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Effect of seed age, stratification, and soaking on germination of wild asparagus (*Asparagus acutifolius* L.)

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ABSTRACT

Wild asparagus (*Asparagus acutifolius* L.) is a widespread species found in all the Mediterranean areas. The spears are highly valued by consumers and owing to its frugality, this species is a feasible new crop with high income potential, especially for Mediterranean marginal areas. Currently, the cultivation of this species is limited because of its low and erratic seed germination that makes difficult the production of seedlings for plant propagation. In this research, non-after-ripened (1 month-old) and after-ripened seeds (dry stored at room temperature for 13 months) were exposed for 30 days in the dark to three moist stratification treatments: cold (5 °C), warm (23 °C) or no stratification; subsequently they were soaked for 12 h in warm water (35 °C) or not soaked. The effect of these pre-germination treatments on three germination parameters (germination percentage, time to 50% of final germination – T_{50} – and germination pattern) was studied, as well as some possible seed dormancy forms involved therein. The 1-year dry storage period proved to be effective in after-ripened seeds by enhancing seed sensitivity to the subsequent pre-germination treatments. After-ripened seeds exhibited higher and more rapid germination compared to non-after-ripened seeds. Soaking, cold or warm moist stratification had similar single effect on non-after-ripened seeds (27% germination). With after-ripened seeds, only soaking or warm stratification were effective (47% germination) when singularly applied, while cold stratification did not improve germination. By combining stratification and soaking treatments, a higher germination for both non-after-ripened and after-ripened seed-lots was achieved. The highest germination was obtained when after-ripened seeds were stratified and soaked (76%), without any significant difference between cold or warm stratification. Single or combined application of moist stratification (regardless of the temperature used) and soaking resulted always in a faster germination compared to that of no-treated seeds and especially with after-ripened seeds (T_{50} = 6 days). A non-deep type 1 physiological dormancy can be hypothesized for the seeds of this species. Low stratification temperature induce secondary dormancy in after-ripened seeds that can be removed by soaking them at 35 °C for 12 h.

1. Introduction

Wild asparagus (*Asparagus acutifolius* L.), former classified in the *Liliaceae* family, has been recently included in the *Asparagaceae* family (Angiosperm Phylogeny Group II, 2003). The centre of origin and the geographic distribution is South Europe, eastwards to southeast Bulgaria (Štajner et al., 2002). Currently, wild asparagus is a common species in all the Mediterranean areas (Sica et al., 2005). In Italy, this species is found mainly in its southern regions, where it grows spontaneously in uncultivated areas, on dry stonewalls fences and specially in the Mediterranean macchia ecosystem. The spears are highly valued and consumed in a vast number of regional dishes (Ghirardini et al., 2007). The market for wild asparagus is based on the harvest from wild, low-yielding plants, whose produce is sold at high prices (15–25 €/kg) and it is restricted to only local and small markets.

Cultivation trials have produced yields of 1.2–1.3 t ha^{-1} over a 2-month harvest (Rosati et al., 2005; Benincasa et al., 2007). The

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species is suitable for low-input growing systems because of its frugality and adaptation to marginal and arid lands. Wild asparagus can become a new crop with high income potential, especially for marginal areas where its cultivation will fit perfectly within a sustainable agriculture framework of both biodiversity and environmental conservation.

Currently, some limitations exist in the cultivation of this vegetable, the most important is related to its low and erratic seed germination that limits seedling or crown production (Rosati, 2001). Chemical scarification techniques to overcome exogenous (physical) dormancy by either the disruption of seed coats and pericarp tissues have proven ineffective in breaking dormancy (Venezia et al., 1993). Therefore, it can be hypothesized that dormancy of this species depends specifically on endogenous factors.

Seed primary dormancy is an intrinsic inhibition of early germination; in freshly harvested, mature, and water permeable seeds it can rely on morphological, physiological, or morpho-physiological factors (Baskin and Baskin, 2004).

The dormancy release is regulated by complex interactions between environmental and genetic factors that are scarcely understood. Morphological dormancy is released within a short period of moist stratification, while physiological dormancy is frequently overcome by after-ripening conditions (dry storage of seeds), warm stratification or cold stratification (chilling) (Baskin and Baskin, 2004).

Physiological dormancy (deep, intermediate, non-deep level) has been related to factors found within the embryo and any embryo-covering structure. It is the most prevalent form of dormancy found in seeds of angiosperms, mainly in the non-deep level (Nikolaeva, 2004), although in *Liliaceae* family also morphological and morpho-physiological dormancy has been reported (Finch-Savage and Leubener-Metzger, 2006).

