

A COMPARATIVE STUDY OF THE SEED GERMINATION CAPABILITIES OF *ANACAMPTIS PALUSTRIS* (ORCHIDACEAE), A THREATENED TERRESTRIAL ORCHID, AND OTHER MORE COMMON *ANACAMPTIS* SPECIES, BY ASYMBIOTIC CULTURE *IN VITRO*

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ABSTRACT

The increasing emphasis on terrestrial orchid conservation has led to conservation actions for a wide range of threatened Mediterranean species. Many terrestrial orchids are currently at great risk for extinction as a result of a multiplicity of threatening processes. We focus on orchid seed germination capabilities *in vitro*, specifically germination capability of a threatened species, *Anacamptis palustris*, compared to three other more common species (*A. laxiflora*, *A. morio*, and *A. papilionacea*), and also discuss its potential impact on orchid distribution and conservation. Asymbiotic germination tests were performed with mature seeds using BM-1 medium. *In vitro* seed germination and protocorm developmental stages were evaluated up to 20 weeks after sowing. Significant differences in seedling development were detected among the species, and a correlation was evident between the rarity of the species and their germinability. Thus, the presence of intrinsic, biological factors that affect and limit the distribution of *A. palustris* may exist.

Keywords: *Anacamptis (Orchis) palustris*, *in vitro* asymbiotic germination, conservation, germination indices, mature seeds, threatened species

Introduction

The increasing emphasis on terrestrial orchid conservation has led to conservation actions for a wide range of threatened Mediterranean orchid species. Terrestrial orchids, which make up one-third of all orchid species, are at great risk for extinction, because of a multiplicity of threatening processes. They are particularly sensitive to habitat fragmentation or changes due to human disturbances and their effects, so much so that many environmental and anthropic factors determine their distribution, decline, and conservation status. In response to threats to orchid species, integrated multidisciplinary conservation approaches, including *in vitro* techniques, are suggested to improve the understanding and modification of processes contributing to orchid rarity, and to facilitate the conservation of some of the most threatened taxa. Several recent reports highlight the need to study both environmental and biological factors linked to habitat changes, such as highly specific mycorrhizal and pollinator relationships, to explain rarity, decline or risk in orchids (Swartz and Dixon 2009; Vereecken et al. 2010). Here, we focus on seed germination capability – another important aspect of orchid biology – and discuss its impact on orchid distribution, abundance and conservation.

Anacamptis palustris (Jacq.) R. M. Bateman, Pridgeon and M. W. Chase (Orchidaceae), the marsh or bog orchid formerly known as *Orchis palustris*, is a Euro-Mediterranean species. It is widely distributed in the western

Mediterranean area, but also has been found (sparsely) in central and northern Europe, as far north as Gotland, and reaching Transcaucasia eastwards (Arduino et al. 1996; Bateman et al. 2003). This species grows exclusively in marsh habitats, typically in retrodunal areas along the coast, with the exception of Central Italy, where a few populations live in highly saline areas, such as sulphuric springs (Cozzolino et al. 2003a,b). Mediterranean coastal marsh habitats have been strongly affected by human activity, and suitable habitats are becoming increasingly reduced in size, fragmented or altered. Thus, the current distribution of *A. palustris* is highly patchy and a severe reduction in habitat extent has been observed, even if some local populations seem quite large. Moreover, this orchid species is genetically depauperate, especially in Italy where genetic variability is very low both within and among populations (Arduino et al. 1996). As a consequence, *A. palustris* is considered to be Endangered in Italy (Conti et al. 1997; Alessandrini and Medagli 2008), and threatened in Austria, Czech Republic, France, Germany, Sweden, Switzerland, and in many other European states (Schnittler et al. 2001).

Since 2006, the Tuscia Germplasm Bank (Botanical Gardens, University of Tuscia, Viterbo, Italy) has been monitoring and studying a small population of *A. palustris* that grows near the Bagnaccio thermal well (Viterbo, Northern Latium, Italy). In the spring of 2008, this population experienced a very strong demographic decline, due to anthropic actions that drastically reduced its habitat, resulting in a dramatic drop in the number of plants

from about 300 to only 10. Since June 2008, *A. palustris* seeds were collected for *ex situ* conservation of the germplasm of this threatened population (Magrini et al. 2011), and for *in vitro* reproduction, because no information exists on mature seed germination and *in vitro* seedling development for this species.

