shazand Arak, Markazi province, Iran; 49° 24′E, 33° 51′N, 2200 m a.s.l.) in 30 October 2011.

Table 1
Crataegus pseudoheterophylla: characteristics of seeds.

Viability (%)	Purity (%)	Moisture (%)	Seed number (per kg)	The weight per 1000 seeds (g)
80.1	99	10.2	6310	158.51

Mean annual rainfall, temperature and humidity are 568.5 mm, 11.6 °C, and 50.5%, respectively. The drought period of the area is approximately 150 days beginning in June and continuing until end of October. The climate is semi-humid, mountainous and cold (Aghakhani and Metaji, 2010). The best time for collecting fruits is from September until October when the fruits are fully ripe. It may last two to three years for the seeds to germinate and the germination rate is less than 10% (Nasri et al., 2010).

2.2. Pulp extracts and determination of seed characteristics

Pulp was removed by wet maceration for tests reported in the present study. Nutlets were extracted from fruit and then seed viability was examined by the tetrazolium test (International Seed Testing Association (ISTA), 1999) and moisture content was determined by the air-oven method (103 °C, 17 h). Evaluations for seed characteristics are shown in Table 1.

2.3. Experiments for improving germination

Seeds with endocarp were soaked in running water for 48 h and then placed on moist sand in perforated plastic boxes and kept in cold storage at −19 °C for 24 h. Seeds were surface sterilized in a 2% sodium hypochlorite (NaOCl) solution for 15 min and rinsed three times with distilled water and subjected to warm stratification with a moist medium in a growth chamber for 1 month at 23 °C. For inoculation, after warm stratification, seeds were soaked for 3 hours in a bacterial suspension containing 10^8 cfu ml^{-1} of 20% gum Arabic as adhesive. Each bacterial strain was inoculated in a 150 ml flask containing 60 ml medium and incubated at 28 ± 1 °C for three days (Gholami et al., 2009). Test treatments were as follows:

2.3.1. Experiment 1: Inoculation with PGPR strains

PGPRs: For inoculation, seeds were rolled into bacterial suspension and a control (without bacteria) treatment, *A. chroococcum* strain 12 (A), *Azospirillum lipoferum* strain OF (A_1), *Pseudomonas fluorescens* strain 169 (P), *Bacillus subtilis* strain FzB24 (B), A + A_1, A + P, A + B, A_1 + P, A_1 + B, P + B, A + A_1 + P + B. For treatments that applied a single, two and four strains, inoculum amounts of 100 ml, 50 ml and 25 ml were used for each PGPR, respectively, with OD 0.5 at 600 nm and inoculums were mixed completely prior to inoculation.

2.3.2. Experiment 2: Stratification

Stratification: Seeds were stratified by 3 stratification regimes: (1) non-stratified regime, (2) constant temperature stratification regime (1 month at 23 °C then 3 months at 4 °C), and (3) alternate temperature regime (1 month at 4 °C then 2 weeks at 23 °C followed by 1 month at 4 °C and 2 weeks at 23 °C eventually 1 month at 4 °C).

2.3.3. Experiment 3: Inoculation with PGPR strains combined with stratification

PGPRs + stratification: seeds were imbibed in PGPR (at the previous suspension) then stratified according to the previously mentioned stratification regimes.

2.4. Preparation of PGPR inoculum

The bacterial inoculums used in these tests were indigenous strains to Iran obtained from the microbial collection of the soil

Table 2
Physicochemical properties of substrate sand:perlite:cocopeat (2:1:1).

Porosity (%)	CEC (C mol kg^{-1})	EC (dS m^{-1})	pH	BD (g cm^{-3})
72	21	0.94	6.7	0. 75

microbiology department of Soil and Water Research Institute (SWRI). They were isolated from rhizosphere of maize and wheat (Hamidi et al., 2011).