Rosati and Falavigna (2000) found that dry storage of *A. acutifolius* seeds for more than 1 year did not completely break dormancy, obtaining a satisfactory germination (~90%) only after moist stratification of freshly harvested seeds in sand for at least 8 months. They also observed that irrespective of dry storage or stratification treatments, germination responses were strongly influenced by ecotype. Other authors, working with different ecotypes, reported 60–70% germination with seeds maintained in open field conditions of a Mediterranean environment under 70% of shading for almost 5 months (January–May: winter–spring period), independently if stratified in sand or directly sown in peat (Fiori et al., 2001).

Soaking seeds in warm water (35 °C, for 12 h) has been reported effective in improving germination of *A. acutifolius* (Piotto et al., 2003). However, the mechanisms to overcome dormancy in *A. acutifolius* seeds are neither clear nor conclusive and deserve further research.

To evaluate the effect on the germination performance as well as to elucidate on the possible seed dormancy class involved for this species, in this paper we report results of two pre-germination treatments (warm/cold moist stratification and soaking) applied on non-after-ripened and after-ripened seeds of *A. acutifolius* collected in Southern Italy.

2. Materials and methods

The seeds used in this study were collected from Daunia Subappennines (Foggia province) in Apulia, the easternmost part of the Italian peninsula. The plants were selected from uncultivated areas of the Mediterranean macchia ecosystem in Orsara di Puglia (41°17′N, 15°16′E). This area is dominated by a Mediterranean macroclimate that becomes more continental at higher altitudes (Macchia et al., 2000); mean minimum and maximum temperatures are 9.3 ± 1.6 and 17.4 ± 2.2 °C, respectively, and mean annual rainfall is 700 mm (Caliandro et al., 2005).

2.1. Seed collection and conditioning

On November 2005 and 2006, wild *A. acutifolius* plant stems with mature green-brown berries were collected. The stems were air-dried for 2 weeks under 90% shading before harvesting the berries. After harvesting, the pericarps were manually removed. The seeds collected in the first year (after-ripened) were dry stored for 13 months in paper bags at room temperature (21 ± 2 °C; R.H. 50 ± 10%).

On December 2006, both seeds collected the month before (non-after-ripened) and after-ripened seeds were treated with a 2% sodium hypochlorite solution for 10 min, then washed in running tap water and air-dried.

2.2. Pre-germination treatments

Non-after-ripened and after-ripened seeds (seed-lots) were subjected to a factorial combination of two treatments: (1) moist stratification: cold (5 °C), warm (23 °C) or no stratification, and allowed by (2) soaking: in warm water or no soaking.

Stratification was carried out in flat aluminium containers (6 cm × 10 cm × 20 cm) filled with a 5 cm layer of washed river-sand saturated with distilled water. The containers were placed in plastic bags to avoid dehydration of the substrate and were stored in the dark in a growth chamber for 30 days. After stratification, soaking treatments were performed with 4 replicates, by putting in 24 vials (50 mL) filled with 25 mL of distilled water, 25 seeds/vial (average weight 1.5 g). The vials were maintained in a thermostatic bath at 35 °C for 12 h in the dark.

After soaking, seeds were washed in running water and then they were air-dried again. The germination test was carried out by placing 25 seeds on moist Whatman No. 1 filter paper inserted in 9-cm diameter Petri dishes (experimental unit). Petri dishes were arranged in a completely randomized design in a growth chamber at 23 °C in the dark. Four replicates were used for each treatment. After the beginning of germination, every 2 days and for a period of 30 days (incubation period), the germinated seeds (young radicles over 5 mm in length) were counted and removed from each Petri dish. Final germination percentage was calculated as number of germinated seeds/25 × 100.

2.3. Statistical analysis

Cumulative proportion of germinated seeds curves for each seed-lot and pre-germination treatment were constructed using Weibull's function with three parameters (Damato et al., 1994):

$$Y = a[1 - 100^{-(X/q)^b}]$$

where Y is the cumulative proportion of germinated seeds at time X, a is the maximum germination that may be reached, b is a shape parameter and q is the time required to achieve 99% of a from the beginning of the germination period.