Asymbiotic seed germination of temperate terrestrial orchids is sometimes difficult, and survival rates of deflasked seedlings even more so (Arditti et al. 1982b; Rasmussen 1995; Miyoshi and Mii 1998). For many orchid species, the green capsule technique, or embryo culture, has a number of advantages: the immature seeds do not need to be sterilized, thereby avoiding possible damage, nor treated for dormancy release, and they yield higher germination rates than the mature seeds (Linden 1980; Arditti et al. 1982a,b; Ballard 1987; Mitchell 1989; De Pauw and Remphrey 1993; Rasmussen 1995; Light and MacConaill 1998; Yamazaki and Miyoshi 2006; Sgarbi et al. 2007). The disadvantages of immature seed techniques are the difficulty to harvest the capsules at the right developmental stage, which requires continuous monitoring of the plant phenology, because the maturation may be faster or slower depending on the species, habitat, climate variability, and other environmental factors, and the need for culture immediately after harvest (Steele 1996; Kitsaki et al. 2004; Hicks 2006). In light of these disadvantages, the use of mature seeds is still generally preferred. In fact, even though they require surface sterilization and seed dormancy must be broken, this method enables collection of a homogeneous sample of mature seeds that remain viable for long periods under refrigeration (Steele 1996; Kitsaki et al. 2004; Seaton and Pritchard 2008; Seaton et al. 2010).

Sodium hypochlorite, which is commonly used for the sterilization of orchid seeds, has also been reported to be effective for breaking seed dormancy and to have a stimulatory effect on seed germination in some orchid species (Miyoshi and Mii 1988; Rasmussen 1995). Surface treatment with sodium hypochlorite until the seeds lose their dark colour, has both sterilizing and scarifying effects: it increases the permeability of the testa and the subsequent germination percentage, allowing access of water to the embryo, a very slow process without mycorrhizal symbiosis, if possible at all (Harvais and Hadley 1967; Lindén 1980). The addition of a detergent such as Tween 80 (a sorbitolanydrate) is necessary for effective chemical treatment of the testa in aqueous solutions, because the detergent contains both hydrophilic and lipophilic groups, thus improving contact between water and lipid surfaces such as the seed coat (Rasmussen 1995).

Most orchid media contain similar components, such as sugars, mineral salts, and agar. However, asymbiotic germination of orchid seeds seems to be influenced principally by the nitrogen source and nitrogen availability (Mead and Bulard 1975, 1979; Van Waes and Debergh 1986; van Waes 1987; Malmgren 1992, 1996; Kauth et al. 2006; Stewart and Kane 2006). This may be because

of enzyme synthesis or activation within the developing protocorms. Thus, ensuring that organic nitrogen can be more readily utilized by young protocorms as available amino acids may bypass certain steps of the nitrogen assimilation process, such as the production of nitrate reductase, which requires several months following seed imbibition (Raghavan and Torrey 1964; Malmgren 1992, 1996).

The aims of this study are 1) to reproduce *in vitro* *Anacamptis palustris* and 2) to assess whether variations in germination capability directly correlate with the distribution, abundance, and frequencies of terrestrial orchid species. Comparisons were made between the germination capability of a rare species of terrestrial orchid, *A. palustris*, and three other more common species: the loose flowered orchid, *A. laxiflora* (Lam.) R. M. Bateman, Pridgeon and M. W. Chase, a species quite common in Italy that grows in the same habitat of *A. palustris* but with larger populations; the green-winged orchid, *A. morio* (L.) R. M. Bateman, Pridgeon and M. W. Chase, and the butterfly orchid, *A. papilionacea* (L.) R. M. Bateman, Pridgeon and M. W. Chase, both chosen for their wide distribution in Italy.

Materials and methods

Collection of seeds

Seeds from 7 wild orchid populations of *Anacamptis palustris* (Jacquin) R. M. Bateman, Pridgeon and M. W. Chase (Orchidaceae), *A. laxiflora* (Lam.) R. M. Bateman, Pridgeon and M. W. Chase, *A. morio* (L.) R. M. Bateman, Pridgeon and M. W. Chase, and *A. papilionacea* (L.) R. M. Bateman, Pridgeon and M. W. Chase, growing in Northern Latium (Italy) were utilized in this study (see Table 1). A total of 10–20 plants from each population were marked during anthesis to make them recognizable during the fruiting phase. Mature capsules were collected after ca. 1 month. A total of 186 capsules were harvested from June to July 2010 from 52 stems, with an average of 3.4 capsules/plant. The capsules were placed in paper envelopes, and taken to the Tuscia Germplasm Bank (Botanical Gardens, University of Tuscia, Viterbo, Italy), where identification codes were assigned to each. There, mature seeds were extracted from opened capsules using sieves with a mesh size of 100 µm, put in 4 ml glass vials, and stored at $T = 5 \pm 1$ °C until use.

Germination tests

Germination tests were started in July 2010. Seeds were surface sterilized in a 5% NaOCl solution (Hicks 2006; Sgarbi et al. 2007) supplemented with a drop of Tween 80 (to improve the efficiency of sterilization and scarification), for a few minutes, until bleaching of the seeds was observed. Seeds were then rinsed three times

Table 1 List of the species studied, with an indication of their rarity in Italy (1: very common; 2: common; 3: very rare) according to Rossi (2002) and GIROS (2009), collection location, and information about the marking and the collection phases; in the last column the Tuscia Germplasm Bank (BGT) accession codes are reported.

