IN VITRO POLLEN GERMINATION OF ORCHIDS TRADITIONALLY USED TO PRODUCE SALEP

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ABSTRACT

In Turkey the tubers of about 120 orchid species are widely collected for manufacturing the traditional drink salep. In this study, we focused on the *in vitro* germination of the pollen of the salep orchid species *Ophrys mammosa*, *Orchis provincialis*, *Anacamptis morio* subsp. *morio*, *Orchis simia* and *Neotinea tridentata* and discussed the potential effects this might have on the conservation of these orchids by reducing the need to collect them in the field. Pollen was sown on different media; Knudson, Orchimax and the medium described by Malmgren, and then incubated at 24 ± 1 °C in darkness for 24 h. Germinated pollen was stained with Brilliant Blue and examined under a stereoscopic microscope. Results of Tukey and Dunnett T3 statistical tests indicated that in terms of percentage germination, the best germination was observed on *O. mammosa* by 55% and Orchimax was the most successful medium by 50.5%. For pollinaria germination, the best rate was observed on *O. mammosa* by 69%. The medium Malmgren was the best germinative by 61.3%. It is clearly seen that difference in germination rates among studied species are achieved using different media. The development of such a method of studied species in this research points to the fact that this is possible and should serve as encouragement for others to devise procedures for other species. These kinds of researches on propagation of orchids would be useful to reintroducing some of the rarer, endangered and endemic species in Turkey such previously succeed for *Orchis militaris* and *Liparis loeselii* in Great Britain.

Keywords: pollinium, pollinarium, orchid, *in vitro* germination, salep, conservation

Introduction

Orchids are cosmopolitan and occur in almost every habitat except in the Polar Regions (Edwards 2007). With more than 1000 genera and at least 25,000 species, the family Orchidaceae is the largest and the most diverse family of flowering plants (Harrap and Harrap 2009). While nearly 70% of orchids live on other plants as epiphytes, they can also grow in soil (25%), and live on rocks and decaying plants (5%) (Arditti 1979; Renz and Taubenheim 1984). In Turkey there are 170 terrestrial orchids belonging to 24 genera (Kreutz 2009).

In addition to their aesthetical and medicinal importance, orchids are ecological indicators (Joshi et al. 2009). Moreover, orchids are used to manufacture a nutritious drink, called salep, which has been very popular for centuries in Anatolian and Arabian cultures and used as an additive in the production of "Maras" ice cream in Turkey (Sezik 1984; Baytop 1999; Kreutz 2009).

Table 1 Tuber weights and tuber counts of orchids per 1000 kg (Sezik 1984).

Commercial name	Average tuber weight (g)	Number of tubers per kg
Muğla Salebi	0.23	4348
Kastamonu Salebi	0.50	2000
Silifke Salebi	0.35	2857
Antalya Salebi	0.21	4762
Maraş Salebi	1.60	625
Van Salebi	1.00	1000

Around 120 orchid species, including the genera; *Aceras, Anacamptis, Balia, Dactylorhiza, Himantoglossum*, *Neotinea*, *Ophrys*, *Orchis* and *Serapias*, are used to make salep in different places in Anatolia. Therefore, orchids belonging to these genera are widely and intensively collected from nature. In this study, we focused on the orchids *Ophrys mammosa* (Desf.), *Orchis provincialis* (Balb.), *Anacamptis morio* (L.) R. M. Bateman, Pridgeon and M. W. Chase subsp. *morio*, *Orchis simia* (Lam.) and *Neotinea tridentata* (Scop.) R. M. Bateman, Pridgeon and M. W. Chase, which have been collected since ancient times in Anatolia and used to make salep (Özhatay et al. 1997; Baytop 1999).

Although orchids are protected worldwide from overexploitation by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (UNEP-WCMC 2013), they are still threatened by the illegal trade in orchids and the collecting of them from nature to produce salep and "Maras" ice cream (Sezik et al. 2007; Kreutz 2009). It is estimated that 20–30 million orchids weighing 20 tons are collected from nature annually for producing salep (See Table 1).