2.5. Determination of physicochemical and nutrient properties of the substrate

Tests were conducted according to a completely randomized design in a factorial arrangement with 3 replications and 50 seeds in each replication. Seed stratification was done in a mixed medium of moist sand:perlite:cocopeat (2:1:1) in polyethylene bags (8 cm diameter, 15 cm height). Polyethylene plastic bags were aerated regularly every week. Evaluations for pH and electrical conductivity (EC) were made for the substrate according to the method cited in Verdonck et al. (1982) (Table 2). Nitrogen was analyzed by Kjeldahal method (Zarinkafsh, 1993), carbon was measured with the Walkly-Black method (Walkey and Black, 1934), and phosphorus was measured by the method cited in Olsen and Sommers (1982). Concentrations of substrate K, Ca, Mg, Fe, Cu, Mn and Zn were measured by atomic absorption (Zarinkafsh, 1993) (Table 3).

2.6. Seed germination

After stratification, the polyethylene plastic bags containing seeds were transferred to a growth chamber with air circulation for germination. Germinated seed were counted at 3-day intervals for 60 days from the beginning of emergence of cotyledon on the soil surface. Final germination percentage was calculated when no further germination had taken place for several days. Germination tests were terminated after 10 months. A tetrazolium test for non-germinated seeds in bacteria treatments showed that all non-germinated seeds were dead. Evaluations for percentage of germination (PG), mean germination time (MGT) and germination speed (SP) were calculated according to Eqs. (1)–(3), respectively (Panwar and Bhardwaj, 2005); where N is total number of sown seeds, n is number of seeds that were germinated on day d, and d is the number of days counted from the beginning of germination.

$$\mathrm{PG} = \frac{\Sigma n}{N} \times 100 \quad (1)$$

$$\mathrm{MGT} = \frac{\Sigma nd}{\Sigma n} \quad (2)$$

$$\mathrm{SP} = \Sigma \left(\frac{n}{d}\right) \quad (3)$$

2.7. Data analysis

Distribution was tested for normality by Kolmogorov–Smirnov and all data were subjected to arc sine ($\sqrt{x}$) transformation before statistical analysis. Equality of variance among treatments was tested using Levene's test for homogeneity of variance. Two-way ANOVA analysis was performed on evaluations for germination percentage, germination speed and mean germination time, considering PGPR inoculants and stratification as fixed variables. Differences among means were analyzed by the Duncan multiple range test ($p = 0.05$), using SPSS 17.

3. Results and discussion

3.1. The effect of inoculation with PGPR strains on percentage of germination and speed of germination

Results showed that the effect of PGPR inoculation treatment was significant ($p < 0.05$) in terms of stimulating percentage of germination, speed and mean germination time (Table 4). In individual of bacterial applications, higher percentages for seed germination and speed were obtained in *B. subtilis* (1% and 0.05 n/d, respectively). *Bacillus* sp. is a phosphate solubilizing bacteria (PSB). PSB bacteria secrete organic acids and convert phosphates into a soluble form. In seed treatments with organic acids, low pH affects breaking physiological seed dormancy and stimulates penetration of gibberellins into cells (Lavakush et al., 2014). Phosphorus affects seed germination by increasing physiological activities in seeds that from water uptake. *B. subtilis* is one of the most widely used bacteria for the production of enzymes such as catalase, protease, cellulase and amylase that can contribute to seed germination by affecting cell wall porosity (Müller et al., 2013). The degradation of bonds between cell walls apparently forces the endocarp to split and thus removes a physical barrier to germination and increases endocarp permeability to water and gases (Müller et al., 2013). In combinations, the treatments that used a combination of all four inocula showed higher percentages for seed germination and speed (5.7% and 0.7 n/d, respectively). These high amounts for seed germination by the combination of all bacterial inocula reflected the synergic effect of uptakes of nutrients and effective hormone synthesis of auxins, gibberellins and a decline of inhibitors such as ABA (Miransari and Smith, 2014).