Species	Rarity index	Collection location (Viterbo, Northern Latium, Italy)	Marking of flowering plant		Collection of mature capsules			BGT code
			Date (2010)	No of plants	Date (2010)	No of plants	No pods /plant	
<i>Anacamptis laxiflora</i>	2	Vetralla, loc. Monte Calvo	May 2	10	July 4	4	2.5	A11710
		Viterbo, loc. Bagnaccio	May 10	20	June 15	10	3.8	A13010
<i>Anacamptis morio</i>	1	Ischia di Castro, loc. Vallerosa	May 18	10	June 15	8	7.6	A10210
		Viterbo, loc. Bagnaccio	May 3	12	June 15	9	3.1	A10310
<i>Anacamptis palustris</i>	3	Viterbo, loc. Bagnaccio	June 15	4	July 14	4	3.0	A13210
<i>Anacamptis papilionacea</i>	1	Monte Casoli Nature Reserve, Bomarzo	May 5	17	June 10	4	4.0	A9710
		Arlena di Castro	April 14	15	June 15	13	2.5	A10510

Table 2 Germination test data: list of the species with BGT and test codes, number of sowed seeds for each test, number and percentages of empty seeds.

Species	BGT code	Test code	No sowed seeds	No empty seeds	% empty seeds
<i>Anacamptis laxiflora</i>	A 117 10	A LAX 1	273	64	23%
	A 130 10	A LAX 2	866	276	32%
<i>Anacamptis morio</i>	A 103 10	A MOR 1	403	176	44%
	A 102 10	A MOR 2	300	29	10%
<i>Anacamptis palustris</i>	A 132 10	A PAL	237	71	30%
<i>Anacamptis papilionacea</i>	A 105 10	A PAP 1	385	91	24%
	A 97 10	A PAP 3	297	52	18%

with sterile distilled water followed by centrifugation at 6000 rpm for 3 min. between rinses.

BM-1 Terrestrial Orchid Medium (*PhytoTechnology Laboratories*® Shawnee Mission, KS, USA) was used as a culture medium: it is a Basic Medium modified according to Van Waes and Debergh (1986) that contains only organic nitrogen, 0.5 g/l casein enzymatic hydrolysate and 0.1 g/l L-glutamine. The pH of the medium was adjusted to 6.3 ± 0.1 before autoclaving at 121°C and 101 kPa for 20 min. The medium was supplemented with 0.6% agar (Plant Tissue Culture Grade, *AppliChem*, Darmstadt, Germany) (Sgarbi et al. 2007) and 0.1% activated charcoal (Plant Tissue Culture Tested, *PhytoTechnology Laboratories*® Shawnee Mission, Kansas, USA) (van Waes 1987). Approximately 100–300 seeds (Table 2) were sown under axenic conditions in each Petri dish (6 cm in diam.), containing 15 ml of BM-1 medium. Three replicate dishes were prepared for each accession of seeds. Germination dishes were sealed with Parafilm® (Pechiney Plastic Packaging, Chicago, IL, USA), wrapped in aluminium foil to exclude light, and incubated at $T = 24 \pm 1^\circ\text{C}$ in darkness (Scrugli and Cogoni 1994; Lauzer et al. 2007).

The number of empty seeds was counted in each dish (Table 2). This number was subtracted from the total number of seeds/dish to calculate the germination indices. Each dish was then examined biweekly for a period of 20 weeks, and all seeds and seedlings were counted using a 1×1 cm grid under a stereoscopic microscope

(Nikon SMZ 1000). The process of seed germination was recorded according to embryonic developmental stage, modified from Miyoshi and Mii (1995) and Yamazaki and Miyoshi (2006): Stage 0, 'No germination' stage. No growth of the embryo occurs; Stage 1, 'Pre-germination' stage. The embryo swells to fill the seed coat; Stage 2, 'Germination' stage. The embryo emerges from the seed coat; Stage 3, 'Protocorm' stage. The embryo is completely discharged from the seed coat; Stage 4, 'Rhizoid' stage. Rhizoids are formed on the protocorm surface; Stage 5, 'Shoot' stage. The shoot is differentiated from the protocorm.

Seedlings with ca. 0.5–0.8 cm long leaves were transferred under axenic conditions to flasks containing fresh medium for continued growth, and incubated at $T = 24 \pm 1^\circ\text{C}$, with a 16/8h photoperiod, under cool-white fluorescent illumination (Osram Dulux L 36W/840 Lumilux, 2900 lm).

