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Breaking seed dormancy in long-term stored seeds from Iranian wild almond species

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Summary

Different treatments for germination of two-year stored seeds have been assayed for some wild almond species including *Prunus scoparia*, *Prunus elaeagnifolia* and *Prunus hausskenchtii*. The treatments included the application under *in vivo* stratification (chill) conditions of distilled water, hydrogen peroxide, and GA_3; and the applications of different concentrations of GA_3 (0.5, 0.75 and 1 mg/l) in agar medium under *in vitro* conditions. Seed germination commenced after 5 and 10 days under *in vivo* and *in vitro* conditions, respectively. Significant differences were observed in the final percentage of seed germination between species and treatments in both experiments and which collectively showed that *P. scoparia* had the best germination capacity. Treatments with H_2O_2 and GA_3 were significantly better in all populations with a higher percentage of germination in the *in vivo* stratification experiments. In addition, seed germination was affected by hormone concentration and revealed a clear difference with increasing GA_3 concentration *in vitro*. A combination of stratification and a pre-treatment with H_2O_2 and GA_3 reduced the germination time and promoted a higher germination rate of the stored seeds in the wild almond species studied.

Introduction

Different wild *Prunus* species close to almond have been described in the mountainous areas of Central Asia from the Tian Shan region in China to Kurdistan (Kester *et al.*, 1991). These wild almond species play a great socio-economical and ecological role. In fact, they have been used for different purposes by native people including consumption, the extraction of oil and the role played by the plants in the reduction of soil erosion (Martínez-Gómez *et al.*, 2007). On the other hand, in cultivated almond germplasm, a limited gene pool restricts production to specific areas and conditions. The introduction of genes from these related species through interspecific hybridization has been used in several rootstock and cultivar breeding programs (Gradziel *et al.*, 2001; Martínez-Gómez *et al.*, 2003).

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Natural regeneration and conservation of these wild almond species occurs only by seed. Their seed can be so used to establish orchards and collections and for *in situ* and *ex situ* conservation. To survive in a particular location, plants have developed mechanisms that regulate seed germination at the most convenient season of the year. One of these mechanisms of seed germination is seed dormancy, which can be defined as the temporary failure of an intact viable seed to complete germination under favourable conditions (Bewley, 1997). In *Prunus* species, seed dormancy is an adaptive mechanism to protect these temperate fruit trees from freeze damage during the winter (García-Gusano *et al.*, 2004). A seed which is dormant will not germinate even when the environment is adequate for germination (Martínez-Gómez and Dicenta, 2001). In *Prunus* seed dormancy is caused by two mechanisms: an external mechanism which inhibits seed germination and is controlled by the endocarp and the seed coat, and an internal mechanism controlled by the embryo which affects later growth of seedlings (Mehanna and Martin, 1985; Mehanna *et al.*, 1985; Seeley *et al.*, 1998; García-Gusano *et al.*, 2004; 2005; 2009). The latter mechanism is the most common type of dormancy in plant seeds of temperate regions and it is essentially governed by antagonistic roles of Abscisic acid (ABA) and Gibberellic acid (GA) that are influenced by environmental factors such as temperature and light (Koornneef *et al.*, 2002, Debeaujon *et al.*, 2000, Hazebroek *et al.*, 1993).

The breakage of these dormancy mechanisms is necessary for the completion of seed germination. A pre-treatment of dormant seeds to force germination could avoid the prolonged duration of seeds in germination beds in nurseries. In this regard, several treatments are used to eliminate seed dormancy such as scarification, stratification, and gibberellic acid (GA_3) and hydrogen peroxide (H_2O_2) application. Stratification has been the method traditionally used to break seed dormancy in *Prunus* species (Grigorian 1972; Zigas and Coombe 1977; Mehanna *et al.,* 1985; Frisby and Seeley 1993; Seeley *et al.*, 1998). In addition, Hilhorst and Karssen (1992) suggested that environmental factors that overcome seed dormancy may be mediated by GA biosynthesis. Instead, seed from GA- deficient mutants of some plants can not germinate in the absence of exogenous GA (Koornneef and Van Der Veen, 1980; Groot and Karssen, 1987). Finally, hydrogen peroxide (H_2O_2) is another compound that has been largely used as a germination stimulant (Fontaine *et al.*, 1994, Chien and Lin, 1994).

In this work we studied the effects of different treatments on the germination of two-year stored seeds from different wild almond species under *in vivo* and *in vitro* conditions.