Higher percentages were recorded for seed germination (18.33%) and speed of germination (4.82 n/d) for seeds co-inoculated by the combination of all 4 bacterial inocula using alternate temperature stratification. In stratification, the highest percentages for seed germination and speed were obtained using an alternate temperature regime (9.8% and 1.33 n/d, respectively). Morpeth and Hall (2000) for *Rosa corymbifera* (95%) and Belletti et al. (2003) for *R. canina* (50.25%) also found that microbial inoculation of seeds increased evaluations for percentage of germination.

The highest percentages for germination and speed were obtained using the combination of all bacterial inoculants (5.7% and 0.7 n/d, respectively). The combination of *P. fluorescens* and *B. subtilis* resulted in 4% germination and 0.35 n/d germination speed (Fig. 1). This might be as a result of an increase in the number of microorganisms in the seed endocarp; as seed germination was facilitated by maceration of the hard, thick seed endocarp (Kazaz et al., 2010).

Results showed that application of tested bacterium individually did not affect germination, but integrated applications led to a significant increase in evaluations for percentage and speed of germination (Figs. 1 and 2). These results confirm the synergistic effect of bacteria. Manero et al. (2001) studied *Bacillus pumilus* and *B. licheniformis* and reported that PGPR produced high amounts

Table 3
Nutrient concentrations properties of substrate sand:perlite:cocopeat (2:1:1).

Organic matter	N	C/N	P	K	Ca	Mg	Fe	Cu	Mn	Zn
	(%)		(mg L^{-1})							
14.3	0.39	21.2	158	1568	414	384	41	0.5	11.6	26

Table 4
Two way ANOVA with PGPRs inoculants and stratification as fixed variables on germination traits of *Crataegus pseudoheterophylla*.

	Source	SS	DF	*F*-test	*p*
PGPRs inoculants	Germination percentage	908.769	11	64.655	*** < 0.002
	Germination speed	64.517	11	79.921	*** < 0.009
	Mean germination time	1415.893	11	1.977	* < 0.043
Stratification	Germination percentage	1206.352	2	472.051	*** < 0.004
	Germination speed	26.875	2	183.106	*** < 0.004
	Mean germination time	261.29	2	2.006	ns 0.142
PGPRs inoculants × stratification	Germination percentage	163.648	22	5.821	*** < 0.009
	Germination speed	26.711	22	16.544	*** < 0.008
	Mean germination time	5853.387	22	4.086	*** < 0.008
Error	Germination percentage	92	72		
	Germination speed	5.284	72		
	Mean germination time	4688.874	72		

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

of physiologically active gibberellins that had a positive effect on seed germination by rupturing testa and endosperm and inhibiting ABA activity. Removal or scarification of the endocarp in seeds played an important role in the process of germination. In nature, pericarp and endocarp are degraded by microorganisms (Morpeth and Hall, 2000). Mafia et al. (2009) reported the highest evaluation for seed germination percentage (62%) in tests on *Eucalyptus grandis* as a result of seed inoculation with *Pseudomonas fulva*, which produced a 462% increase compared to the control. *Bacillus subtilis* was the most effective treatment for *E. globulus*, promoting a significant increase in seed germination (79%). *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). It has been reported that germination stimulators such as compost activator (Garotta) that constitutes a mixture of some micro-organisms and nutrients applied to speed up breakdown of the endocarp. Cullum and Wood (1998) for *R. corymbifera* and Belletti et al. (2003) for *R. canina* reported high evaluations for seed germination (95% and 50.25%, respectively) when Garotta was incorporated into the stratification mixture due to degradation of cellulose and loosening of the bonds between cell walls. Inoculation with PGPR could be due to high levels of N and its accelerating mineralization processes on the surface of the endocarp that by changing/lowering the C/N ratio allows fungi or other microorganisms to break down the endocarp tissue. N compounds can enhance seed germination through increased amylase activity, adjusting the K^+/Na^+ ratio, increasing levels of ATP production and seed respiration and decreasing the level of ABA in seeds (Zheng et al., 2009). The reported improvement in evaluations for germination percentage in the present study might also be due to increased synthesis of the hormone gibberellin, which stimulated activity of α-amylase and other enzymes that ate specific to germination such as protease and nuclease that are involved in hydrolysis and starch assimilation (Gholami et al., 2009).