Germination indices

The germination capability of tested seeds from the different species was calculated and analyzed using 9 germination indices: germinability or final germination percentage (G), recorded as the total percentage of Stages 2–5 after 20 weeks; elapsed time until the first germinant is observed (T_1), recorded as the number of days required to reach Stage 2; time required to reach 50% germination

(T_{50}), where $T_{50} = t_i + [(N/2 - n_i) (t_j - t_i)] / (n_j - n_i)$, N is the final number of germination, and n_i, n_j are the cumulative number of seeds germinated from adjacent counts when $n_i < N/2 < n_j$; time to reach maximum germination (T_{max}); mean germination time (MT); coefficient of variation of the germination time (CV_t); mean germina-

tion rate or germinants per day (MR); uncertainty of the germination process (U), a measure of the uncertainty associated with the distribution of the relative frequency of germination, $0 \leq U \leq \log_2 N$, where N is the total number of germinated seeds; synchrony of the germination process (Z), an index of the degree of germination over-

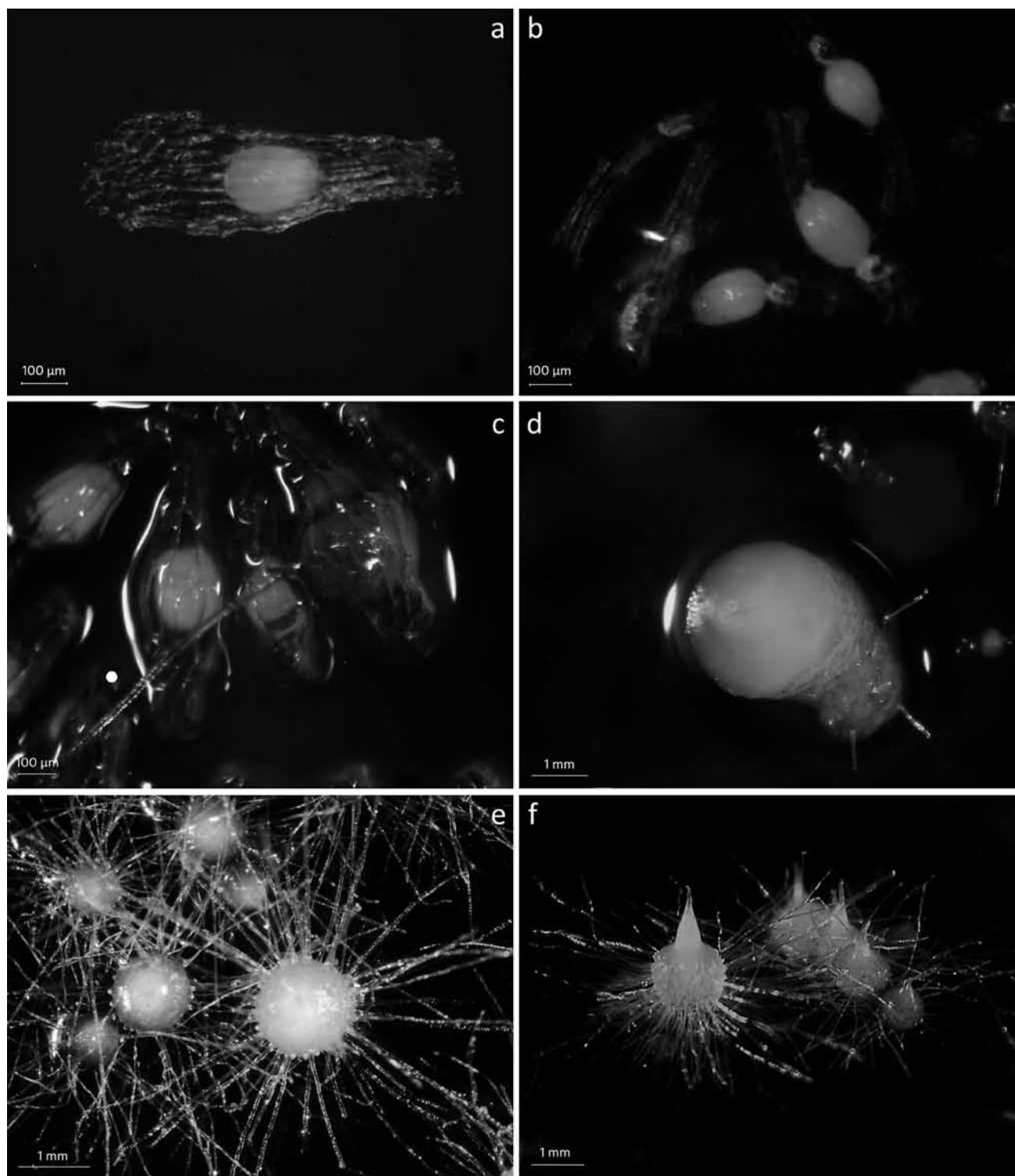


Fig. 1 Successive developmental stages of mature seeds of *Anacamptis palustris*, from germination to seedling formation, by asymbiotic culture *in vitro*. (a) Stage 0: 'No germination stage', no growth of embryo occurs. (b) Stage 1: 'Pre-germination stage', embryo swells in the width of the seed coat. (c) Stage 2: 'Germination stage', embryo emerges from the seed coat. (d) Stage 3: 'Protocorm stage', embryo completely emerges from the seed coat. (e) Stage 4: 'Rhizoid stage', rhizoids are formed on the surface of the protocorm. (f) Stage 5: 'Shoot' stage. The shoot is differentiated from the protocorm.

lapping ($0 \leq Z \leq 1$). The last 5 indices were calculated as described by Ranal et al. (2006).

Germination data were analyzed by one-way ANOVA using GraphPad Prism 5.0, followed by Tukey's Multiple Comparison Test to test for significant differences among orchid species. Pearson's Correlation Test was performed to test for correlation between the rarity index and all other germination indices at 95% confidence interval (95% CI).

