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Seed banking is the most appropriate way of ex situ conserving orchid species. This entails selection, storage and germination testing of seed samples as well as regeneration of plants from the embryos. Many epiphytic orchids germinate and grow easily in asymbiotic culture, on a nutrient medium. However, in species with complicated dormancy patterns and a pronounced dependency on the symbiotic fungus, like many terrestrial species, it is difficult to develop a sound storage protocol because there is little correspondance between viability and germination capacity. An independent seed viability test is difficult to perform in a reproduciple way. Problems pertaining to the germination in vitro and to the regeneration of plants in symbiosis with a fungus are discussed.

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Introduction

The basic problems in ex situ conservation of wild species are the selection and storage of a genetically representative sample, the regeneration of plants from the material in storage, and the cultivation until new storage units can be harvested.

Somatic tissues (callus– and suspension cultures) in long term storage are of limited value in the conservation of genetic diversity of species, because each sample is genetically homogeneous. On the other hand, regeneration of orchid plants from such tissues causes few problems (Morel 1974). Storage of somatic tissue is standard procedure in maintaining selected clones (Griesbach 1986) and may be applicable also for the conservation of species in cases where it is difficult to obtain seeds.

Pollen is a space-effective way to store a vast amount of haploid genomes, and the orchid pollinia survive storage at −196°C for at least one year if not to strongly desiccated (Pritchard & Prendergast 1989). However, at present direct regeneration from immature pollen is possible only in rather few species, none of them orchids (Han & Hongyuan 1986 and references herein). In order to make use of a pollen bank for later seed production, it is necessary to keep a corresponding collection of potential mother plants, with all the problems associated with maintaining a living plant collection. According to recent investigations, self-incompatibility is much more common in the orchids than previously assumed (Agnew 1986; Clifford & Owens 1988; Johansen 1990). This fact makes the selection of appropriate specimens for a stock collection more critical.

The ideal representation of a wild species must be heterozygous material with high genetic diversity. Therefore, the most appropriate way of ex situ conservation is a seed collection. Orchid seeds are relatively easy to store, being so small that one milligram may represent one thousand genotypes. They survive fairly well under certain storage regimes. However, the regular viability testing required in rational seed banking always causes difficulty in species with complicated dormancy patterns or special germination requirements. In this respect many orchid species are difficult to handle. A symbiotic relationship with a fungus plays a role both in the germination and in the seedling development. Chemical additions to the culture media may to some extent and in some species mimic the stimulatory effects of the fungus, but a general biological understanding of the orchid mycorrhiza has not yet been fully achieved.

In many cases, orchid embryos excised from the green capsules germinate more readily than mature seeds, presumably because dormancy is induced during the final desiccation of the maturing seeds. Defined storage methods for immature embryos have not yet been developed.

Germination technique

Most tropical epiphytic orchids are easily germinated. They can be sown on a sterile medium containing a carbon source and different salts. The establishment of

