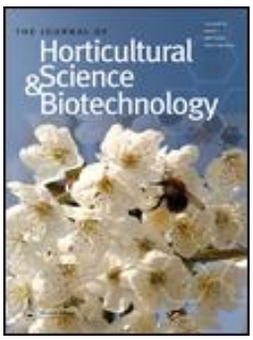

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Seed washing, exogenous application of gibberellic acid, and cold stratification enhance the germination of sweet cherry (*Prunus avium* L.) seed

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SUMMARY
Seed germination in sweet cherry (*Prunus avium* L.) is a slow and lengthy process which has delayed breeding efforts. In this study, seed from ripe fruit of the sweet cherry cultivar 'Lambert' were collected and, after removing the endocarp, various dormancy-breaking treatments such as seed washing, the application of exogenous gibberellic acid (GA_3), or cold stratification were evaluated for their ability to enhance the percentage and rate of seed germination. The results indicated that seed washing was necessary to break dormancy in sweet cherry. The seed germination percentage and rate improved to 26.5% and 1.17 seed d^{-1} simply by washing the seed for 24 h. The application of up to 500 mg l^{-1} GA_3, in addition to a seed washing treatment, further improved both the seed germination percentage and rate to 47.1% and 1.9 seed d^{-1}, respectively. Although the seed germination percentage improved as a result of 6 weeks of cold stratification, a longer cold period (8 weeks) was required to obtain the maximum rate of germination. The application of higher concentrations of GA_3 and longer periods of cold storage did not result in further improvements in the seed germination percentage or rate. The highest germination percentage (61.2%) was obtained following seed washing for 24 h, followed by 500 mg l^{-1} GA_3 treatment, then 6 weeks of cold stratification, which was higher than the germination percentage in the control treatment (0%). The highest rate of seed germination was observed following 24 h of seed washing, then 1,000 mg l^{-1} GA_3 treatment and 8 weeks of cold stratification (3.8 seed d^{-1}), but this combined treatment did not differ significantly ($P \leq 0.05$) from seed washing, 1,000 mg l^{-1} GA_3, and 6 weeks of cold stratification (3.6 seeds d^{-1}).

Sweet cherry (*Prunus avium* L.) is an important fruit species cultivated in temperate zones. Sweet cherry orchards are usually established by planting rootstocks that are then used to produce grafted trees. The rootstocks are grown from seedlings in nurseries. To propagate rootstocks it is often necessary to stimulate cherry seeds to enhance their rate and percentage of germination. However, the seeds of most *Prunus* species, including *P. avium*, exhibit deep dormancy, which needs to be overcome. *Prunus* seed dormancy can be broken in various ways, and the best method depends on the species (Heidari *et al*., 2008). Seed dormancy is an adaptive mechanism that *Prunus* species use to protect their seedlings from frost damage during harsh Winters. The dormancy mechanism inhibits seed germination until the seeds have achieved the required extent of cold stratification, even when the moisture, oxygen, and temperature conditions are otherwise favourable (Frisby and Seeley, 1993). Therefore, it is a major challenge to obtain abundant germinated seeds for breeding purposes (Schmidt and Ketzel, 1994).

A diversity of methods have been used to break seed dormancy. These often differ in the duration of pre-treatment within and among various cherry species and cultivars (Garcia-Gusano *et al*., 2004; Çetinbafl and Koyuncu, 2006). Traditionally, cold stratification has been used to break seed dormancy in *Prunus* spp. (Garcia-Gusano *et al*., 2004). In some studies, cold stratification was complemented by other treatments such as the application of gibberellic acid (GA_3) to speed-up dormancy breaking and to accelerate seed germination (Iglesias and Babiano, 1997). GA_3 is one of the plant hormones that has been suggested to control primary dormancy by inducing seed germination (Iglesias and Babiano, 1997). Seed washing is another effective treatment to break seed dormancy as it removes any potential inhibitors from the seed coat and enhances germination (Gosling, 2007). The effects of seed washing on dormancy-breaking in different species such as hawthorn (Mirzadeh Vaghefi *et al*., 2010), and jackfruit (Maiti *et al*., 2003) have been reported.

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The aim of this research was to evaluate the effectiveness of several methods capable of stimulating and enhancing seed germination in *P. avium* L.