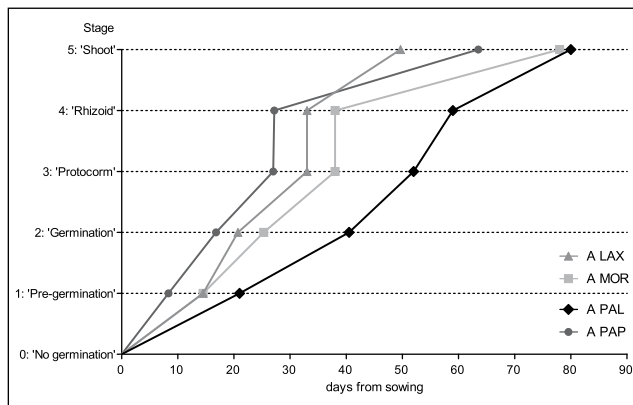


Fig. 2 Comparison of *in vitro* asymbiotic germination trends in *Anacamptis* species. The graph shows the average elapsed time before first observation of each developmental stage for the 4 species: A LAX: *A. laxiflora*; A MOR: *A. morio*; A PAL: *A. palustris*; A PAP: *A. papilionacea*.

Results

Mature seeds of *Anacamptis palustris* germinated and grew to all five developmental stages on the asymbiotic medium (Fig. 1), but two months were required before Stage 4 seedlings were observed. In contrast, seeds from the other three more common *Anacamptis* species attained Stages 3–4 after only one month *in vitro* (Figs. 2–3), and shoots (Stage 5) were visible after two months. *Anacamptis papilionacea* displayed higher total germination values for all observation periods (Fig. 3). Total germination percentages for *A. papilionacea* were: $48.2 \pm 16.0\%$, $75.5 \pm 13.7\%$, $83.2 \pm 6.1\%$, and $84.3 \pm 4.9\%$, at 1, 2, 3, and 4 months, respectively. *Anacamptis laxiflora*, on the other hand, was found to display higher percentages of protocorms with shoots (Stage 5), even after only 1 month (Stage 5 percentages were $0.3 \pm 0.5\%$, $13.2 \pm 0.3\%$, $35.9 \pm 17.1\%$, and $53.6 \pm 26.1\%$ after 1, 2, 3, and 4 months, respectively). In contrast to these species, *A. palustris* was observed to display the lowest total germination percentages ($13.1 \pm 7.9\%$, $29.1 \pm 6.1\%$, $31.8 \pm 7.3\%$, and $52.0 \pm 12.7\%$ after 1, 2, 3, and 4 months, respectively). In fact, the germination trend for *A. palustris* (Fig. 2 and 3) is significantly different from *A. papilionacea* (Tukey's Test; $P < 0.01$) and *A. morio* (Tukey's Test; $P < 0.05$).

After 4 months, the seedlings with the longest (ca. 0.5–0.8 cm) hyaline leaves were transferred to flasks containing fresh BM-1 medium (160 of *A. laxiflora* and 60