3.2. The effect of inoculation with PGPR strains on mean germination time

Mean germination time in *A. chroococcum* was significantly higher (38 d) than that determined in other treatments (Fig. 3). *Azotobacters* are aerobic, free-living soil microbes that play an important role in the nitrogen cycle in nature and *Azospirillums*

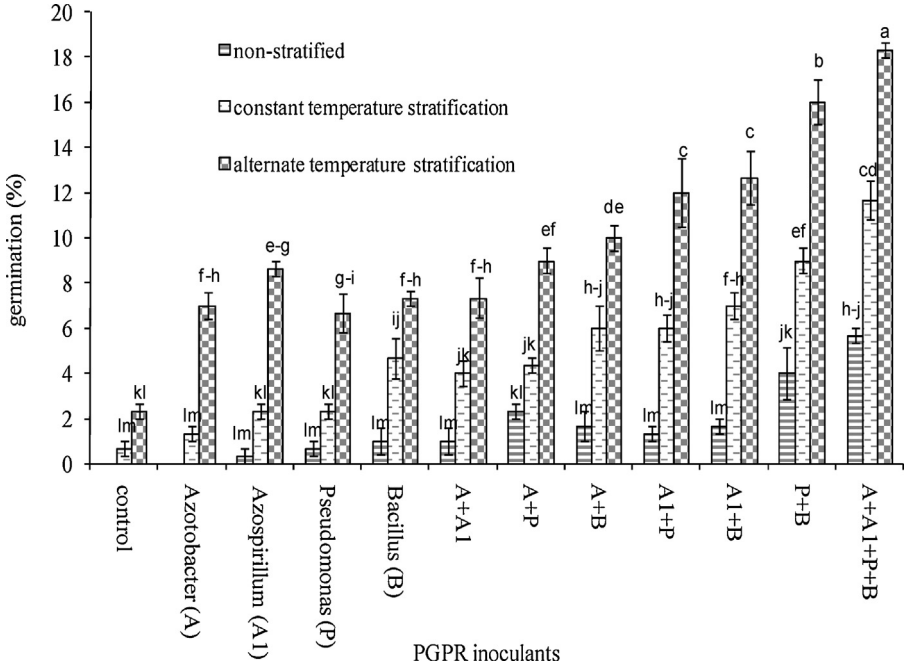

Fig. 1. Effects of bacteria inoculation and stratification on germination percentage of *Crataegus pseudoheterophylla* seeds. Error bars represents standard error of the mean. T1 = control, T2 = *Azotobacter chroococcum* 12, T3 = *Azospirillum lipoferum* OF, T4 = *Pseudomonas fluorescens* 169, T5 = *Bacillus subtilis* FzB24, T6 = *A. chroococcum* + *A. lipoferum*, T7 = *A. chroococcum* + *P. fluorescens*, T8 = *A. chroococcum* + *B. subtilis*, T9 = *A. lipoferum* + *P. fluorescens*, T10 = *A. lipoferum* + *B. subtilis*, T11 = *P. fluorescens* + *B. subtilis*, T12 = *A. chroococcum* + *A. lipoferum* + *P. fluorescens* + *B. subtilis*.