Material and methods

Plant material

Mature fruits of three wild almond species including *Prunus scoparia* (Spach) C.K. Schneid., *P. elaeagnifolia* (Spach)*,* and *P. haussknechtii* C.K. Schneid. endemic of the Irano-Afghan plate were collected in the province of Esfahan (Central Iran). Wild almond seeds with shells (endocarp) were stored at room temperature for two years. After this period, seeds with shells were surface-sterilized for 20 minutes in sterile beaker containing

full-strength commercial sodium hypochlorite (40 g/l of active chloride). Following surface-sterilization, seeds with shells were rinsed three times for 2 minutes each in sterile distilled water. To maintain the sterility of the seeds, the shells were kept out in all the experiments using strong sterilized scissors, treating latterly the seeds with the different treatment assayed.

Germination treatments

The *in vivo* (stratification) experiment tested four different treatments before stratification. Seed lots were soaked in sterile solutions of distilled water (for 24h) (control), hydrogen peroxide H_2O_2 (0.5%, 24h), GA_3 (1 mg/l, 30min) and combined hydrogen peroxide H_2O_2 (0.5%, 24h) and GA_3 (1 mg/l, 30min). After treatment the seeds were put on sterile vermiculate in Petri dishes (figure 1) and maintained at stratification, chilling temperature of 4°C in a cold chamber. Four other treatments were assayed in the *in vitro* experiments.

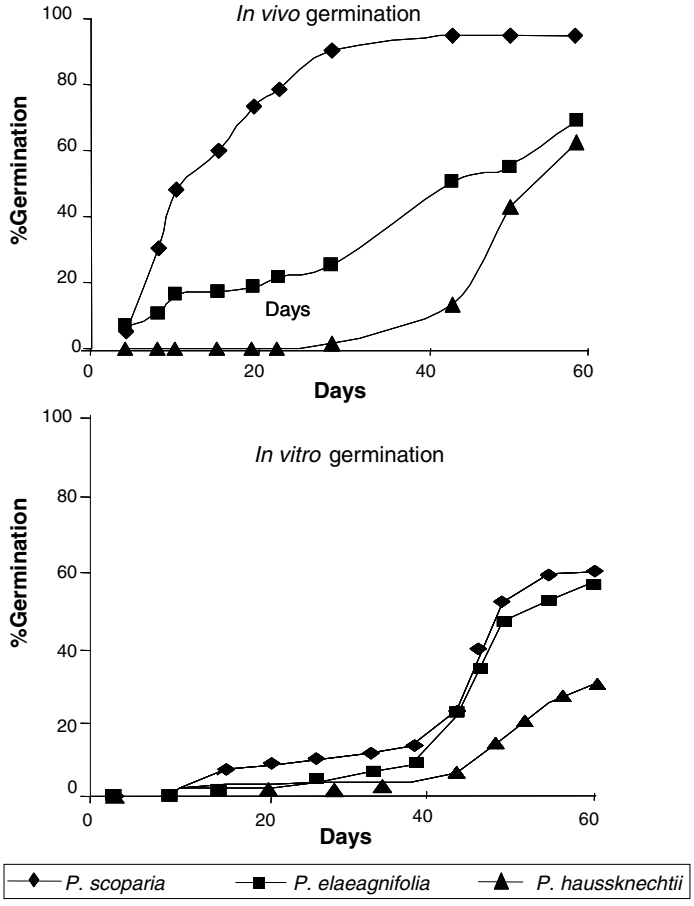

Figure 1. Evolution of mean values for seed germination in the wild almond species studied under *in vivo* (conventional stratification) and *in vitro* (without stratification) conditions.

The germination media used in these experiments were full strength MS medium containing 30 g/l of sucrose and different concentrations of GA_3 [0 (control), 0.5, 0.75 and 1 mg/l]. The medium was solidified by 7 g/l of agar and the pH was adjusted to 5.8 before autoclaving for 20 minutes at 120°C. The medium was then poured into tubes at the rate of 12 ml/tube. The seeds were transferred directly to the medium after their extraction from the shells (figure 1). Germination was carried out in a growth chamber at 25°C under a light irradiance of 19 μmol m^{-2} s^{-1} and a 16/8 h (day/night) photoperiod.