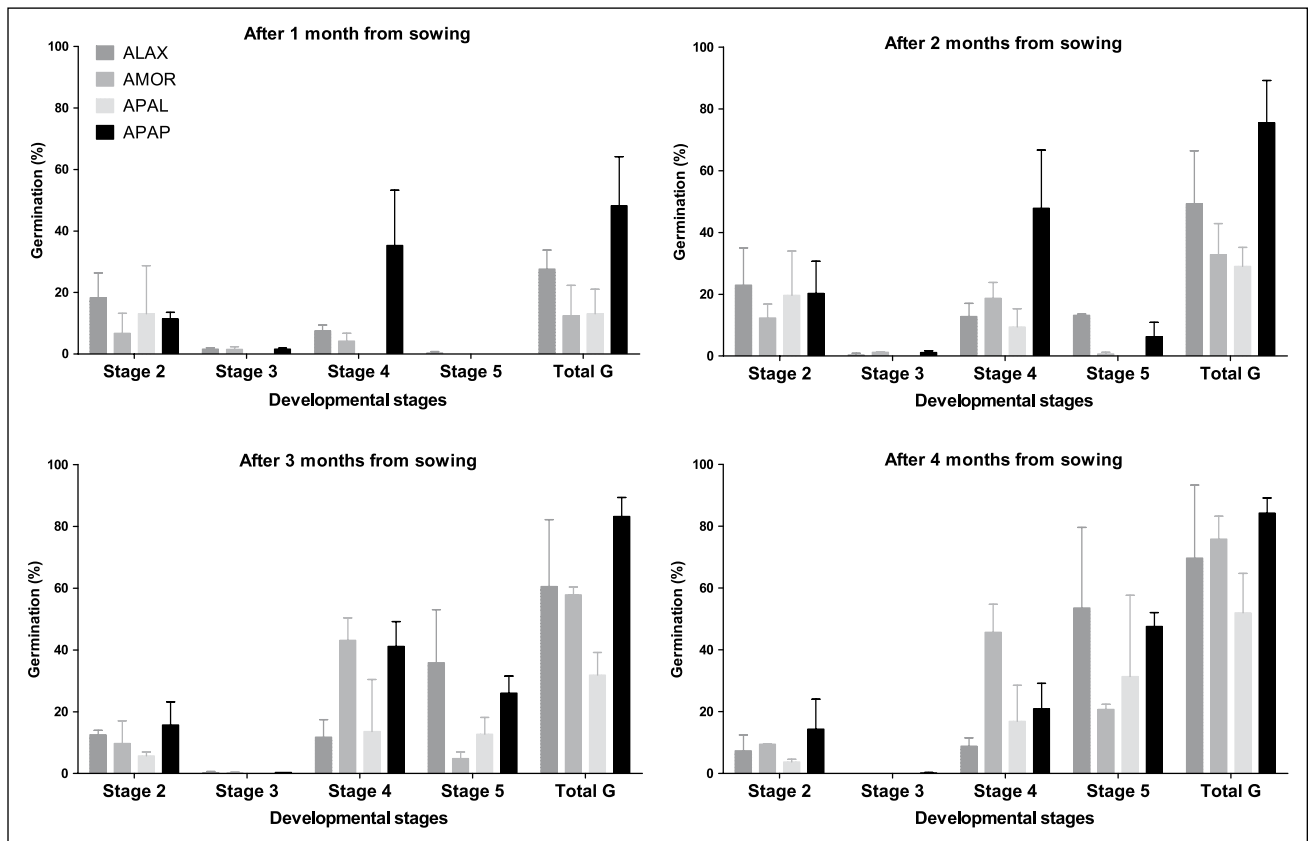


Fig. 3 Germination percentages (mean \pm SD) of the 4 *Anacamptis* species for each developmental stages, recorded 1, 2, 3, and 4 months after sowing (A LAX: *A. laxiflora*; A MOR: *A. morio*; A PAL: *A. palustris*; A PAP: *A. papilionacea*).

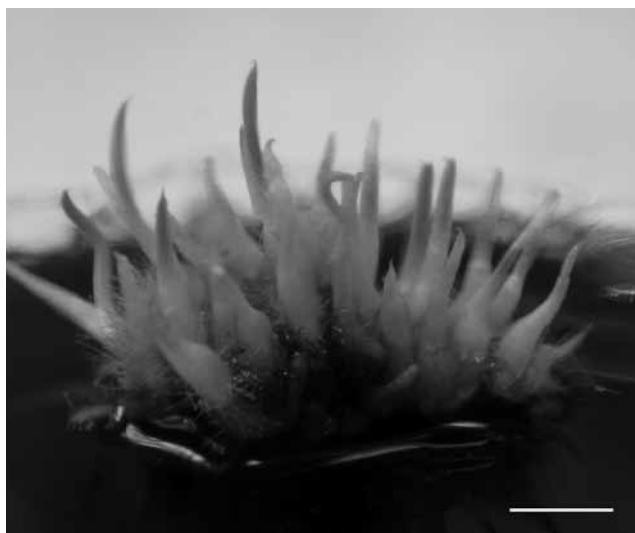


Fig. 4 *Anacamptis papilionacea* seedlings with green leaves and roots, transferred to flasks containing fresh BM-1 medium (scale bar = 1 cm).

of *A. papilionacea*). After 1 week of culture, the leaf color became green, and all of the seedlings showed continued growth and leaf elongation (Fig. 4).