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Fig. 1

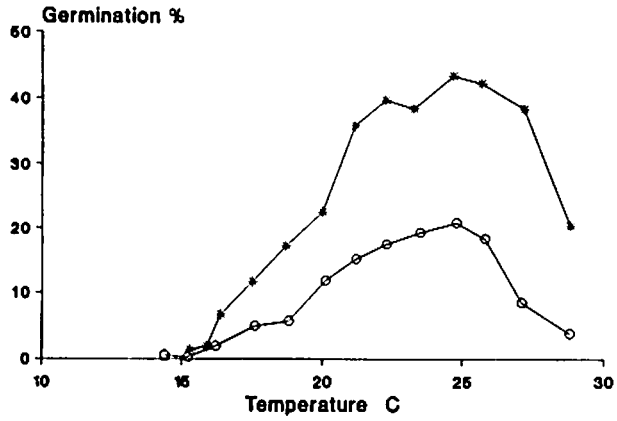


Fig. 2

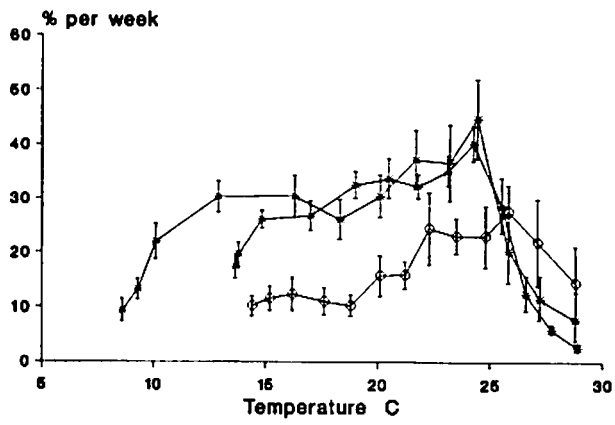


Fig. 3

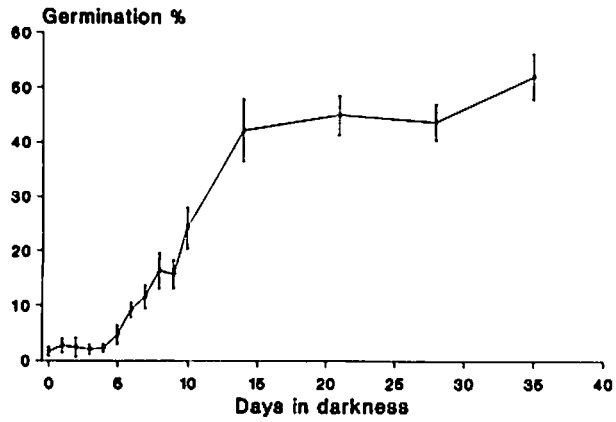
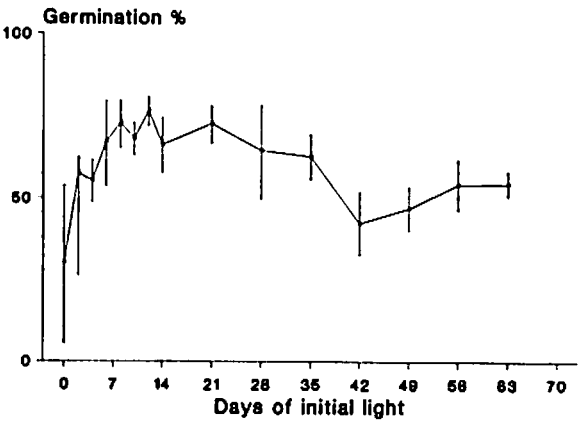


Fig. 4



Figs 1–4. *Dactylorhiza majalis*. – Fig. 1. Germination percentage function of temperature. Symbiotic germinations (*) with a strain of *Tulasnella calospora* (D47–7) on oats medium. Asymbiotic germinations (○) on Mead & Bullard (1979) medium. Germination percentage measured after 42 d in darkness. Data pooled from several germination dishes, each point based on c. 600 seeds. After Rasmussen et al. 1990a. – Fig. 2. Relative length increase per week as a function of temperature. Symbiotic cultures (*) (two consecutive experiments), asymbiotic cultures (○). Means of 16, 40 (symbiotic experiments) or 20 seedlings (asymbiotic experiment) with 95% confidence intervals. After Rasmussen et al. 1990a. – Fig. 3. Symbiotic germination percentage as a function of initial darkness for 0–35 days followed by fotoperiods until day 42. Less than 14 days of initial darkness inhibited germination. Means of 10 replicates with 95% confidence intervals. After Rasmussen et al. 1990b. – Fig. 4. Symbiotic germination percentage as a function of initial light treatment. All initial light periods were followed by 28 d of darkness. Up to fourteen (16 h) photoperiods increased germination significantly. Means of 13 replicas with 95% confidence intervals. After Rasmussen et al. 1990b.

a symbiotic relationship is not needed for germination or continued growth. However, many of the terrestrial and temperate orchids are very difficult to germinate and to grow, and they seem to depend heavily on their symbiotic fungus in the first stages of seedling development.

For our experiments, fresh seeds from species of *Dactylorhiza*, *Orchis*, *Ophrys*, and *Serapias* and several others are each year harvested from nature and from the living collection grown in the Botanical Garden of the University of Copenhagen. Most of our studies of germination and seedling biology have been made on one test species: *Dactylorhiza majalis* (Rchb. f.) Hunt & Summerhayes, which can be germinated both asymbiotically and symbiotically. It is a rather common plant in Northern Europe, and we have isolated the symbiotic fungus from several different populations.

