

SEED DORMANCY AND GERMINATION REQUIREMENTS IN *ANGELICA PALUSTRIS* (BESSER) HOFM., A CRITICALLY ENDANGERED PLANT

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Abstract

The phenology of *Angelica palustris* seeds including maturation, germination requirements, and dormancy class, is still unknown. In opposite to the results reported from other species of *Angelica*, present findings showed that *A. palustris* produced seeds with embryo underdeveloped (the ratio between the embryo and the whole seed length was between 0.19 and 0.12) and physiologically dormant which corresponded to Morphophysiological Dormancy class. Dormancy breakdown requires a post maturation period (at least 30 days) at 18 - 20°C for a complete embryo development, and also up to 30 days of cold stratification at 4°C. The best germination indices were obtained when fruit was removed. Germination energy (Ge) was achieved within 19 days after imbibition and was 25.8 ± 0.03 and germination per cent (Gp) was achieved within 28 days and was 64.7 ± 0.14 . Fruit structure (lateral wings with air cavities) and physiology (essential oil production) are adaptations to facilitate seed dispersal and dormancy/germination balance.

Introduction

Current rate of plant diversity loss is of global concern. Many plant species are endangered by habitat loss and subsequent reduction of population viability and size. One of the most exposed habitats to human induced pressure is wetlands due to water drainage, excessive cutting, grazing, sowing, fertilization, etc. As a result, natural swamps and swamp meadows, which are specific for many plant species including marsh angelica, encompass only small and fragmented areas with adjacent small and vulnerable population (Dittbrenner *et al.* 2005). Presently, marsh angelica - *Angelica palustris* (Besser) Hoffm. (Apiaceae) – became an endangered species in whole Europe, being listed in Annex II of the Habitats Directive (1992). As listed in Annex II (b) is considered a plant species of European Community interest whose conservation requires designation of special areas of conservation (Natura 2000 sites). At national level is included in Critically Endangered (CR) zoological category (Dihoru and Negrean 2009) and indicated from 7 sites of Natura 2000 network and 18 localities respectively, from which was confirmed within recent years only from 5 localities (Sârbu *et al.* 2007).

Species survival requires appropriate conservation strategies that could not be taken without reliable information about species ecology and biology. Reproduction biology and habitat requirements are most important in this respect. Pollination strategies, germination behaviour and seed dispersal, seems to play a crucial role in population fitness and long term survival of species. Moreover, seed morpho-anatomical characteristics, germination preferences and dormancy patterns are recognised as valuable indicators of species adaptation to environment and ultimately for understanding evolutionary patterns (Baskin and Baskin 1998). Unfortunately, there is a lack of information about germination ecology of many endangered wild plant. But due to their medicinal uses, there are some reports upon seed germination requirements on a few *Angelica*

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species (Butola and Badola 2004, Kandari *et al.* 2007, Vashistha *et al.* 2009, Pop *et al.* 2011) but none on *A. palustris*. Moreover, the pattern of seed germination seems to be particularly irregular in *Angelica* species (Butola and Badola 2004). Therefore, studies conducted upon unstudied *Angelica* species will complete the figure of the germination peculiarities within the genus. This study aims to shed light upon *A. palustris* germination requirements and limitations and also upon dormancy breakdown in correlation with fruit morpho-anatomical characteristics and plant ecology.

Materials and Methods

Inflorescences bearing mature fruits of were collected in middle September (2013) from a naturally growing population in a marsh called Bahna Mare (near Balanesti, Neamt County, Romania – N: 46.96781, E: 26.61275). In order to ensure uniformity in seed quality, for observation and germination experiments only the fruits/seeds from the primary umbel were considered.

Mature fruits bearing seeds (mericarps) were detached only from the primary umbel and air dried for 5 days and stored in airtight bags. The subject of the experiments were the whole fruits and the bare seeds, post matured (at 18 - 20°C) and cold stratified at 4°C as shown in Table 1. Taking into account that a period of 30 days of cold stratification is enough to break dormancy in a range of species the experimental variants were designed considering intervals of time around 30 days. Similarly, 30 days of post ripening period is required in most species for embryo complete maturation, therefore we have tested the maturation period around this value (Table 1). For germination experiments the samples were soaked in distilled water for 24 hrs at room temperature, placed on Petri dishes (9 cm diameter) on Whatman No.1 wet filter paper and then kept 16 hrs light and 8 hrs dark at 20°C conditions. Seeds were considered to have germinated upon the initiation of radicle. Number of seeds germinated was counted daily until no further germination was found. Germination parameters were calculated using two indices, the Germination percentage ($G_p = (\text{Number of seed germinated during the test period} / \text{total number of seeds}) \times 100$) and the Germination energy ($G_e = (\text{Number of seed germinated in the shortest time} / \text{total number of seeds}) \times 100$).