The three parameters of Weibull's function for each treatment was estimated through PROC NLIN (SAS Institute, 1999). Time to 50% cumulative germination (T_{50}) was calculated using the following formula derived from Weibull's function:

$$T_{50} = \left\{\log_{10}\left[\frac{-a/(Y-a)}{2}\right]\right\}^{(1/b)} q$$

Table 1
Significance of *F*-test for stratification and soaking treatments on the germination and on the time required to achieve 50% cumulative germination (T_{50}) in after-ripened and non-after-ripened seeds (seed-lots) of *Asparagus acutifolius*

Factor	Germination (%)	T_{50} (days)
Seed-lot (SL)	***[a]	***
Stratification (ST)	***	***
Soaking (SK)	***	ns
ST × SK	ns	**
ST × SL	ns	***
SK × SL	*	*
ST × SK × SL	*	***

[a]ns, * and ***, not significant or significant at $P < 0.05$ or 0.001, respectively.

Data were subjected to ANOVA and mean separation was carried out using $LSD_{0.05}$ test. The values expressed as percentages were transformed by arcsin $\sqrt{x}$ before data analysis.

3. Results

At the end of the pre-germination treatments none of the seeds had germinated, and at the end of the incubation period, all seeds were totally imbibed even if they had not germinated.

3.1. Germination percentage

Dry storage, stratification and soaking interacted significantly in affecting seed germination (Table 1).

Non-treated seeds of both seed-lots showed the lowest germination (12%, on average) (Fig. 1). In non-after-ripened seeds the application of soaking and stratification (cold or warm) improved germination up to 27%, on average. In after-ripened seeds, singly soaked or stratified at 23 °C, germination averaged 47%, while with the only stratification at 5 °C germination was 16% and not significantly different from that obtained in non-treated seeds. With the combination of both stratification (cold or warm) and soaking, germination was higher than 76% and 56% on average, for after-ripened and non-after-ripened seeds, respectively, without any significant difference between the temperatures used in the stratification.

3.2. Time to 50% cumulative germination (T_{50})

T_{50} was significantly influenced by the interaction among the three experimental factors (Table 1).

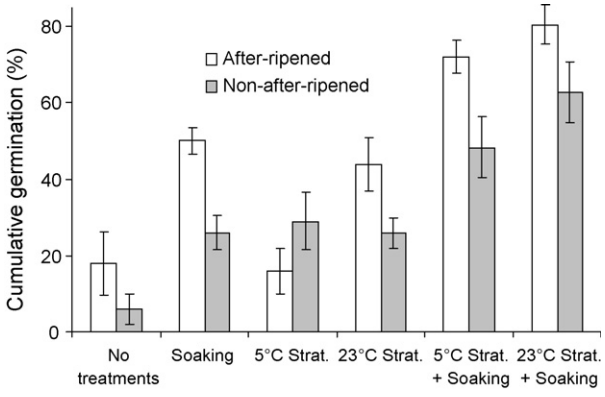

Fig. 1. Cumulative germination in non-after-ripened or after-ripened *Asparagus acutifolius* seeds untreated or treated with stratification for 1 month at 23 or 5 °C or soaking for 12 h in 35 °C water or with the combination of stratification followed by soaking for 12 h in 35 °C water. (Vertical bars represent ±S.E. of means.)

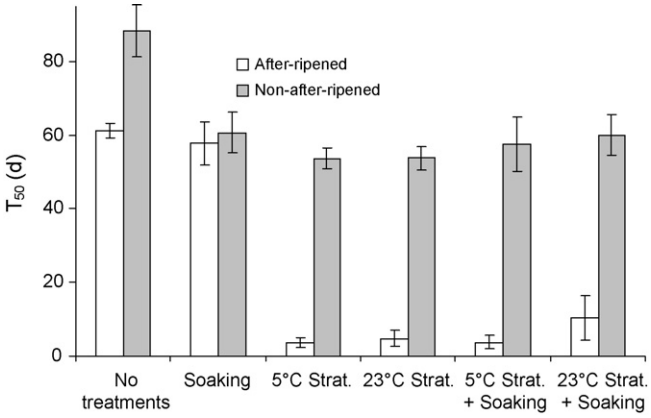

Fig. 2. Time required to reach 50% cumulative seed germination (T_{50}) in non-after-ripened or after-ripened *A. acutifolius* seeds non-treated or treated with stratification for 1 month at 23 or 5 °C or with soaking for 12 h in 35 °C water or with the combination of stratification followed by soaking treatments. (Vertical bars represent ±S.E. of means.)

With the non-after-ripened seeds, when no pre-germination treatments were applied, T_{50} was 88 days, while it decreased to about 60 days when these seeds were only soaked, stratified, or subjected to both treatments (Fig. 2). With the after-ripened seeds, when not treated or only soaked, T_{50} was about 60 days, while by stratifying these seeds for 1 month, T_{50} was reduced to about 6 days, irrespective of stratification temperature or post-stratification soaking.