Most of germination indices (Table 3) were statistically significantly different between these orchid species (T_1 : $F_{3,20} = 28.03$, $P < 0.0001$; T_{50} : $F_{3,20} = 20.28$, $P < 0.0001$; T_{max} : $F_{3,20} = 3.97$, $P < 0.05$; MT : $F_{3,20} = 33.90$, $P < 0.0001$; CV_t : $F_{3,20} = 3.72$, $P < 0.05$; MR : $F_{3,20} = 39.10$, $P < 0.0001$). First seed germination (T_1) was scored for *Anacamptis papilionacea* (16.8 days after sowing) (Fig. 2, Table 3), that displayed also the shortest time to 50% germination (T_{50}) and the shortest maximum germination (T_{max}). In contrast, *A. palustris* displayed the longest T_1 and T_{max} times, with extremely significant differences for T_1 (40.5 days,

Tukey's Test; $P < 0.001$) and significant differences for T_{max} (134.0 days, Tukey's Test; $P < 0.05$). The maximum T_{50} was observed for *A. morio* (69.5 days), which was significantly different from *A. papilionacea* and *A. laxiflora* (Tukey's Test; $P < 0.001$), as well as from *A. palustris* (Tukey's Test; $P < 0.01$).

At T_{max} , maximum germinability (G) was recorded for both *A. papilionacea* (84.6%, in the shortest time, 113.8 days), and *A. morio* (75.9%). These germinability values are significantly greater (Tukey's Test; $P < 0.05$) than the values recorded for *A. palustris* seeds, which show the minimum germinability (55.7%) with the longest germination time (134.0 days).

The lowest mean germination time (MT) was recorded for *A. papilionacea* (47.4 days; Tukey's Test; $P < 0.001$). In contrast, longer mean germination times were recorded for *A. morio* (81.6 days) and *A. palustris* (79.4 days), which in addition showed the lowest coefficient of variation of germination time (CV_t), and the same mean germination rates (MR, 0.01 germinants/day).

The low Z values recorded for all of the species (0.2–0.3, much less than 1) indicate a very low degree of synchrony between the seeds during germination. This lack of synchrony was further confirmed by the low U values observed, which suggest a low degree of germination spreading over time.

The results of the Pearson's Correlation Test (95% confidence interval, significant correlation for $\alpha = 0.05$) performed between the rarity index of the species (Table 1) and the germination indices (Table 3) highlight a significant correlation between the rarity of the species and their germinability (G) (Pearson's R: -0.9537 ; p -value (two-tailed): 0.0231^* ; $R^2 = 0.9096$). No statistically significant correlation was observed with the other germination indices.

Table 3 Germination indices (mean \pm SD) of seeds from the 4 *Anacamptis* species studied.

Species	G (%)	T_1 (days)	T_{50} (days)	T_{max} (days)	MT (days)	CV_t (%)	MR (germinants/day)	U (bit)	Z
A LAX	69.7 \pm 3.9a,b	20.7 \pm 3.6a,b	46.9 \pm 2.9a,b	129.3 \pm 7.2a,b	62.9 \pm 1.0a	49.0 \pm 2.8a	0.02 \pm 0.00a	2.5 \pm 0.4a	0.2 \pm 0.0a
A MOR	75.9 \pm 6.4a	25.3 \pm 8.8a	69.5 \pm 13.1c	134.0 \pm 7.7a	81.6 \pm 1.6b	37.4 \pm 6.4b	0.01 \pm 0.00b	1.9 \pm 0.4a	0.2 \pm 0.1a
A PAL	55.7 \pm 15.6b	40.5 \pm 0.7c	51.0 \pm 0.2b	134.0 \pm 0.1a	79.4 \pm 0.5b	47.6 \pm 0.5a,b	0.01 \pm 0.00b	1.9 \pm 0.5a	0.2 \pm 0.0a
A PAP	84.6 \pm 4.3a	16.8 \pm 1.2b	38.1 \pm 5.1a	113.8 \pm 20.9b	47.4 \pm 0.8c	40.5 \pm 2.0a,b	0.02 \pm 0.00c	1.9 \pm 0.1a	0.3 \pm 0.1a
P value	<0.05	<0.0001	<0.0001	<0.05	<0.0001	<0.05	<0.0001	ns (0.1259)	ns (0.0908)
$F_{3,20}$	5.770217	28.03085	20.27945	3.969223	33.90178	3.722474	39.10458	2.149257	2.478038
R^2	0.4639600	0.8078633	0.7525927	0.3731914	0.8356687	0.3583044	0.8543481	0.2437926	0.2709807
B (P)	5.641232 (0.1304)	30.10315 ($P < 0.0001$)	37.42419 ($P < 0.0001$)	34.02461 ($P < 0.0001$)	29.19681 ($P < 0.0001$)	25.78486 ($P < 0.0001$)	31.31544 ($P < 0.0001$)	3.420312 (0.3312)	14.28993 (0.0025<0.05)