MATERIALS AND METHODS

Preparation of plant material

Ripe fruit of *P. avium* L. 'Lambert' were collected in June 2011. After removing the fleshy parts of each fruit, the mature seeds from open-pollinated sweet cherries were washed and dried for 3 d at room temperature. The outer shell of each seed was removed, as experience had indicated that removing the shells (i.e., the stony endocarps) from *Prunus* seed resulted in higher and more rapid rates of germination (Garcia-Gusano *et al*., 2004; Heidari *et al*., 2008).

Experimental details

To evaluate the response of sweet cherry seed to different dormancy-breaking treatments, a three-factorial experiment was conducted in June 2011. The treatments applied consisted of two levels of seed washing (i.e., no washing or seed washing for 24 h under running tap water), four concentrations of GA_3 (0, 250, 500, or 1,000 mg l^{-1}), and three durations of cold stratification (4, 6, or 8 weeks). A randomised complete block design, with four replications of each treatment applied to 20 seeds, was employed to analyse the data.

All 1,920 seeds were sterilised in 1% (v/w) sodium hypochlorite for 20 min and rinsed three-times with distilled water prior to each experimental treatment. Half of the seeds (n = 960) were placed under running tap water for 24 h, while the other 960 seeds were left unwashed. Subsequently, seeds from each washing method were divided into four groups for treatment with exogenous GA_3 (Merck, Darmstadt, Germany). Seeds were soaked in 250, 500, or 1,000 mg l^{-1} GA_3 for 12 h. One group of seeds was soaked in distilled water (0 mg l^{-1} GA_3) for 12 h.

The seeds (n = 20) in each replication were placed in a freezer bag containing abundant wet perlite to maintain high humidity. The bags were then placed in the dark in a cold chamber at 4º ± 0.5ºC for 4, 6, or 8 weeks of cold stratification. After cold stratification, the freezer bags containing the seeds plus wet perlite were placed in a growth chamber at 13º ± 0.5ºC for 21 d.

Data collection

Germination tests were performed according to the methods of the International Seed Testing Association (ISTA, 1996). Germination percentages and rates were measured every 2 d when the seeds were in the growth chamber after cold stratification. Seeds were considered to have germinated when the tip of the radicle had grown free of the seed coat (ISTA, 1996). The initial germination percentage was recorded at the end of each period of cold stratification, before transferring the seeds to the growth chamber. The final germination percentage was calculated based on the number of germinated seeds after 21 d in the growth chamber divided by the number of seeds in each replication (20).

The germination rate (GR) was calculated using the numbers of germinated seeds counted at 2 d intervals, as follows:

$$GR = \Sigma_{n=1}^{21} (\textit{number of seeds germinating by } t - 1)/t$$

where t denotes the number of days in the growth chamber.

Data analysis

Before analysing the data, all germination percentages were transformed using:

$$y = \arcsin\sqrt{p}/100$$

where p denotes the germination percentage.

The data are presented as means ± standard errors (SE). The protected Least Significant Difference (LSD) test was used to separate means ($P \leq 0.05$). All statistical analyses were performed using SAS software (SAS Version 9.1; SAS Institute Inc., Cary, NC, USA).

RESULTS

Interaction between washing treatment and the application of exogenous GA_3 on dormancy-breaking in sweet cherry seed

The percentages and rates of germination of sweet cherry seed responded positively to washing treatment and to the application of GA_3 (Figure 1 A,B). The average germination percentage of seed without washing or GA_3 was only 1.66%. Washing seeds for 24 h significantly ($P \leq 0.05$) increased the germination percentage (Figure 1A) and the rate of germination

