S. Oikonomidis, K. Koutsovoulou & C. A. Thanos

Germination of *Neotinea maculata* (*Orchidaceae*) in nutrient media and water agar

Abstract

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The asymbiotic seed germination of orchids is a complex process with considerable technical difficulties, due to the natural requirement of a plant-fungus symbiotic relationship under field conditions. Only on rare occasions, orchid seeds are known to readily germinate in water. *Neotinea maculata* is a relatively common orchid species with its main distribution around the Mediterranean Sea. Germination of *N. maculata* seeds was tested in both nutrient media and water agar, in asymbiotic culture. Full germination was achieved both in nutrient media (96.1%) and water agar (84.0%) in darkness at 20°C. In this study, apart from presenting the protocols of germination for *N. maculata* in asymbiotic culture, we also furnish data for the other members of the genus *Neotinea*.

Key words: asymbiotic germination, Greece, orchid, photoinhibition.

Introduction

Orchidaceae is one of the richest, in number of species, flowering plant families of the world, comprising about 28,000 species and 800 genera (Govaerts & al. 2019); it is also one of the oldest Angiosperm families in an evolutionary perspective (Givnish & al. 2015). Members of the family can be found almost everywhere, throughout all continents except Antarctica (Christenhusz & al. 2017).

Orchid seeds are among the smallest-sized ones. They consist of a spindle-shaped, thin seed coat that encloses the underdeveloped embryo. Due to the rudimentary structure of the orchid embryo, comprised by just a cluster of undifferentiated cells, there are no well-shaped embryonic organs and thus germination cannot be defined by the concept of radicle emergence from the seed coat. A system of developmental stage evaluation has been put forward for the assessment of orchid seed germination (Zettler & Hofer 1998) and it is commonly used in most relevant studies. Therefore, in this work, according to Zettler & Hofer (1998), germination is considered to take place as soon as the rhizoids have started to develop.

Neotinea maculata (Desf.) Stearn, commonly known as the dense-flower orchid, is a relatively common species with its main distribution around the Mediterranean Basin. In

Greece, it is found growing in numerous small populations, mostly in grassland and coniferous forest habitats, from sea level up to 2000 m of elevation.

N. maculata is one of the four members of the genus *Neotinea*. While the other species (*N. lactea*, *N. tridentata* and *N. ustulata*) are allogamous, relying on insects for pollination, *N. maculata* is an autogamous species with only rare occasions of insect pollination (Claessens & Kleynen 2018).

Regarding seed germination data, the *Neotinea* genus shows a widely diverse picture: on the one end, no records of successful germination for *N. tridentata* and *N. lactea* exist, while minimal germination (1%) has been reported for *N. ustulata* (Ponert & al. 2011); on the other hand, *N. maculata* seeds germinate relatively easily, in asymbiotic nutrient media culture, with percentages ranging between 59% and 80% (Calevo & al. 2017; Ponert & al. 2011). In this work, we further investigated the germination capacity of *N. maculata* seeds both in nutrient media and in water agar (the latter tested for the first time), at a range of constant temperatures under two light regimes; we also compared the relatively easy germination exhibited by *N. maculata* with those of the other genus members.

23. *Neotinea maculata* (Desf.) Stearn (*Orchidaceae*)

Accession data

Gr: Mt. Hymettus (Athens), loc. Aesthetic Forest of Kaisariani (WGS84: 37.96305556°N, 23.79638889°E), 314 m a.s.l., 26 Mar 2017, *S. Oikonomidis* (NKUA/GR-Acc. 0206, Seed Bank of National and Kapodistrian University of Athens).

Germination data

Pretreatments: Disinfection with: **a**) 10% bleach solution (0.5-1% NaOCl) for 15 min followed by three rinses in sterile distilled water, **b**) 5% $Ca(OCl)_2$ + 1% Tween 80 solution for 15 min followed by three rinses in sterilized distilled water, **c**) no pretreatment.