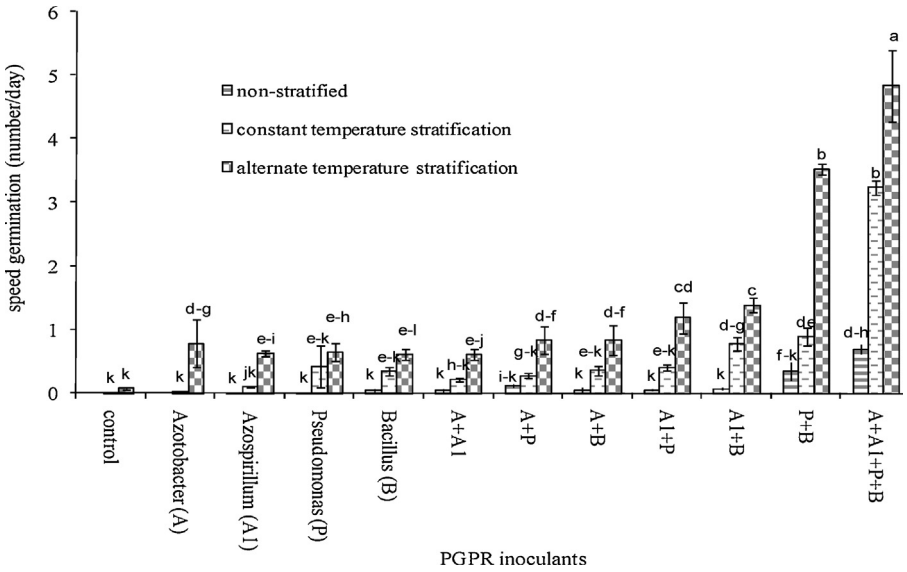

Fig. 2. Effects of bacteria inoculation and stratification on germination speed of *Crataegus pseudoheterophylla* seeds. Error bars represents standard error of the mean. T1 = Control, T2 = *Azotobacter chroococcum* 12, T3 = *Azospirillum lipoferum* OF, T4 = *Pseudomonas fluorescens* 169, T5 = *Bacillus subtilis* FzB24, T6 = *A. chroococcum* + *A. lipoferum*, T7 = *A. chroococcum* + *P. fluorescens*, T8 = *A. chroococcum* + *B. subtilis*, T9 = *A. lipoferum* + *P. fluorescens*, T10 = *A. lipoferum* + *B. subtilis*, T11 = *P. fluorescens* + *B. subtilis*, T12 = *A. chroococcum* + *A. lipoferum* + *P. fluorescens*+ *B. subtilis*.

are free-living nitrogen-fixing bacteria. *P. fluorescens* and *B. subtilis* are reportedly effective phosphate solubilizers. The individual form of *Azotobacter* produces IAA, gibberellin, kinetin, siderophore, polysaccharide, ammonia and antifungal metabolites such as HCN (Ahemad and Kibret, 2014). Therefore, it can be assumed that a single form of bacteria is not enough for cracking or weakening the seed coat to allow the seed to begin its germination process.

The co-inoculants and combinations of bacteria enhance the uptake of nutrients compared to individual forms of bacterium. Bacterial inoculation in combination using all four combinations of inocula at constant temperature stratification showed a lower mean germination time (8.7 d). In the tested PGPR strains, this can be attributed to increased hormone synthesis (IAA), siderophore production and solubilization of phosphorus. IAA is able to stimulate the production of ethylene (Arteca and Arteca, 2008). Subsequently, ethylene breaks seed dormancy by regulating the expression of cysteine-proteinase genes, and its protein complex, proteasome and interacting with the inhibitory effects of ABA. Moreover, it participates in the production of reactive oxygen species and serves to facilitate release of seed dormancy by regulating cell development and cell redox conditions (Graeber et al., 2010). Siderophores produced by bacteria act as iron (Fe) solubilizing agents and Fe is required as a co-factor to activate numerous enzymes in seed germination (Indiragandhi et al., 2008). A combination of all bacteria showed a significant effect on uptakes of P, N and K that has been advocated by Lavakush et al. (2014) due to the synergistic relationship of nitrogen-fixing bacteria with phosphate solubilizing bacteria. Belletti et al. (2003) reported that different