Morphological observations and the photographs were made under a Zeiss Stemi 2000C stereo microscope equipped with an AxioERc5S digital camera.

Histological observations were made on samples prepared by standard sectioning method using a hand microtome (Ruzin 1999). Longitudinal section through the seeds (of about 25 - 30µm) and superficial section through the fruit, were analyzed, under a Nikon Eclipse E200 microscope, and micrographs were recorded with a Nikon Coolpix 5400 digital camera.

There were ten mainly experimental variants, thrice replicated in a randomized design, each sample consisted of 20 fruits or seeds. Means and standard errors were calculated for germination percentages. ANOVA with mean separation by protected least significant difference ($p = 0.05$) was used to assess treatment differences. Total germination percentage was calculated by linear interpolation from the two germination values closest to median germination.

Results and Discussion

As general morphology the fruit is a schizocarp (cremocarp) made of two mericarps, each bearing a single seed. Each mericarp (5 - 5.5 mm length and 3 - 3.7 mm width) shows two lateral thin wings and two surfaces: the commissural surface, slightly concave with a filiform and divided carpophore (Fig.1a), and a dorsal surface convex, with three raised ridges over the vascular

bundles (Fig.1b). The seeds, of about 3.5 - 4 mm length and 1.5 - 2 mm width, follow the general fruit morphology except the wings and the stylopod.

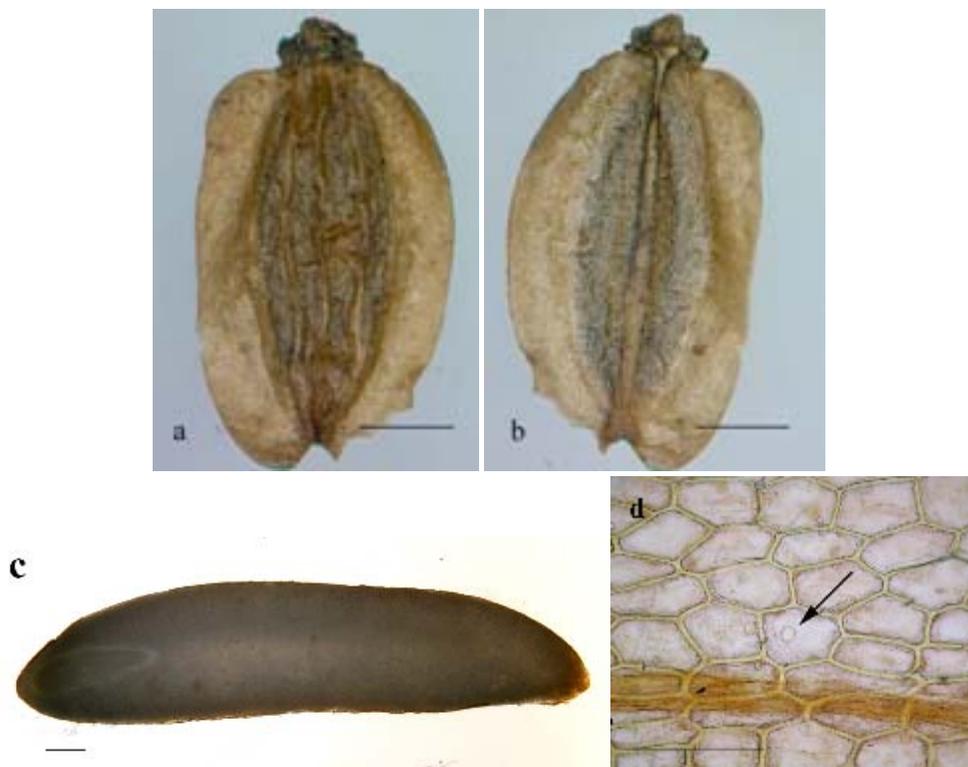


Fig. 1. *A. palustris* mericarps and seed. a. Mericarp commissural. b. Mericarp dorsal surface (bar: 1 mm), surface (bar: 1 mm). c. Seed with underdeveloped embryo (bar: 0.25 mm). d. Mericarp structure - cell with oil drops (arrow) (bar : 50 μ m).

Dormancy is a seed characteristic, the degree of which defines the conditions necessary for seed germination. In this respect, dormancy, is not due to the absence of environmental factors required to initiate germination, is considered separately from germination requirements. Therefore, we have designed our experimental variants in order to understand if the seeds are truly dormant, if yes, to what class of dormancy belongs, and which are their germination requirements.