The seeds are surface sterilized and sown in vitro on semisolid nutrient medium (Rasmussen et al. 1990a). In the symbiotic experiments the culture vessels are inoculated with an isolate of an appropriate symbiotic fungus. Cultures are normally kept at fixed temperature

Fig. 5

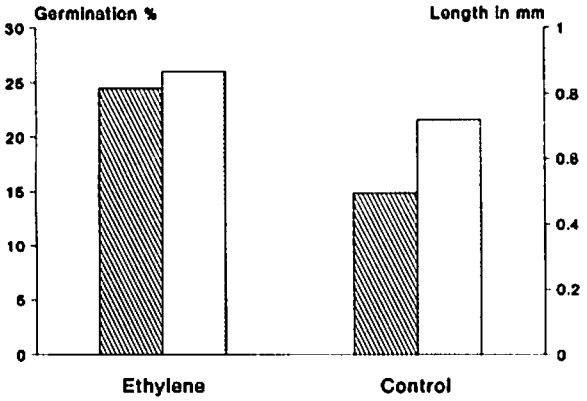


Fig. 6

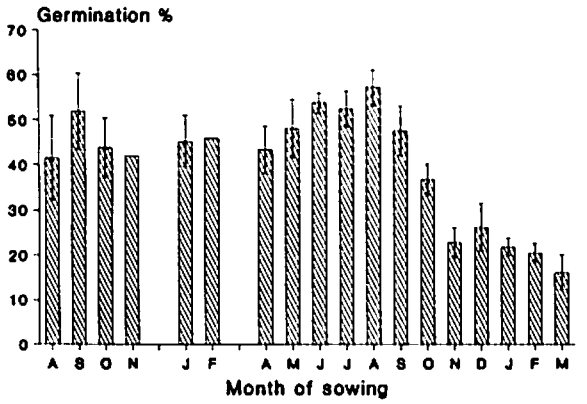
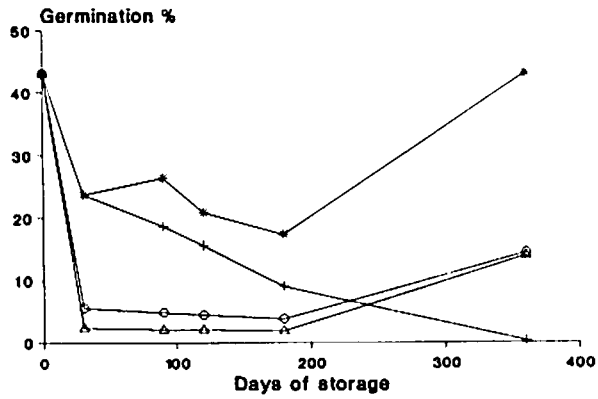


Fig. 7



and light conditions but a temperature gradient ranging from about 10°C to 30°C with steps of 1.5°C was used for optimizing temperature conditions (Rasmussen et al. 1990a). The composition of the atmosphere around the germination dishes was manipulated by placing the Petri dishes in air tight jars in which specified amounts of various gasses were injected.

Germination

The seeds of *Dactylorhiza majalis* are rather typical orchid seeds. The embryo is 0.002–0.004 mm^3 and comprises about 200 undifferentiated cells stocked with proteins and lipids (Rasmussen 1990). There are no other storage tissues. The water content in orchid seeds is about 11% (Pritchard 1984). During germination proteins are hydrolysed, the cells vacuolate, and starch begin to accumulate. The infection should occur at this stage before the reserve nutrients are exhausted. The endosymbiotic fungus identified as *Tulasnella calospora* (Andersen 1990) enters through the first developed roothairs.

In the infected cells of the seedling the fungus produces hyphal coils which are digested by the orchid (Bernard 1904; Burgeff 1936). After lysis of the first hyphal coil, the cell may become reinfected. A meristem differentiates in the chalazal part of the seedling which never becomes infected, and after several months this region produces a green shoot. Until then, the seedling has been heterotrophic and totally dependent upon the fungus. Some orchid species, such as *Neottia nidus-avis*, remain chlorophyll free and feed on the fungus during their entire life (Fuchs & Ziegenspeck 1924).

Obviously, such a close relationship between orchid and fungus already at the seedling stage may cause some difficulties for the regeneration of plants in vitro.