Germination media: **1)** modified (type 1) Malmgren (**Mm**) medium (Malmgren 1996 modified by Kitsaki & al. 2004) consisting of 75 mg/L $(Ca)_3PO_4$, 75 mg/L KH_2PO_4, 75 mg/L $MgSO_4(H_2O)_7$, 10 mg/L sucrose, 50 mL/L coconut water, 6 g/L agar and 0.5 g/L charcoal, at pH 5.8; **2)** modified (type 2) Malmgren (**M+**) medium (Calevo & al. 2017) consisting of 75 mg/L $(Ca)_3PO_4$, 75 mg/L KH_2PO_4, 75 mg/L $MgSO_4(H_2O)_7$, 100 mg/L NH_4NO_3, 10 g/L sucrose, 50 mL/L coconut water, 8 g/L agar and 0.5 g/L charcoal, at pH 5.8; **3)** Murashige & Skoog (**MS**) medium (Murashige & Skoog 1962); **4)** 2% agar (**WA**). All germination media were autoclaved at 0.1 MPa and 121°C for 20 min.

Sample size: 140 seeds (70 × 2 replicates) in nutrient media; 500 seeds (100 × 5 replicates) in water agar.

Germination	Thermoperiod	Pretreatment /Medium	Photoperiod [light/dark]	T_1 [d]	T_{50} [d]	T_{max} [d]	MTG [d]
96.1%	constant 20°C	a/Mm	0/24h	11	16	54	12

89.3%	constant 20°C	b/M+	0/24h	11	18	66	18
86.6%	constant 20°C	a/M+	0/24h	8	14	42	24
84.0%	constant 20°C	c/WA	0/24h	13	16	50	28

Observations

Mature seed pods were collected in March 2017 from a wild population (26 mature individuals) of *N. maculata* in Mt. Hymettus. A total of 104 seed pods from 13 individuals (8 per plant) were collected and they were taken in the Seed Bank of National and Kapodistrian University of Athens where seeds were extracted and placed in glass vials, in laboratory conditions until the end of the experiments. After the end of the experiments (July 2018), the seeds were deposited for long term storage in the NKUA Seed Bank at -20°C.

N. maculata is a relatively easy species to germinate (Calevo & al. 2017; Ponert & al. 2011). In our study, optimal germination results, higher than previously reported, were reached in three media with two pretreatments. Final germination of seeds pretreated with 10% bleach for 15 min (96.1%) was slightly higher than seeds pretreated with 5% $Ca(OCl)_2$ + 1 Tween 80 (89.3%) in Mm; the same observation was also made in the case of MS where final germination for bleach pretreated seeds was higher (60.8%) than the $Ca(OCl)_2$ pretreated seeds (14%). In the nutrient media experiments, optimal final germination was obtained in Mm (96.1%) and M+ (86.6%) while MS gave the lowest germination (60.8%). Germination of *N. maculata* has been previously tested on Michl-5 by Ponert & al. (2011) and on M+ by Calevo & al. (2017); final germination on Michl-5 was 80% but this medium was not tested in our study. In the present work, final germination in M+, after 15 min of pretreatment with bleach, yielded a considerably higher final germination (86.6%) than previously reported by Calevo & al. (2017) who achieved 59% of germination in the same medium, with seeds pretreated with NaOCl for 20 min, with the main difference being in the shorter duration of the pretreatment.

In addition, it is for the first time that germination of *N. maculata* is observed in water agar. Optimal germination (84.0%) was achieved with no pretreatment at 20°C, when seeds were imbibed on 2% agar (no nutrients added). Regarding temperature, 20°C was found to be optimal for *N. maculata* germination while final germination (similarly without pretreatment) at 10°C, 15°C, 25°C, and 30°C was 39%, 66%, 10%, and 0%, respectively. Pretreating the seeds with NaOCl for 15 min resulted in lower final germination levels compared to untreated seeds (53%, 66%, 61%, 3%, and 0% at 10, 15, 20, 25, and 30°C, respectively).

Despite the fact that germination of small seeds is usually promoted by light, an increasing number of orchids have been reported to have seeds with photoinhibited germination (Carta & al. 2017; Rasmussen 1995). *N. maculata* is an addition to the short list of the *Orchidaceae* species with photoinhibition. Germination of *N. maculata* seeds was fully inhibited by white light (photoperiod of 12/12h) at 20°C.

For the other three species within the genus *Neotinea*, very low germination data for only *N. ustulata* have been reported, so far (Ponert & al. 2011). Therefore, in our lab, we tried to germinate asymbiotically *N. lactea* and *N. tridentata* seeds. Final germination values in Mm were relatively low, 15% and 10% for *N. lactea* and *N. tridentata*, respectively; in both species, seed germination was obtained with longer scarification time: 30 min for the former and 60 min for the latter.