Statistical analysis

The germination response was scored daily and seeds were counted as germinated when a 2 millimetre length radicle had emerged from the seed (figure 1). Germination was recorded over 2 months (60 days). The variables studied included final seed germination (percentage), germination capacity (germinated/non germinated seeds), mean germination time for maximum seed germination (days), germination rate (coefficient of velocity of germination) and coefficient of uniformity of germination calculated according to the formulas reported by Ranal and Santana (2006). Germination experiments were analyzed in a factorial model based on completely randomized design (CRD) with 3 levels of species, 4 levels of treatments and 30 replications in both *in vivo* and *in vitro* experiments. The significance of treatment means were tested by the factorial model based on CRD analysis on the transformed Arcsin square root of germination percentages using MSTATC and SPSS (9.0). Means were tested by LSD test when significant differences were identified. A significance level of 5% was used for all statistical analysis and the results obtained from experiments were analyzed separately.

Results

Regarding *in vivo* germination experiments, significant differences were observed in the final percentage of seed germination between species, treatments and their interaction. There were significant differences for final germination in the 3 species studied with *P. scoparia* being the most vigorous (94.6% of mean in all the *in vivo* treatments), germination capacity (13.6) and germination rate (3.6). This species also showed the fastest germination time (55.3 days of mean germination time) with a good uniformity (0.012). On the other hand, treatment with H_2O_2 and GA_3 resulted significantly better in the 3 species studied with a higher percentage of germination (91.6% of mean) and a better germination capacity (10.0) and germination rate (3.2%). These results were positive when taken in the context of the long period of room-temperature storage of these seeds. On the other hand, the treatments with H_2O_2 and GA_3 also showed a faster germination time (31.8 days) (table 1). Germination was first observed under our *in vivo* conditions after 5 days in *P. scoparia* and *P. elaeagnifolia* and 25 days in *P. hausskenchtii* reaching their maximum at 40 days in the case of *P. scoparia* and 56 days in *P. elaeagnifolia* and *P. hausskenchtii* in all the *in vivo* treatments assayed (figure 1).

As in the case of the *in vivo* germination assays, significant differences were observed in the final percentage of seed germination between species and treatments *in vitro*

Table 1. Germination of seeds in the wild almond species studied under *in vivo* (conventional stratification) and *in vitro* (without stratification) conditions.

		In vivo germination					*In vitro* germination				
		Control	H_2O_2	GA_3 (1 mg/l)	$GA_3 + H_2O_2$	Mean	Control	GA_3 (0.5 mg/l)	GA_3 (0.75 mg/l)	GA_3 (1 mg/l)	Mean
Seed germination (%)	*P. haussknechtii*	64.3 b	59.3 b	39.3 c	78.4 b	*65.0*	16.0 b	18.9 c	22.3 b	39.3 b	*28.0*
	P. elaeagnifolia	60.2 b	72.2 ab	51.2 b	72.4 b	*68.3*	43.3 a	45.3 b	60.2 a	68.1 a	*57.0*
	P. scoparia	89.3 a	94.3 a	83.3 a	96.3 a	*94.6*	56.2 a	62.3 a	64.3 a	72.3 a	*59.5*
	Mean	*68.6*	*81.7*	*49.3*	*91.6*	***74.0***	*34.1*	*36.0*	*39.1*	*55.3*	***44.8***
Germination capacity	*P. haussknechtii*	2.5 b	2.3 b	1.6 b	3.2 b	*2.5*	0.3 a	0.3 a	0.2 a	0.6 b	*0.4*
	P. elaeagnifolia	2.0 b	3.9 a	2.0 ab	5.6 b	*2.9*	0.6 a	0.8 a	0.5 a	1.2 a	*1.1*
	P. scoparia	3.9 a	4.6 a	2.6 a	13.6 a	*7.8*	0.8 a	0.6 a	0.7 a	1.4 a	*1.2*
	Mean	*2.6*	*4.4*	*1.9*	*10.0*	***4.3***	*0.5*	*0.5*	*0.6*	*1.2*	***0.7***
Germination time (days)	*P. haussknechtii*	50.3 a	53.2 a	46.5 a	45.3 a	*50.5*	45.3 a	46.9 a	42.0 a	38.6 ab	*42.5*
	P. elaeagnifolia	52.2 a	49.3 a	46.3 a	43.6 a	*44.8*	42.3 a	40.5 b	43.3 a	41.2 a	*39.4*
	P. scoparia	27.9 b	28.3 b	26.8 b	23.5 b	*25.3*	44.2 a	39.6 b	38.2a	30.1 b	*36.4*
	Mean	*40.9*	*36.2*	*33.5*	*31.8*	***36.5***	*45.5*	*41.0*	*41.6*	*33.5*	***40.6***
Germination rate	*P. haussknechtii*	2.0 b	2.4 b	1.5 c	1.9 c	*1.9*	2.2 a	2.3 a	1.9 b	2.2 b	*2.2*
	P. elaeagnifolia	2.5 a	2.3 b	2.2 b	2.7 b	*2.4*	2.6 a	2.5 a	2.6 a	2.6 b	*2.5*
	P. scoparia	2.8 a	3.5 a	3.3 a	3.8 a	*3.6*	2.5 a	2.6 a	2.5 a	3.2a	*2.7*
	Mean	*2.6*	*2.9*	*2.1*	*3.2*	***2.8***	*2.2*	*2.4*	*2.4*	*2.9*	***2.5***
Coefficient of uniformity	*P. haussknechtii*	0.003 a	0.003 a	0.004 a	0.005 a	*0.005*	0.002 b	0.006 a	0.002 b	0.012 a	*0.009*
	P. elaeagnifolia	0.005 a	0.003 a	0.007 a	0.008 a	*0.006*	0.032 a	0.008 a	0.009 ab	0.005 b	*0.008*
	P. scoparia	0.003 a	0.003 a	0.003 a	0.012 a	*0.012*	0.009 b	0.007 a	0.016 a	0.009 a	*0.010*
	Mean	*0.003*	*0.003*	*0.003*	*0.003*	***0.003***	*0.034*	*0.007*	*0.010*	*0.006*	***0.024***