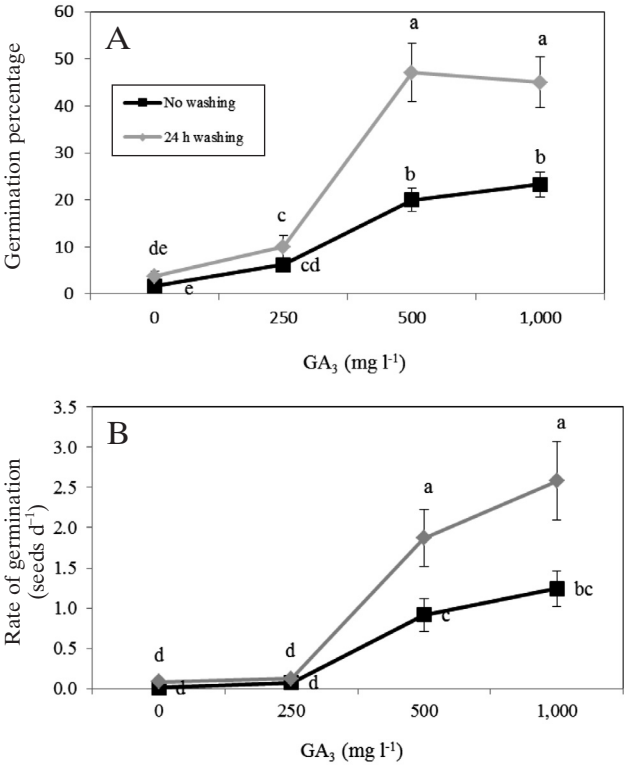

FIG. 1
Interactive effect of washing treatment and GA_3 concentration on the germination percentage (Panel A) and germination rate (Panel B) of sweet cherry (*Prunus avium* L.) 'Lambert' seeds. Means were separated using the protected LSD test. Mean values in each Panel with the same lower-case letters do not differ at $P \leq 0.05$. Error bars represent ± standard errors.

(Figure 1B). With or without a washing treatment, an increase in GA_3 concentration was associated with increases in the percentage and rate of germination of sweet cherry seed, especially in the range of 250 – 500 mg l^{-1} GA_3. Washing seeds for 24 h, in combination with 500 mg l^{-1} GA_3, enhanced the average germination percentage and rate of germination from 1.66% and 0.016 seeds d^{-1} in the untreated controls, to 47% and 2.58 seeds d^{-1}, respectively. The positive effects of the application of GA_3 on the percentage and rate of germination of sweet cherry seeds was therefore enhanced when it was preceded by a washing treatment. The differences in the percentages and rates of germination between 500 and 1,000 mg l^{-1} GA_3 following either washing treatment were not significant.

Interaction between washing treatment and the duration of cold stratification on breaking seed dormancy in sweet cherry seed

Prolonging the period of cold stratification from 4 to 8 weeks caused significant increases in the percentages and rates of germination after either washing treatment (Figure 2A, B). The highest germination percentage (35.3%) was observed when seeds were washed for 24 h, then kept at + 4ºC for 8 weeks. It should be noted that, following either washing treatment, no significant difference in germination percentage was observed between 6 and 8 weeks of stratification (Figure 2A). Also, the highest rate of seed germination (1.76 seeds d^{-1}) was achieved after a water washing treatment and 8 weeks of cold stratification. This did not differ significantly from a washing treatment plus 6 weeks of cold stratification (Figure 2B).

Interaction between the application of exogenous GA_3 and the duration of cold stratification on dormancy breaking in sweet cherry seed

The seed germination percentage did not increase significantly with an increase in GA_3 concentration from 0 to 250 mg l^{-1}, but when the concentration of GA_3 increased to 500 mg l^{-1}, a notable increase in germination percentage was observed (Figure 3A). The increase in germination percentage was more pronounced when the application of exogenous GA_3 was followed by cold stratification. However, no significant difference was observed between 6 and 8 weeks of cold stratification. Also, 1,000 mg l^{-1} GA_3 was not significantly more effective than 500 mg l^{-1} GA_3. The highest germination percentage (44.3%) was observed following 1,000 mg l^{-1} GA_3 and 8 weeks of cold stratification (Figure 3A). However, treatment with 500 mg l^{-1} GA_3 and 6 weeks cold stratification was sufficiently effective to break seed dormancy and induce the germination of sweet cherry seeds, saving both hormone and storage time.