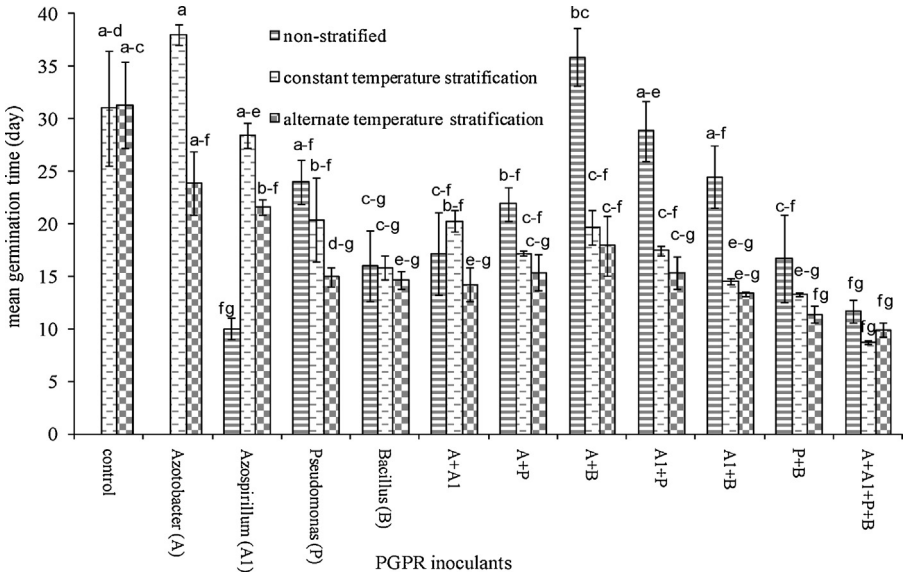

Fig. 3. Effects of bacteria inoculation and stratification on mean germination time of *Crataegus pseudoheterophylla* seeds. Error bars represents standard error of the mean. T1 = Control, T2 = *Azotobacter chroococcum* 12, T3 = *Azospirillum lipoferum* OF, T4 = *Pseudomonas fluorescens* 169, T5 = *Bacillus subtilis* FzB24, T6 = *A. chroococcum* + *A. lipoferum*, T7 = *A. chroococcum* + *P. fluorescens*, T8 = *A. chroococcum* + *B. subtilis*, T9 = *A. lipoferum* + *P. fluorescens*, T10 = *A. lipoferum* + *B. subtilis*, T11 = *P. fluorescens* + *B. subtilis*, T12 = *A. chroococcum* + *A. lipoferum* + *P. fluorescens*+ *B. subtilis*.

doses of Garotta in *R. canina* further shortened mean germination time from 8.48 to 9.64 d over the control.

El-Refaey and El-Dengawy (2005) reported that chilling treatment affected phosphate metabolism in seeds. Moist-chilling may increase levels of organic phosphates such as fructose 2,6-biphosphate, ATP and nucleotides (Bewley and Black, 1994). Also, during chilling treatment, a significant increase in the level of phosphate pathway enzymes coincided with breaking seed dormancy prior to seed germination (Gosling and Ross, 1980). For this reason, in the present study, phosphate-solubilizing bacteria had a greater impact than nitrogen fixation bacteria on *P. fluorescens* and *B. subtillis*. Also, germination was variable among different species and strains of bacteria. Singh et al. (2011) demonstrated that there was no universal strain of PGPR for every plant species, thus the first step is to select the most appropriate PGPR for inoculation of a particular plant species.

3.3. Effect of stratification on evaluations for percentage and speed of germination

Stratification had a significant effect on evaluations for percentage of germination and speed of germination for *C. pseudoheterophylla* seeds ($p < 0.004$) (Table 4). Non-stratified seeds did not germinate in the control and Azotobacter treatments (Fig. 1). This result confirms the importance of stratification because those seeds that did not germinate (*Crataegus* sp.) had hard and thick endocarp Morgenson (2000). Results showed that the highest percentage of germination and speed were obtained using the alternate temperature regime (9.8% and 1.33 n/d, respectively). Pawłowski (2010) reported that stratification triggers physiological changes in protein expression and synthesis and energy and methionine metabolism that are necessary processes for breaking seed dormancy. Han et al. (2010) demonstrated that stratification overcame physiological and morphological embryo dormancy of *Michelia yunnanensis* through stimulation of gibberellins, ethylene synthesis and a decline in ABA. Seed stratification facilitated hydrolyzation of storage proteins and removal of metabolic barriers to seed germination.