Because of the threat from logging, mining, urbanisation as a consequence of the increase in the human population, agricultural activities and the collecting of orchids from nature for the manufacture salep it is important to develop new and advanced techniques for their sustainable usage (Kreutz 2009; Cribb 2011). This has resulted in improvements in the *in vitro* propagation of orchids in order to reduce the need to collect orchids from nature and make it possible to conserve the most threatened species (Magrini et al. 2011). Thus, in this

Table 2 List of the species studied, locations, dates of collection and numbers of pollinaria collected.

Species	Collection location (Çanakkale, Turkey)	Collection of pollinaria	
		Date (2014)	Number of pollinaria
Anacamptis morio subsp. *morio*	Kilitbahir	April 15	210
Neotinea tridentata	Onsekiz Mart University Terzioğlu Campus	April 20	211
Ophrys mammosa	Kilitbahir	March 31	201
Orchis provincialis	Denizgöründü village	April 21	328
Orchis simia	Denizgöründü village	April 21	199

study, we developed a new method for the *in vitro* propagation using the pollen of orchids used in the manufacture of salep.

Although there are studies on the evolution (Bradshaw and Schemske 2003), ecology (Desrochers and Rieseberg 1998), pollen ovule aging (Proctor 1998; Bellusci and Musacchio 2010), morphology and ultra-structure of orchids (Feijo and Pais 1989; Pacini and Michael 2002) the germination pollen has not been previously studied (Pritchard and Prendergast 1989; Aybeke 2002; Pacini and Michael 2002).

While many orchid genera have pollinia, which is an adhesive mass of pollen, there are a few genera that produce single pollen grains. Therefore, orchids differ greatly in terms of whether they produce a mass of sticky pollen or individual grains of pollen (Aybeke 2002). While the pollen of *Pterostylis plumosa*, *Pterostylis concinna*, *Neottioids*, *Neuwiedia*, *Cypripedium acaule*, *Cypripedium calceolus*, *Apostasia wallachii* consists of individual grains, that of *Epipactis microphylla*, *Bletilla striata*, *Neottia*, *Cleistes divaricata*, *Neottia nidus-avis, Epidendrum scutella*, *Epidendrum ibaguense*, *Loroglossum hircinum*, *Pleurothallis eumecocaulon* and *Calypso bulbosa* consists of groups of four pollen grains (Pacini and Michael 2002). Using light-microscopy we observed that *O. mammosa*, *O. provincialis*, *A. morio* subsp. *morio*, *O. simia* and *N. tridentata* produce tetrad groups of pollens contained in pollinia.

The aims of this study are to: (1) determine whether it is possible to germinate the pollen of *O. mammosa*, *O. provincialis*, *A. morio* subsp. *morio*, *O. simia* and *N. tridentata*; (2) whether there differences in percentage germination between species and (3) the effects of KN, ORC and SV media on percentage germination.

Material and Methods

Collection of Study Materials

Pollinia were collected from 5 species of orchids: *Ophrys mammosa* (Desf.), *Orchis provincialis* (Balb.), *Anacamptis morio* (L.) R. M. Bateman, Pridgeon and M. W. Chase subsp. *morio*, *Orchis simia* (Lam.) and *Neotinea tridentata* (Scop.) R. M. Bateman, Pridgeon and M. W. Chase, growing wild in the centre of Çanakkale city and its surroundings (Turkey) (See Table 2 and Fig. 1).

Plants were identified using "Flora of Turkey and the East Aegean Island" vol. 8 (Renz and Taubenheim 1984) and vol. 11 (Kreutz 2000), "Orkidelerimiz" (Sezik 1984) and "Türkiye Orkideleri" (Kreutz 2009).

In vitro Germination of Pollen

Germination tests were started in March and April 2014. The surfaces of pollinia were sterilized by placing them in a 0.5% solution of NaOC1 for 5 minutes after which they were rinsed three times in sterile distilled water and between rinses they were broken into pieces in a vortex mixer.