The rate of seed germination followed a similar trend, where the highest rate of germination (2.77 seeds d^{-1}) was observed following 1,000 mg l^{-1} GA_3 then 6 weeks of

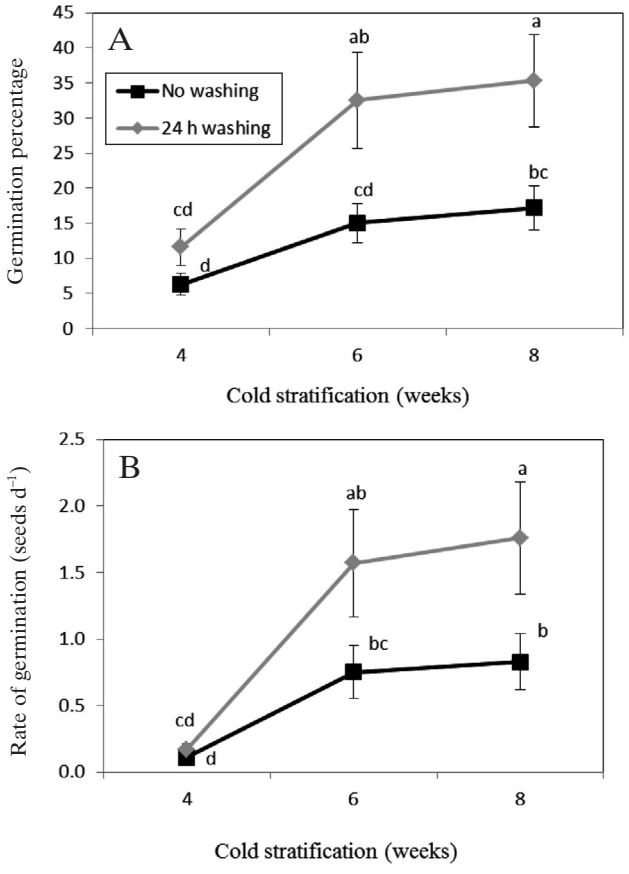

FIG. 2
Effect of washing pre-treatment and the duration of cold stratification on the germination percentage (Panel A) and germination rate (Panel B) of sweet cherry (*Prunus avium* L.) 'Lambert' seed. Mean values in each Panel were separated using the LSD test. Mean values with the same lower-case letters do not differ at $P \leq 0.05$. Error bars represent ± standard errors.

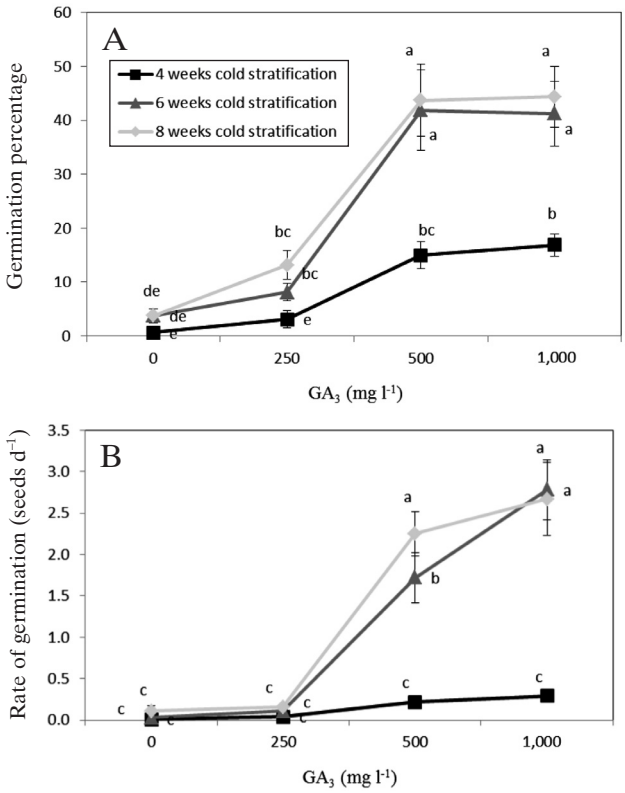

FIG. 3
Effect of the duration of cold stratification and GA_3 concentration on the germination percentage (Panel A) and germination rate (Panel B) of sweet cherry (*Prunus avium* L.) 'Lambert' seeds. Means values were separated using the LSD test. Mean values in each Panel with the same lower-case letters do not differ at $P \leq 0.05$. Error bars represent ± standard errors.

cold stratification. However, this treatment did not differ significantly from 1,000 mg l^{-1} GA_3 plus 8 weeks of cold stratification (2.66 seeds d^{-1}), or 500 mg l^{-1} GA_3 plus 8 weeks of cold stratification (2.44 seed d^{-1}; Figure 3B).