[a] Values followed by the same letter show no significant differences by LSD range test ($P<0.05$)

although their interaction was not significant. As in the *in vivo* experiments *P. scoparia* had the best percent of seed germination (59.5%) with a higher germination capacity (1.2) and rate (2.7); and a faster germination time (36.4 days). However, differences were smaller than in the case of *in vivo* stratification assays. Lowest seed germination occurred in *P. haussknechtii* species (table 1). On the other hand, seed germination was affected by hormone concentration with a clear difference with increasing GA_3 concentration *in vitro*. Among all *in vitro* media and studied species, seed germination was significantly higher (55.3%) on a concentration of 1 mg/l GA_3 with a better germination capacity (1.2) and germination rate (2.9%); and a faster germination time (33.5 days) (table 1). Seeds became swollen after one week incubation on culture media, and germination commenced after 10 days in *P. scoparia* and 30 days in *P. haussknechtii* and *P. elaeagnifolia*. The maximum germination occurred in *P. scoparia* after 48 days and 56 days in *P. elaeagnifolia* and *P. hausskenchtii* (figure 1).

In general the *in vivo* assays using stratification treatments showed a higher percentage of germination (74.0%) in comparison with *in vitro* assays (44.8%) with a better germination capacity (4.3) and germination rate (2.8%); and a faster germination time (36.5 days) (table 1; figure 1). In both experiments, seedlings were transferred to pots in a growth chamber without any apparent stress and all pre-treatment did not show any detrimental effect on seedling growth.

Discussion

All *Prunus* species showed significant different germination requirements under both *in vivo* and *in vitro* conditions. *P. scoparia* germinated better than *P. eleagnifola* and *P. hausskenchtii* in both experiments indicating a significant advantage in arid and semi-arid conditions and may help explain this plant broader geographic range. The seeds of *P. elaeagnifolia* exhibit different behaviour from *P. hausskenchtii*. We hypothesize that these results may be caused by different chilling (stratification) requirements, the thickness of the seed integuments, and the concentration of germination inhibitors and percentage of kernel. Such inhibition due to the seed integuments was verified in woody species such as *Pinus taeda* (Cooke *et al.*, 2002).

Long-term stored seeds of all species showed different responses to stratification and the *in vitro* germination tests. The stratification experiment had a dramatic effect on germination percentages. This variation in final and speed of germination could be due to amount of water absorbed by the kernels and the degree of aeration of the germination substrates. Under *in vivo* conditions aeration and water availability in vermiculite are more important than within the agar solidified medium in *in vitro* conditions. Many studies reported the importance of oxygen in seed germination. During seed germination an increase in respiration processes has been reported which implies the importance of supplementing with enough oxygen during the process (Ogawa *et al.*, 2001). Many studies have also reported the reduction in germinability of seeds as a result of low oxygen concentration than the surrounding air (Eldestein *et al.*, 2008). Maximum germination of crops such as wheat, sorghum, corn, soybean and sunflower was also reported when oxygen concentration was about the atmosphere (Arya, 2005). The effect of carbon

dioxide in seed germination is opposite to that of oxygen. Many studies have stated that increasing the concentration of CO_2 beyond that of the surrounding air decreases the seed germination of most plant species (Arya 2005).