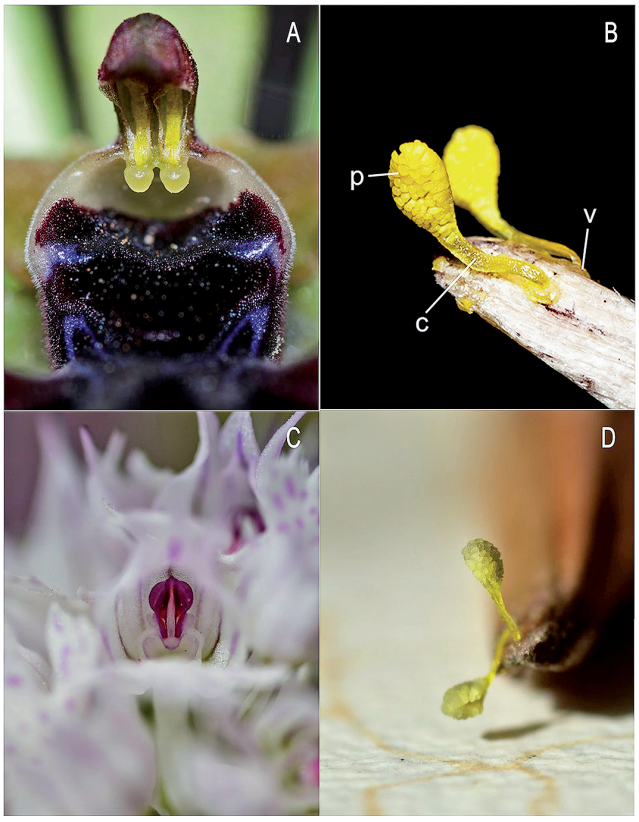

Fig. 1 Detailed images of flowers and pollinaria of *N. tridentata* and *O. mammosa*. Reproductive parts of *Ophrys mammosa* (A) and *Neotinea tridentata* (C). Pollinarium of *Ophrys mammosa* (B) and *Neotinea tridentata* (D); p: pollinium, c: caudicle, v: viscidium.

Table 3 Species studied, their codes used in subsequent tables and numbers of sown pollinia and pollinaria on individual media.

Species	Code	Number of sown pollinia			Number of sown pollinaria		
		KN	ORC	SV	KN	ORC	SV
Anacamptis morio subsp. *morio*	A MOR	71	107	111	79	76	55
Neotinea tridentata	N TRI	82	65	72	76	76	59
Ophrys mammosa	O MAM	165	237	738	67	62	71
Orchis provincialis	O PRO	119	84	121	113	123	92
Orchis simia	O SIM	72	75	81	50	67	82

Two different commercial media (Orchimax Orchid Medium "ORC", O0257 and Knudson C Orchid Medium "KN", K0215, Duchefa Biochemie BV, Haarlem, the Netherlands) and one special medium (SV) (Malmgren 2006) were used. KN and ORC media were supplemented with 20 g/l sucrose, 1 g/l activated charcoal and 6 g/l agar. SV was prepared with; 90 mg/l $Ca_3(PO_4)_2$, 90 mg/l KH_2PO_4, 90 mg/l $MgSO_4$, 20 g/l sucrose, 1 g/l activated charcoal, 6 g/l agar and 30 ml/l pineapple juice. The pH of the medium was adjusted to 5.75 ± 0.1 before autoclaving at 121 °C and 101 kPa for 20 min. Approximately 50–200 pollinia (Table 3) were sown under axenic conditions in each Petri dish (10 cm in diam.), containing 25 ml of either KN, ORC or SV medium. There were five replicates for each accession of pollinia. The Petri dishes were sealed with stretch film, wrapped in aluminium foil to exclude light and incubated at 24 ± 1 °C in darkness for 24 h.

The pollen was stained with Brilliant Blue R (Sigma-Aldrich B7920) and the number that had germinated counted under a stereoscopic microscope (Olympus SZ-51).

Statistical Analyses

Results were analyzed using one-way ANOVA (IBM SPSS Statistics ver. 22), followed by Tukey's HSD and Dunnet T3 tests to determine whether the results differed significantly for the different orchids and media.

Results

We observed that germination occurred solely between connecting surfaces of pollinia to pollinaria and pollen tubes were elongated such fringes from pollen grains while there was no germination observed on outer surfaces of pollinia (Fig. 2). We also investigated Species + Media interaction effects on germination rate and performed statistical analyses. Statistical analyses showed that Species + Media interaction was significant at $p < 0.05$.