Interaction between washing treatment, the application of exogenous GA_3, and the duration of cold stratification on dormancy breaking in sweet cherry seed

Although the interaction effect of washing × GA_3 concentration × cold stratification period on sweet cherry seed germination was not significant, seed washing for 24 h followed by 500 mg l^{-1} GA_3 and 6 weeks of cold stratification resulted in the highest germination percentage (61.2%), higher than the germination percentage in the control treatment (0%; Table I). The highest rate of germination was observed following seed washing, 1,000 mg l^{-1} GA_3 and 8 weeks of cold stratification (3.8 seeds d^{-1}), but this treatment did not differ significantly ($P \leq 0.05$) from seed washing, 1,000 mg l^{-1} GA_3 and 6 weeks of cold stratification (3.6 seeds d^{-1}; Table I). Regardless of treatment, the rate of seed germination was positively correlated ($P \leq 0.01$; R^2 = 0.89) with the germination percentage in sweet cherry seeds. Thus, more rapid seed germination was associated with a higher germination percentage (Figure 4).

DISCUSSION

Successful seed germination represents the first essential step for efficient crop establishment

TABLE I

*Interactive effects of seed washing, GA_3 concentration, and duration of cold stratification on the germination percentage and rate of germination of sweet cherry (*Prunus avium*) 'Lambert' seed*

Treatment				
Washing	GA_3 (mg l^{-1})	Cold stratification (weeks)	Germination percentage (%)	Germination rate (seeds d^{-1})
No washing	0	4	0.00 (± 0.00)†	0.00 (± 0.00)
		6	2.50 (± 1.44)	0.03 (± 0.01)
		8	2.50 (± 1.44)	0.03 (± 0.01)
	250	4	2.50 (± 2.50)	0.03 (± 0.03)
		6	7.50 (± 2.50)	0.11 (± 0.04)
		8	8.75 (± 1.25)	0.10 (± 0.02)
	500	4	10.00 (± 2.04)	0.14 (± 0.03)
		6	22.50 (± 1.44)	0.95 (± 0.08)
		8	27.50 (± 2.50)	1.66 (± 0.22)
	1,000	4	12.50 (± 1.44)	0.27 (± 0.05)
		6	27.50 (± 3.23)	1.93 (± 0.16)
		8	30.00 (± 2.04)	1.53 (± 0.16)
Washing in tap water	0	4	1.25 (± 1.25)	0.01 (± 0.01)
		6	5.00 (± 2.04)	0.04 (± 0.02)
		8	5.00 (± 2.04)	0.20 (± 0.14)
	250	4	3.75 (± 2.39)	0.05 (± 0.03)
		6	8.75 (± 2.39)	0.12 (± 0.04)
		8	17.50 (± 4.33)	0.21 (± 0.04)
	500	4	20.00 (± 2.89)	0.30 (± 0.03)
		6	61.25 (± 3.15)	2.48 (± 0.13)
		8	60.00 (± 5.40)	2.84 (± 0.24)
	1,000	4	21.25 (± 2.39)	0.32 (± 0.05)
		6	55.00 (± 5.40)	3.63 (± 0.31)
		8	58.75 (± 2.39)	3.80 (± 0.11)
Source of Variation				
Washing treatment (A)			**	**
GA_3 concentration (B)			**	**
Cold stratification period (C)			**	**
A×B			**	**
A×C			ns	**
B×C			*	**
A×B×C			ns	**

†Values are means (± SD) of four replicates. Means were separated by the LSD test at $P \leq 0.05$.

**, * and ns, significant at $P \leq 0.01$, $P \leq 0.05$, or not significant, respectively.

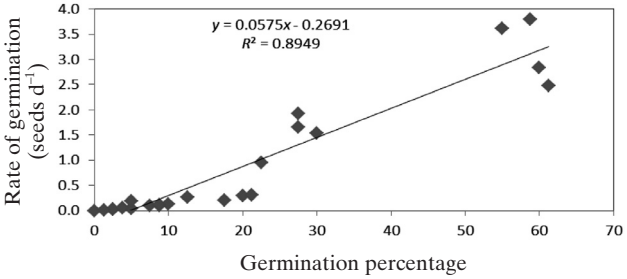

FIG. 4

Relationship between the germination percentage and the germination rate of sweet cherry (*Prunus avium* L.) 'Lambert' seeds after the various treatments applied.