Conclusions

Orchid minuscule seeds with their underdeveloped embryos lack sufficient nutrient to support germination and early seedling development. In most cases, in nature, a fungus symbiont provides nutrients to the seedling in the first stages of its development while in the laboratory, nutrients are provided by the culture media used. Relatively few species of the family *Orchidaceae* can readily germinate in water and the final germination level is usually low (Rasmussen 1995). In this study we present a successful protocol for germination of *N. maculata* which yielded higher final germination than previously reported in nutrient media; we also demonstrate, for the first time, the ability of the species to readily germinate in water agar without any nutrients added, while we additionally reveal the inhibition of seed germination by light for this species. Seeds sown in nutrient media reached a stage 4-5 in the Zettler & Hofer scale (Zettler & Hofer 1998), while seeds imbibed in water agar reached stage 1-2 in the same scale (Fig. 1).

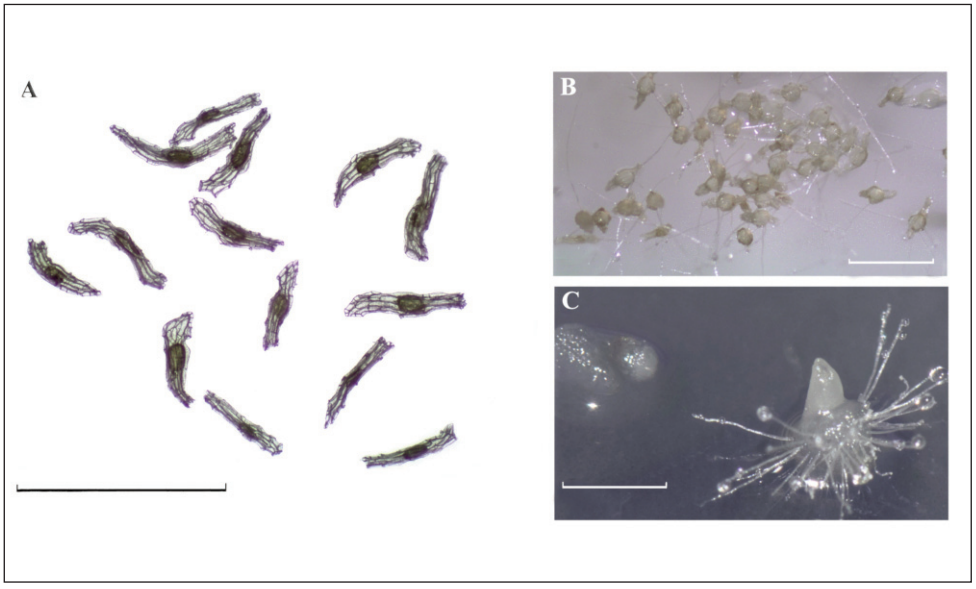

Fig. 1. *Neotinea maculata*. A: dry seeds; B: germinated seeds in water agar (WA) at the developing rhizoids stage; C: germinated seed in modified (type 1) Malmgren (Mm) at the protocorm plus initial shoot stage. Scale bars = 1 mm.

The optimal germination temperature was 20°C and the highest, reported so far, germination (96.1%) was achieved in Mm medium. High germination (84.0%) was also achieved in water agar with no seed pretreatment, a result that seems to indicate the water permeability of the seed testa in this species. *N. maculata* is one of the few orchid species known (Rasmussen 1995) to readily germinate in water with almost full germination. Seeds of *N. maculata* do not require scarification in order to germinate while such a pretreatment, even for a short duration, may lead to negative results in seed viability and subsequent germination.

Comparing the germination behaviour of the four species, we can assume that *N. maculata* is the only member of the genus with a water-permeable testa, while the other species have an impermeable seed coat, which might be the result of the accumulation of water-repelling substances (e.g. lignin, waxes etc.), thus requiring chemical degradation in order for water to be absorbed and the germination process to start.

Species which readily germinate in water like *N. maculata* can be utilized as comparative means for further understanding the germination physiology of species which exhibit hindered germination either in common orchids (e.g. in the genus *Neotinea*) or in rare and endangered ones (e.g. in the genus *Dactylorhiza* where germination in water was also observed in several members of the genus, Rasmussen 1995).

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Addresses of the authors:
Spyridon Oikonomidis[1], Katerina Koutsovoulou[1, 2], Costas A. Thanos[1],
[1]Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, 15784 Athens, Greece. E-mails: soikonomidis@biol.uoa.gr, kkoutsovoulou@biol.uoa.gr, cthanos@biol.uoa.gr
[2]Green Fund, 14561 Athens, Greece.