The best germination rates of pollinia for A MOR and N TRI were on the medium KN, for O MAM and O PRO on the medium ORC and for O SIM on the medium SV (Table 4). The germination rates, however, did not statistically differ between A MOR and O PRO, but there were significant differences between N TRI, O MAM and O SIM (Table 4). Overall germination rates of individual media for pollinia are shown in Table 5. There were no significant differences in germination rates of pollinia among media (Table 5). Overall pollinia germination rates for individual species are shown in Table 6. The differences in germination rates between species were not statistically significant (Table 6).

We observed germination within the inner sides of pollinaria where pollinia were connected. Pollen tubes increasingly elongated from inside to outside (Fig. 3). Statistical analyses confirmed significant differences of species-media composition and species-media interaction on germination rates ($p < 0.05$).

The best germination rates of pollinaria for O PRO and O SIM were on the medium KN, for N TRI and O MAM on the medium SV, for A MOR on the medium ORC (Table 7). There were statistically significant differences between individual media for O MAM, A MOR and N TRI, but there was none between O PRO and O SIM (Table 7). Overall germination success rates of pollinaria for individual media are shown in Table 8; there was no significant difference between the media. The overall germination rates of pollinaria for individual species are shown in Table 9. There were no statistically significant differences between these.

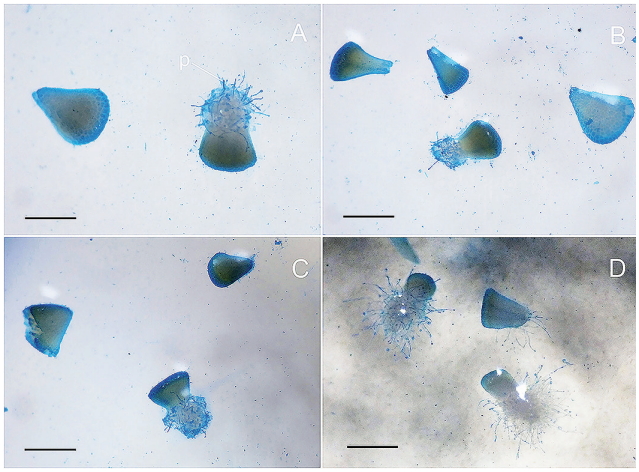

Fig. 2 Pollen tube (p) development on pollinia of *Ophrys mammosa* (A), *Orchis simia* (B), *Neotinea tridentata* (C) and *Orchis provincialis* (D). Scale bars: 1 mm.

Table 4 Germination rates of pollinia (mean ± SD) of individual species on each medium.

	KN	ORC	SV	p value	$F_{2,12}$
A MOR	39.6 ± 5.5	32.7 ± 5.3	36.9 ± 11.6	0.413	0.952
N TRI	51.2 ± 4.3	40.9 ± 6.3	44.4 ± 6.8	0.049	3.903
O MAM	54.4 ± 3.6	65.6 ± 8.7	52.3 ± 3.0	0.007	7.760
O PRO	42.0 ± 9.8	46.6 ± 12.1	37.1 ± 15.6	0.508	0.716
O SIM	28.2 ± 9.3	46.8 ± 7.2	50.6 ± 8.4	0.002	10.367

Table 5 Overall germination rates of pollinia (mean ± SD) on each medium.

	mean ± SD	p value	$F_{2,12}$
KN	43.4 ± 10.9	0.885	0.124
ORC	46.6 ± 11.0		
SV	44.4 ± 9.5		

Table 6 Overall pollinia germination rates (mean ± SD) for individual species.

	mean ± SD	p value	$F_{4,10}$
A MOR	36.1 ± 3.4	0.060	3.233
N TRI	46.2 ± 4.9		
O MAM	56.7 ± 5.5		
O PRO	42.0 ± 6.1		
O SIM	42.5 ± 4.9		

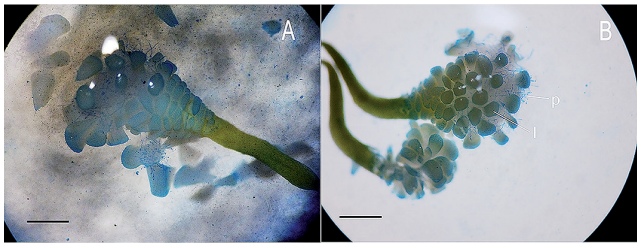

Fig. 3 Pollen tube (p) development and pollinium (l) view on pollinarium of *Ophrys mammosa* (A) and *Anacamptis morio* subsp. *morio* (B). Scale bars: 1 mm.