3.3. Germination pattern

When after-ripened seeds were stratified (at 5 or 23 °C) and regardless of subsequent soaking, germination started in only a few hours after placing the seeds in Petri dishes; while germination started after 44 and 52 days when these seed were only soaked or not treated, respectively (Fig. 3A and B). When non-after-ripened seeds were stratified and/or soaked, germination started between the 45th and the 52nd day (Fig. 3A and B) and after 83 days for non-treated seeds (Fig. 3C).

With 5 °C stratification, after-ripened seeds showed a fast start of germination and quickly reached a plateau. The level of the plateau was quite high when the seeds were even soaked (72%) or very low if they were not (16%) (Fig. 3B).

Excluding the 5 °C stratified after-ripened seeds, in all the other cases (irrespective of stratification or after-ripening) soaking improved the germination pattern, showing an increasing trend, so that germination appeared not completed at the end of observation period (Fig. 3A, B and C).

4. Discussion

Since seeds appeared fully imbibed at the end of the pre-germination, it points to the absence of physical dormancy, as already noted by other authors (Rosati and Falavigna, 2000).

Morphologic dormancy seeds are characterized by embryos too immature to germinate immediately and it can be released in a short time by submitting seeds to specific conditions (Baskin and Baskin, 2004) such as stratification (warm or cold depending on species) or gibberellic acid treatments (Geneve, 2003). Physiological dormancy can have an embryo and/or coat (testa, endosperm, pericarp) component; and their sum and interaction determine the degree of seed physiological dormancy (deep, intermediate, non-deep level). Embryo dormancy is characterized by inhibition of extension growth, while coat dormancy manifested as mechanical

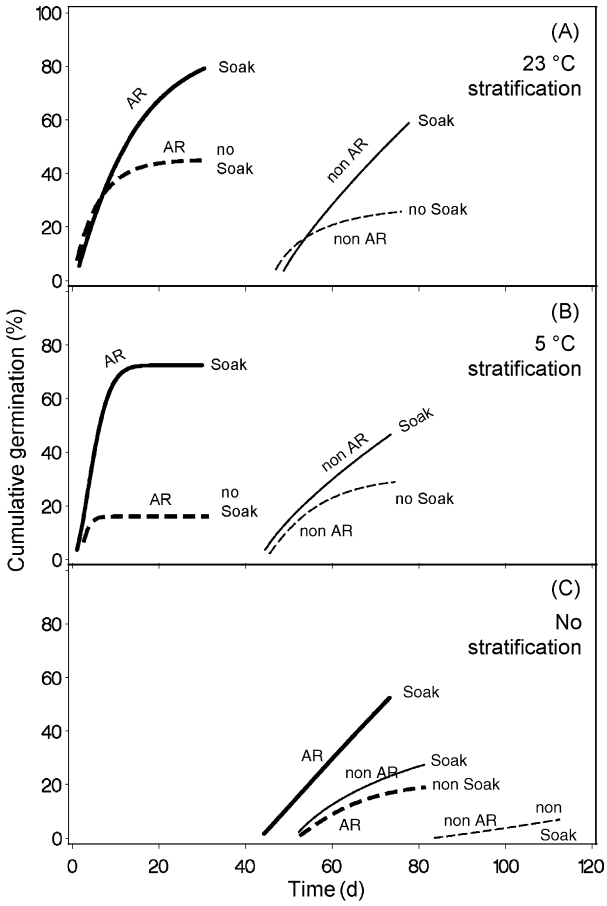

Fig. 3. Germination during the 30-day period following the beginning of the germination test in non-after-ripened (non AR) or after-ripened (AR) *Asparagus acutifolius* seeds as affected by seed stratification for 1 month at 23 °C (A), 5 °C (B), or no stratification (C), and a subsequent soak for 12 h in 35 °C water (Soak) or without soaking (no Soak)

resistance from testa and endosperm to embryo growth (Finch-Savage and Leubener-Metzger, 2006), or as chemical dormancy due to presence of inhibitor compounds in the covering layers of the seeds (Baskin and Baskin, 2004). After-ripening and moist stratification affect metabolic and physiologic changes in seeds that involve both the embryo and its covering layers (Leubner-Metzger, 2005; Bair et al., 2005) linked to a rapid decline in the abscisic acid content of imbibed dormant seeds (Gluber et al., 2005).