A LAX: *A. laxiflora*; A MOR: *A. morio*; A PAL: *A. palustris*; A PAP: *A. papilionacea*; G: percentage of germinating seeds; T_1 : First germination time; T_{50} : Time to reach 50% germination; T_{max} : Time to reach maximum germination; MT: mean germination time; CV_t : coefficient of variation of the germination time; MR: mean germination rate; U: uncertainty of the germination process; Z: synchrony of germination process. For each parameter, the values followed by the same letters are not significantly different at the 5% level of probability, as determined by Tukey's test. (P: probability; F: F-test distribution value, boldfaced values indicate statistically significant difference among matrixes (ANOVA; $P < 0.0001$); R^2 : is the fraction of the overall variance attributable to differences among the group means, boldfaced values indicate great variance among the species; B: Bartlett's test for equal variances; boldfaced values indicate significant difference among the variances (ANOVA; $P < 0.0001$).

Discussion

This is the first report of successful asymbiotic germination of mature seeds of *Anacamptis palustris*, a threatened species of the European flora. *In vitro* techniques have found increasing use in the conservation of threatened orchids (Fay 1992; Vaasa and Rosenberg 2004; Znaniecka and Lojkowska 2004; Viswambharan et al. 2006). In fact, there are many recent reports of the successful *in vitro* asymbiotic reproduction of terrestrial orchids, which also used mature seeds from the *Anacamptis* species. *Anacamptis laxiflora* (Mead and Bulard 1975, 1979; Scrugli and Cogoni 1994), *A. morio* (van Waes and Debergh 1986; McKendrick 1996; Znaniecka and Lojkowska 2004), and *A. papilionacea* (Pedroso and Pais 1992) were cultured on different media. However, none of these previous reports include precise data on the germination or subsequent growth of protocorms, or the application of germination indices to analyze and compare the germination process of the species.

The choice of BM-1 as culture medium (van Waes and Debergh 1986) yielded good results, supporting rapid asymbiotic germination and advanced protocorm development in *Anacamptis* species. In all of the cultures, we could observe all germination stages, including the development of seedling with shoots, albeit with significant differences in times and percentages between the species. All of the orchids showed first germination within 16–40 days of sowing, and after 38–80 days they all had developed protocorms with rhizoids and shoots, for total germination percentages of 56% and 85%. The asymbiotic germination of mature *A. palustris* seeds is an important result, even if this species displayed the longest germination times and lowest germination percentages, especially when compared to results from previous studies (Magrini et al. 2011) that provide no germination data for mature seed cultures on MS medium (Murashige and Skoog 1962) after 10 months, dramatically longer first germination times (6 months), and lower total germination percentages (6% after 6 months, and 35% after 10 months), using immature seeds.

Germination test results show significant differences between the germination trend found in the four *Anacamptis* species and most germination indices, especially germinability and first germination times, supporting the hypothesis that the presence of intrinsic, biological factors affect and limit the distribution of *A. palustris*. This correlation was confirmed by Pearson's Correlation Test results, which highlight a very significant correlation between the rarity of the analysed species and germinability. Short germination times and high germination percentages provide these orchids with many advantages to improve their dispersal efficiency and colonization ability, which in turn make them more adaptable to changing environmental conditions.

Implications for conservation

We highlight the need to consider the physical and biological factors which determine orchid rarity and conservation, as well as human disturbances and their effects. As highlighted by other authors (Swartz and Dixon 2009; Vereecken et al. 2010), extrinsic rarity in orchids is a reflection of both anthropogenic processes directly limiting or reducing the distribution and abundance of a species, and their biological ability to adapt themselves to environmental changes. In this study, we pointed out that germination capability can be one of the many causes of terrestrial orchid rarity. Although *A. palustris* and *A. laxiflora* grow in the same habitat, often together, they show different behaviours during the germination process that reflect the differences in their distribution and abundance.

Furthermore, we emphasize the importance of *in vitro* conservation efforts for threatened orchids. In fact, although the use of *in vitro* techniques in germplasm conservation is increasing, there is still great potential for further development of these methods, which could allow investigators to obtain plant material for DNA analyses, for the definition of reproduction protocols, and for auto-ecological or biological studies.

Future research should focus on improving the efficiency of *A. palustris* seed germination, especially testing the use of mycorrhizal fungi, on the acclimatization, and on the establishment of *in situ* methodologies, to further progress in the development of conservation techniques for rare and threatened orchids.

Acknowledgements

The authors thank S. Buono (GIROS – Italian Group for Research on Wild Orchids), for the harvesting of mature seeds of *Anacamptis laxiflora*.

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