(Radosevich *et al*., 1997). Many *Prunus* species, including *P. avium* L., exhibit two different types of dormancy: internal (or embryo) dormancy and external (or endocarp) dormancy (Ghayyad *et al*., 2010). The results of this study indicated a strong promoting effect of seed washing treatment on breaking dormancy in sweet cherry seed. Similarly, Nadjafi *et al*. (2006) reported that washing seed for a period of 14 d resulted in increased germination rates and percentages in *Ferula gummosa*. Washing is a routine procedure used to enhance the germination of dormant seeds (ISTA, 1996). Washing seeds under tap water or soaking seeds in water helps to soften the seed coat, removes any inhibitors of germination, reduces the time required for germination, and increases the germination percentage (Kumar *et al*., 2012). The results of the present investigation agree with previous reports on enhancing seed germination by washing (Maiti *et al*., 2003; Mirzadeh Vaghefi *et al*., 2010).

The positive effect of a water washing treatment on the percentage and rate of germination of sweet cherry seeds was more pronounced when it was followed by the application of GA_3. Previous work indicated that the application of exogenous GA_3 resulted in release from seed dormancy (Grappin *et al*., 2000; Pipinis *et al*., 2012). The gibberellin content is low in dormant seed, but increases gradually prior to, or at dormancy break (Nagar and Anil, 2000). The physiological role of GA_3 in regulating dormancy is not fully understood (Suttle, 2000). However, it has been shown that the gibberellin-mediated hydrolysis of storage materials precedes growth processes in the embryonic axis (Arias *et al*., 1976). Exogenous gibberellins also promoted the expression of hydrolase and protein-kinase genes associated with mobilising storage materials and sprouting (Alvarado *et al*., 2000). In addition, GA_3 has an active role in the synthesis of hydrolytic enzyme in seeds and, consequently, in releasing metabolites and transporting substances to the growing embryo (Kucera *et al*., 2005). The effect of the application of GA_3 on breaking seed dormancy depends on its concentration. Among the concentrations of GA_3 tested in this study, 500 mg l^{-1} GA_3 resulted in the highest germination percentage. A further increase to 1,000 mg l^{-1} GA_3 did not differ significantly from 500 mg l^{-1} GA_3. Similar results have been reported in other species, including *F. gummosa* (Nadjafi *et al*., 2006) and *Sesamum indicum* (Kyauk *et al*., 1995).

Cold stratification is commonly applied to enhance seed germination in different plant species (Bewley and Black, 1994; Hartmann *et al*., 2002). In this study, cold stratification up to 6 weeks clearly increased the

percentage and rate of germination in sweet cherry seed. The efficacy of cold stratification on seed germination has been demonstrated in other *Prunus* species such as *P. persica* (Frisby and Seeley, 1993) and *P. dulcis* (Garcia-Gusano *et al*., 2004). The effect of cold stratification on enhancing dormancy-breaking is due to the physiological changes which occur at low temperatures and act as cues for germination. These changes can probably be attributed to adaptations to the natural environmental conditions encountered by this species (Manjkhola *et al*., 2003). Our results agreed with previous reports by Yang *et al*. (2007) and Nadjafi *et al*. (2006) in other species. Frankland and Wareing (1962) reported that cold stratification increased endogenous gibberellin levels and decreased inhibitor concentrations in the seed, which, in turn, increased germination. Martinez-Gomez and Dicenta (2001) concluded that the enhancement effect of cold stratification on seed germination could be further improved when combined with other dormancy breaking treatments such as the application of hormones. According to this study, cold stratification followed by the application of GA_3 was more effective at breaking seed dormancy than cold storage alone.

CONCLUSIONS

Washing sweet cherry seeds in running tap water increased both the germination percentage and the rate of germination by > 50%. Although the application of 500 mg l^{-1} GA_3 significantly improved seed germination, the combination of washing and 500 mg l^{-1} GA_3, and/or cold stratification, were even more effective at breaking dormancy. The highest germination percentage (61.2%) was obtained from the triple combination of seed washing, followed by 500 mg l^{-1} GA_3 treatment and 6 weeks of cold stratification. The highest rate of seed germination was obtained by washing, 1,000 mg l^{-1} GA_3 and 8 weeks of cold stratification.

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