Table 7 Germination rates of pollinaria (mean ± SD) for each medium and species.

	KN	ORC	SV	p value	$F_{2,12}$
A MOR	38.4 ± 8.2	66.4 ± 12.9	59.9 ± 17.6	0.016	5.973
N TRI	52.1 ± 6.0	54.6 ± 8.2	69.2 ± 8.6	0.009	7.135
O MAM	51.6 ± 19.8	49.9 ± 6.9	81.7 ± 14.7	0.015	6.041
O PRO	57.5 ± 13.7	52.5 ± 15.9	48.4 ± 4.7	0.524	0.683
O SIM	61.9 ± 11.2	56.7 ± 13.1	51.2 ± 5.9	0.309	1.297

Table 8 Overall pollinaria germination rates (mean ± SD) on each medium.

	mean ± SD	p value	$F_{2,12}$
KN	57.2 ± 13.0	0.661	0.428
ORC	56.8 ± 6.1		
SV	62.7 ± 13.3		

Table 9 Overall pollinaria germination rates (mean ± SD) for individual species.

	mean ± SD	p value	$F_{4,10}$
A MOR	59.3 ± 10.4	0.588	0.737
N TRI	46.2 ± 4.9		
O MAM	68.3 ± 14.9		
O PRO	54.5 ± 5.6		
O SIM	56.6 ± 5.4		

Discussion

Although *in vitro* symbiotic and asymbiotic seed germination has become a favoured and useful technique for orchid propagation and use in plant reintroduction to nature, *in vitro* pollinium or pollinarium germination has not been studied widely in terrestrial orchids. This is especially important for species used for commercial salep production. Therefore, here we provide the first successful *in vitro* pollinia and pollinaria germination and comparison report of salep orchids *Ophrys mammosa*, *Orchis provincialis*, *Orchis simia* and *Neotinea tridentata*.

All plants, which have pollinium instead of pollen grains, need water for hydration of complex pollen clusters (Marginson et al. 1985). During pollinium development of *Epidendrum ibaguense*, plasmodesmata and thin cytoplasmic channel formations enclose tetrads, and consequently proximal sides of each tetrad have thinner walls (Yeung 1987). This structural differentiation helps water to be absorbed easier, which explains germination success of inner parts of pollinaria. As observed recently (Yeung 1987; Feijo and Pais 1989; Aybeke 2002), for both pollinia and pollinaria, only inner surfaces where pollinia were attached to pollinaria, germinated and pollen tubes were developed. It can be explained by hydration advantages of pollen tetrads at broken parts of pollinium during the breaking processes of pollinaria, and by thinner walls of proximal sides of tetrads. Therefore, before sowing process, breaking pollinaria into pollinia and even smaller pieces can increase the success of germination significantly. These results are parallel with other *in vivo* results regarding germination differences between inner and outer walls of tetrads (Swamy 1947; Poddubanya-Arnoldi 1976). Our comparison of pollinia and pollinaria germination supports those results.

It is known that sugar level of germination medium is very important for germination as well. While Pfundt

(1909) recommends sucrose level by 5–20% (w/v), another researcher found orchid pollen germinates best on the medium with a range of 3–10% (w/v) sucrose level (Miwa 1937). Relatively recently, 1–10% (w/v) sugar level was suggested (Pritchard and Prendergast 1989). Considering this and according to our pilot experiments we decided 2% (w/v) sucrose level as the optimum for each germinating medium in our study.

We show that Svante medium can be used as a basic germinating medium for orchids *Ophrys mammosa*, *Orchis provincialis*, *Anacamptis morio* subsp. *morio*, *Orchis simia* and *Neotinea tridentata*. Future studies can develop germination medium with different combinations of supplements and investigating medium success on more orchid species. Future studies must be aimed to ensure further development of the plants until adulthood for further reintroduction to nature and manufacturing salep from their tubers.

Acknowledgements

The authors wish to thank Cathy Seither for linguistic improvement of this manuscript and Svante Malmgren for sharing his own-developed medium receipt.

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