Based on colleted data in the current experiments, seed germination was affected by H_2O_2 treatment and there was no significant difference between H_2O_2 and (H_2O_2 + GA_3). This indicates that there is no synergistic effect of these two compounds. The exogenous application of H_2O_2 or its accumulation by using catalase inhibitors promote the germination of dormant seeds of many species (Hendricks and Taylorson, 1975; Reuzeau and Cavalie, 1995; Ogawa and Iwabuchi, 2001). In this situation the mechanism of action was explained by the activation of the oxidative pentose phosphate pathway, via a coupling involving the glutathione oxido-reductase cycle. The NADPH produced reduces the thioredoxin protein which activates some enzymes of carbohydrate metabolism such as alpha amylase and pyrophosphate fructose 6-phosphate, 1-phosphotransferase (Fontaine *et al.*, 1994, Buchanan *et al.*, 1994). According to our result H_2O_2 can be selected as the most economic and best forcing agent for a large scale application in seed germination of these wild almond seeds. Indeed, it has been used as both a sterilizing and a forcing agent to improve the germination of numerous herbaceous and woody species (Fontaine *et al.*, 1994, Chien and Lin, 1994). Furthermore, H_2O_2 could be used as an alternative aggressive antiseptic or fungicide (Garcia-Gusano *et al.*, 2004) for seed sterilization.

Stratification and GA_3 treatment did not stimulated seed germination like H_2O_2 in stratification experiments but a stimulation of "Halfold" peach cultivar seeds by GA was observed by Mehanna *et al.* (1985). Nevertheless, results of *in vitro* conditions showed that seed germination was affected by hormone concentration and revealed a clear difference with increasing GA_3 concentration. More seed germination was produced with GA3 (1 mg/l). This means that the concentration and the duration of the pre-treatment were not sufficient to break the dormancy of seeds in stratification experiment. This phytohormone was previously proven to be efficient in the breaking dormancy of dormant seed due to its antagonistic effect against abscisic acid (ABA) (Mehanna *et al.*, 1985; Bhargava 1997; Chung and Pack 2003; Kucera *et al.*, 2005). In fact, the treatment with this growth regulator has shown an increase of percentage of seed germination of *Tripsacum dactyloides* (Rogis *et al.*, 2004). Also, Calvo *et al.* (2004) observed a drastic increase in *Fagus sylvatica* seeds germination when treated with GA_3 and this was reverted by paclobutrazol, a well known GA_3 biosynthesis inhibitor. Those authors concluded that GA_3 biosynthesis regulate the processes from seed dormancy to germination.

Stratification (chilling) treatments under *in vivo* conditions were more efficient in breaking dormancy in these two-year stored seeds in comparison with hormonal treatment under *in vitro* conditions. In agreement with these results, the standard method for providing germinable *Prunus* seed is stratification at 7°C for at least two months (Kester *et al.*, 1977; Mehanna *et al.*, 1985; Seeley *et al.*, 1998; García-Gusano *et al.*, 2004; 2005). A combination of stratification and a pre-treatment with H_2O_2 reduced the germination time and promoted germination percentage in these wild species. The reduced effect of stratification-GA_3 pre-treatment seemed to be due to the concentration and incubation time used. Increasing these two factors could give best results. Also the removal of seed tegument could ameliorate the time and percentage of germination.

Conclusions

This work constitutes the first deep investigation comparing *in vivo* and *in vitro* conditions germination in wild almond species. It provides an efficient and economic protocol for forcing germination of the studied species combining scarification, stratification, and H_2O_2 and GA_3 treatments. From the three wild almond species studied *P. scoparia* showed the best percent of germination and germination capacity. The obtained results were positive when considering the long period of room-temperature storage of these seeds. *In vivo* assays using stratification treatments showed a higher and faster germination. A combination of stratification and a pre-treatment with H_2O_2 and GA_3 under *in vivo* conditions in Petri dishes reduced the germination time and promoted the higher germination rate in these wild almond species. This protocol could be feasible at a commercial scale in nurseries.

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