From all the tested experimental variants, only those which involve both post maturation and cold stratification proved to be successful to achieve germination. These results show that *A. palustris* exhibit deep seed dormancy in opposite with other previously tested species of *Angelica* where germination could be achieved at a few days after harvesting (Butola and Badola 2004, Kandari *et al.* 2007, Vashistha *et al.* 2009, Pop *et al.* 2011). This behaviour confirms that dormancy and germination requirements are largely irregular within *Angelica* genus as previously suggested by Butola and Badola (2004). The best germination indices obtained for the variants for fruit was removed, respectively for V8' variant Ge was achieved within 19 days after imbibition and was 25.8 ± 0.03 and Gp was achieved within 28 days and was 64.7 ± 0.14 , compared with the similar variant where the fruit encloses the seed (V8) where Ge was achieved within 34 days and

was 16.3 ± 0.05 and Gp was achieved within 43 days and was 39.6 ± 0.18 . Extending periods for both post-maturation and cold stratification do not result in an increase in germination indices (Table 1).

Table 1. Experimental variants used for dormancy breakdown.

Experimental variant	Post maturation (days)	Cold stratification (days)	Ge days	Gp days
V1 Whole fruit	20	-	0	0
V1' Bare seed			0	0
V2 Whole fruit	30	-	0	0
V2' Bare seed			0	0
V3 Whole fruit	-	20	0	0
V3' Bare seed			0	0
V4 Whole fruit	-	30	0	0
V4' Bare seed			0	0
V5 Whole fruit	20	20	0	0
V5' Bare seed			0	0
V6 Whole fruit	20	30	2.3 ± 0.08 after 36	6.4 ± 0.12 within 45
V6' Bare seed			5.1 ± 0.02 ,, 25	8.2 ± 0.07 ,, 30
V7 Whole fruit	30	20	4.8 ± 0.04 ,, 38	11.6 ± 0.03 ,, 42
V7' Bare seed			7.5 ± 0.12 ,, 23	16.1 ± 0.13 ,, 32
V8 Whole fruit	30	30	16.3 ± 0.05 ,, 34	39.6 ± 0.18 ,, 43
V8' Bare seed			25.8 ± 0.03 ,, 19	64.7 ± 0.04 ,, 28
V9 Whole fruit	50	30	7.3 ± 0.05 ,, 34	23.1 ± 0.13 ,, 40
V9' Bare seed			11.2 ± 0.15 ,, 21	26.1 ± 0.09 ,, 32
V10 Whole fruit	30	50	4.1 ± 0.08 ,, 31	7.1 ± 0.14 ,, 44
V10' Bare seed			3.3 ± 0.08 ,, 23	5.8 ± 0.07 ,, 31

The requirement for both post-maturation and cold stratification to overcome seed dormancy and to achieve germination, suggests that even though the seed seems matured, the embryo is still underdeveloped and needs to pass a period to mature. Our observations led to the conclusion that at harvesting time *A. palustris* have seeds with a well developed endosperm but with embryos in the middle of developmental stages (torpedo), the ratio between the embryo and the whole seed length (E/S) being between 0.19 and 0.12 (Fig. 1c). A low value of E/S ratio characterizes the seeds with immature embryos. Moreover, recent findings of Vandeloos (Vandeloos *et al.* 2012) showed that genera with similar E/S tend to co-occur and genera with low E/S dominate wet habitats, like the case of *A. palustris*. During the embryo maturation, the seed does not enlarge but the endosperm is diminished and the ratio between the embryo and the seed lengths rises. According to Baskin and Baskin (2004) when the embryo development is triggered by cold stratification, the dormancy belongs to Physiological Dormancy (PD) class. Moreover, according to the same classification system, when in mature seeds the embryo is small (underdeveloped) and requires a post-harvesting period of time to mature, the dormancy belongs to Morphological Dormancy. class. In the case of *A. palustris* seeds, both types of dormancy occur, so we may consider that it belongs to a combinational class of dormancy named Morphophysiological

Dormancy (MPD) in which the embryo is both underdeveloped and physiologically dormant. This class of dormancy is considered to be primitive among the angiosperms (Forbis *et al.* 2002). This character of primitivism confirms the hypothesis that *A. palustris* is a glacial relict which managed to survive in some wet habitats, despite of gradual warming of the climate. Seed dormancy enhances seedling survival by preventing germination under unfavorable conditions being a critical regulator of the seasonal timing of germination. After ripening and cold stratification requirements prevent seed germination after dispersal in early autumn and trigger the germination process in warmer and wet conditions of late spring.