The non-after-ripened seeds of *A. acutifolius* given warm or cold stratified for 1 month exhibited low germination (25%) (Fig. 1), moreover the first germinated seeds appeared only after 44–45 days (Fig. 3A and B). Dry storing *A. acutifolius* seeds for 13 month (after-ripened) did not improve their germination. When no pre-germination treatments were applied no differences in germination percentage were found (12%, on average) between after-ripened and non-after-ripened seeds (Fig. 1), even if germination evolved more rapidly in after-ripened (T_{50} = 60 days) than in non-after-ripened seeds (T_{50} = 88 days) (Fig. 2). However, dry storage was crucial for dormancy release; in fact, dry-stored seeds subjected to only one or to both pre-germination treatments (except only stratification at 5 °C) always had greater germination compared to the non-after-ripened seeds given the same treatments (Fig. 1). Moreover, moist stratification strongly decreased T_{50}, but only with after-ripened seeds. With non-after-ripened, stratified and/or soaked seeds, T_{50} was about 60 days (Fig. 2). Thus the 1-year dry storage (after-ripening) enhanced seed sensitivity to factors that relieve dormancy and stimulated radicle protrusion as reported elsewhere (Finch-Savage et al., 2007).

Both in non-after-ripened or after-ripened seeds, the singly applied treatments enhanced germination. Soaking or cold or warm moist stratification had similar single effect on non-after-ripened seeds (27% germination), while on after-ripened seeds only soaking or warm stratification were effective (47% germination), while cold stratification did not improve the germination percentage (Fig. 1). In after-ripened seeds 23 °C stratification for 1 month or during the 44-days long period of incubation in the Petri dishes was necessary to start germination in only soaked seeds (Fig. 3A and C), while conversely the 5 °C-stratification inhibited germination (Fig. 3B). After-ripened seeds given 23 °C-stratification were released from primary dormancy (conditional dormancy) but 5 °C-stratification induced a secondary dormancy. Other authors have reported no effect on germination of sand stratification at +5 °C on different *A. acutifolius* ecotypes (Fiori et al., 2001).

When stratification and soaking treatments were combined, the increase in germination of non-after-ripened and after-ripened seeds was higher than when these treatments were applied singly, but their effect were not additive. Moist stratification and soaking seem to act on different mechanisms of dormancy relief, but some of them could be in common. This behaviour is in agreement with the hypothesis reported for the seeds of *Arabidopsis thaliana* Cvi, subjected to different treatments for the relief of dormancy (after-ripening, cold, nitrate, light), according to which the genetic controls of dormancy relief mechanisms are different, but they can partially overlap (Cadman et al., 2006; Finch-Savage et al., 2007).

Singly or combined application of stratification (regardless of temperatures) and soaking resulted always in a more earlier germination compared to non-treated seeds. Also, soaking allowed a higher, but gradual, seed germination. Soaking may have reduced inhibitor compounds in the covering layers of the seeds. Moreover soaking seeds in water at 35 °C for 12 h, may have helped seeds to overcome secondary dormancy promoted by the cold stratification (Fig. 3B).

These findings firmly support the hypothesis that it should not be a morphological dormancy and on the whole of the above considerations we propose that *A. acutifolius* seeds fit the characteristics a non-deep type 1 physiological dormancy according to the dormancy classification of Baskin and Baskin (2004).

5. Conclusions

The investigated genotype of *A. acutifolius* seems to have type 1, non-deep physiological dormant seeds. However, further investigations should be carried out in order to confirm this hypothesis (i.e. testing the response to gibberellic acid, and to a wider range of germination temperatures). The non-deep physiological dormancy could be interpreted as a form of *A. acutifolius* adaptation to the Mediterranean climate (dry, warm summer and rainy, cold winter) for preventing germination under unfavourable conditions.

The 1-year period of dry storage allowed seeds of this genotype to after-ripen, as revealed by the increase of their sensitivity to the subsequent pre-germination treatments.

In after-ripened seeds, stratification increased germination except when cold stratified, and stratification at either temperature was strongly effective in speeding germination. Soaking at 35 °C for 12 h improved germination to the same extent as of stratification, but it had a low effect on the rapidity of germination. Combined treatments were more effective than singly applied treatments in improving germination. Soaking was crucial in removing secondary dormancy of after-ripened cold stratified seeds.

As expected *A. acutifolius* seeds showed the variability in the depth of dormancy that is normally present within a seed-lot, linked to the strong interaction between environmental conditions and genotype.

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