3.4. The effect of stratification on mean germination time

No statistical difference in terms of mean germination time was found between stratification treatments and the control (Table 4). This is in agreement with results of research by Bujarska-Borkowska (2006) reporting that non-stratified seeds of *Crataegus* did not exhibit imbibition or germination because of a hard seed endocarp. Hardness and impermeability of the seed coat as an inhibiting factor in seed germination has been studied for seed of several *Crataegus* species (Yahyaoglu et al., 2006; Bujarska-Borkowska, 2007). Accordingly, cold stratification (20, 40, 60 and 90 d) alone, 60 and 90 d cold stratification with submersion in sulphuric acid (H_2SO_4) for different durations (30, 75, 105, 120, 150 and 180 min), and autumn sowing did not promote germination of *Crataegus microphylla*, *C. monogyna*, *C. pontica*, and *C. pseudoheterophylla* seeds (Yahyaoglu et al., 2006). Germination occurred only in *C. monogyna* subsp. azarella seeds and the highest evaluation for germination percentage was 17.5%.

Stratification stimulates structural GA_3 synthesis. During cold imbibitions, genetic changes in mRNA and protein levels occur. Particularly notable is an increased expression of genes that control biosynthesis of gibberellic acid (Bujarska-Borkowska, 2007). Although, at low temperatures, germination may be inhibited, progress toward germination is made via changes in enzyme production and concentrations. Stratification may lower rates of enzyme reactions within seeds, and may cause differential changes in enzyme concentrations or in enzyme production (Bewley and Black, 1994; Garcia-Gusano et al., 2004).

3.5. Effect of alternate and constant temperature stratification on germination

Evaluations for seed germination percentage for stratification in the alternate temperature regime were higher at constant temperature (Fig. 1). These results confirm importance of the warm period that interrupts cold stratification in speeding up embryonic growth. The biological system of a seed moves from a dormant state to an active state when seeds are stratified at varying temperature. Exogenous dormancy is released during warm stratification, as demonstrated by splitting of the endocarp and endogenous dormancy is broken during cold stratification (Finch-Savage, 2001; Alirezaie Noghondar et al., 2011). Other authors have also recommended periods of warm stratification for selected species (Young and Young, 1992; Morgenson, 2000). Stratification by alternate temperature regime is believed to lead to degradation and softening of the endocarp, leakage of growth inhibitors, and development of the embryo (Baskin and Baskin, 2001).

3.6. Effect of inoculation with PGPR strains combined with stratification

Table 4 shows a significant ($p < 0.01$) interaction for effects of concentration of PGPR inoculant and stratification. Results showed higher percentages for germination (18.33%) and speed of germination (4.84 n/d) by seeds treated with the combination of all bacterial inoculants under stratification with the alternate temperature regime. Results of these tests did not demonstrate a high rate of seed germination, even under inoculation of a combination PGPR strains with alternate temperature stratification. This result highlights complexity of the dormancy mechanism of *C. pseudoheterophylla* seed.

4. Conclusion

In the current study, seeds of *C. pseudoheterophylla* were confirmed as in a state of dormancy. Overall, results indicated that concentration of PGPR inoculant and stratification treatment and their interaction had a positive affect on seed germination. These results confirm that PGPR should be used in combination with stratification to overcome seed dormancy. Adaptation and practical application of these findings could be applied to reduce production costs for nurseries. Further studies are in progress to increase the rate of germination of *C. pseudoheterophylla* in order to improve propagation protocol.

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