The tested experimental variants showed that the fruit removal enhances germination indices significantly, but only when the embryo is completely developed. The ecological significance of the fruit in this case, is to protect the seed from mechanical injuries but also to prevent seed imbibition prior the embryo maturation. Histological observations of the fruit showed a one layer fruit structure on each side, except the wings where the structure is double layered. The layers are made by large cells with moderately thick walls (Fig. 1d), very dried and practically dead when fruit is matured. At full maturity fruit cells acts like air cavities thus improving seed floatability and anemochory. Moreover, this cells stores essential oils that make the fruit waterproof and prevents from consumers or pathogens attack (Fig. 1d). As long as the fruit matures and dries the oils are released and becomes almost depleted when the seed/embryo are matured, allowing imbibition and subsequent germination. Furthermore, it seems to be a positive correlation between the fruit oil content and plant ecology. Species of *Angelica* with the same ecology have similar fruit oil content. Table 2 summarised data concerning fruit essential oils content in some *Angelica* species as reported in the monographic book of Azimova (Azimova *et al.* 2012) compared to species ecology after Shishkin (Shishkin *et al.* 1950). For example, *A. palustris*, *A. cincta*, *A. decurrens*, and *A. tenuifolia* (Group 1, Table 2), species of alluvial meadows and alder swamps, fruit essential oil content is approximately the same, around 2%. Likewise, if compared *A. archangelica* (wild specimens), *A. purpurascens*, *A. komarovii*, *A. sylvestris*, and *A. daurica* (Group 2), which are mountain species found on riverside, fruit essential oil content is very similar, with values around 0.5%, but not as raised as marsh *angelica* species. The same figures can be found for species which grows in more extreme environments like *A. brevicululis* from gravels near glaciers and *A. litoralis* (Group 3) from costal dunes, where fruit essential oil content is approximately 1%. For species more adaptable to various environments, the essential oils from fruit are also variable in a larger range, like it is in *A. anomalia* (Group 4) where fruit essential oil content varies from 0.3 to 3.1%. These findings suggest that environmental condition are correlated with fruit essential oil content and that in wet habitats the oil content of *Angelica* fruits is significantly raised as a result of plant adaptation to environmental conditions.

Another important subject, for both conservation and species restoration is to distinguish dormant seeds from non-viable seeds. If seed dormancy is not correctly diagnosed and understand, in the absence of germination, seed viability can be underestimated (Baskin and Baskin 2004). Our study reveals that even completely matured, about 35 - 40% of seeds are not viable. Investigation upon ungerminated seeds showed that many of them were with underdeveloped endosperm, sterile or the seed was completely consumed by insect larvae which develop inside the dried fruit. These findings are important for species conservation initiatives, both *in situ*, for reinforcement of existing population and, also *ex situ*, to establish new population in habitats less exposed to endangering factors.

The reported results lead us to the conclusions that, *A. palustris* produce dormant seeds. The dormancy belongs to Morphophysiological Dormancy class and is considered primitive. This type of dormancy supports the hypothesis that *A. palustris* is a glacial relict where dormancy

Table 2. Fruit essential oil content and ecology of some *Angelica* species.

Group	Species	Fruit essential oil content (%) (after Azimova 2012)	Species ecology (after Shishkin 1950)
	<i>A. cincta</i> Boissieu	1.96	Marshes and edge of marshes
1	<i>A. decurrens</i> (Ledeb.) B. Fedtsch.	2.7	Banks of rivers and bogs; flooded meadows
	<i>A. palustris</i> (Bess.) Hoffm	1.97	Marshes, damp stream banks; damp meadows
	<i>A. tenuifolia</i> (Pall. ex Spreng.) M. Pimen.	2.15	Marshes, herbaceous bogs; swampy beds of rivers and streams
2	<i>A. archangelica</i> L.	0.5	Marshy spruce, shrubs near ditches; sedge bogs
	<i>A. daurica</i> (Fisch ex Hoffm.) Benth. et Hook. Jol. ex Franch. et Savat.	0.55	Banks of rivers and edges of marshes
	<i>A. komarovii</i> (Schischk.) V. Tichomirov	0.5	Banks of mountain stream
3	<i>A. purpurancens</i> (Ave-Lall.) Gilli	0.41	Subalpine meadows; edge of mountain forest
	<i>A. sylvestris</i> L.	0.3 - 0.79	Inundated meadows; edge of marshes
	<i>A. brevicaulis</i> (Rupr.) B. Fedtsch	0.9 - 1.14	Banks of streams in mountain ravines, moist taluses; gravels near glaciers
	<i>A. litoralis</i> Fries.	0.9 - 1.22	Alluvial soil along riverbanks; costal dunes
4	<i>A. anomala</i> Ave-Lall.	0.36 - 3.1	Banks of rivers and streams; damp forest plots

breakdown requires both cold stratification (to trigger embryo development) and a post maturation period (at least 30 days to undergo all the embryonic stages). These two requirements are considered adaptation of germination to temperate climate. Fruit removing enhances germination indices. Fruit structure and physiology is highly correlated with seed germination and physiological significance of oil content is different in fruit (protection) compare to those of seed endosperm (reserve).

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