Figs 5–7. – Fig. 5. Symbiotic germination percentage (hatched bars) and maximal length of protocorms (dotted bars) after 28 days of germination in normal and ethylene enriched (0.5%) atmosphere. In the presence of ethylene the seed showed higher germinability and developed longer seedlings. Means of 8 replicates. Orig. – Fig. 6. Germinability of a seed sample of *Dactylorhiza majalis* tested every month from August 1988 until March 1990 (except December 1988 and March 1989). The germinability is almost constant at 50% until August 1989 where a decline to about 17% in March 1990 occurs. Means of 8–10 replicates with 95% confidence intervals. Orig. – Fig. 7. Germinability of a seed sample of *Dendrobium linguella* stored at different temperatures over NaI as a function of time. 20°C (+), 6°C (*), −18°C ( ), −80°C (○). Until day 180 the germinability was scored 28 d after sowing. The point at day 360 was scored 46 d after sowing. Only the seeds stored at 20°C lost germinability through the entire experiment. The germinability of seeds stored at 6°C, −18°C and −80°C for 360 d could be improved by extending the test period, indicating that part of the seeming viability loss was a loss of vigour causing the germination process to decelerate. After Jørgensen 1990.

Factors affecting germination and growth

Several physical and chemical factors affect the germinability and the possibility of inducing a stable symbiotic relationship in vitro (Rasmussen et al. 1989, 1990a, 1990b). An optimized germination protocol for *Dactylorhiza majalis* may give us a key to the regulation of germination and seedling growth of orchids in general.

Temperature

The optimum temperature for symbiotic germination of *Dactylorhiza majalis* is about 21°C to 25°C (Fig. 1), roughly the same as for asymbiotic germination. However, the symbiotic germination percentage is much higher than the asymbiotic. It is remarkable that maximum germination takes place at this relative high temperature, since it is assumed that the seeds germinate at temperatures below 20°C in nature (Rasmussen et al. 1990a).

Seedling growth is also markedly affected by temperature (Fig. 2). The symbiotic seedlings grow best near 24°C, the asymbiotic seedlings possibly have a slightly higher optimum. Temperatures beyond the optimum result in much slower growth of the seedlings, suggesting that the temperature conditions should be carefully observed during regeneration of symbiotic plants.

Chilling at 5°C for two to three months will induce leaf and tuber development in many species after the temperature is raised again (Borris 1970; Hadley 1970; Mittchell 1989). Even rhizomatous species like *Goodyera repens* and *Epipactis gigantea* respond positively to this treatment (unpubl. obs.). Without this cold treatment the seedling growth stagnates and the seedlings eventually die.

Light and darkness

Many terrestrial species do not germinate in continuous light (e.g., Fast 1980). *Dactylorhiza majalis* needed about two weeks of darkness to reach maximum germination percentage under a given set of test conditions (Fig. 3) (Rasmussen et al. (1990b)). Even green light at low intensity caused a significant depression of germination (0.02 W m^2, 540 nm, unpubl. data).

Although darkness is necessary for germination in the vast majority of European orchid species, a pretreatment in the light can increase the germination percentage in at least one species (Rasmussen et al. 1990b). Ten to fourteen days of light given before the darkness period increased the germination of *Dactylorhiza majalis* from about 30 to 75 percent. If light was given over a longer period, a lower germinability was observed (Fig. 4). In order to get comparable results, the germination vessels should therefore be subjected to exactly the same light regimes.

Atmosphere

An atmosphere with added ethylene significantly increases the germination (about 25% with 0.1% ethylene compared with 15% in the controls, Fig. 5). Seedlings were also significantly larger in the ethylene treated dishes (Fig. 5), which could be due to faster germination. Our latest observations indicate that the CO_2 concentration also alters germinability. In this way sealing and ventilation of the culture containers may affect the results considerably.

These factors – and probably others which are less obvious should be optimized for each species in order to give a realistic estimate of the viability and survival of seeds under storage.

Dormancy

A seed collection of *Dactylorhiza majalis* from 1988 showed a very stable germination capacity of about 75%, which suddenly declined after 1.5 years of storage (Fig. 6). The seeds had been dried over silicagel after harvest and were stored at room temperature until germination test (2000–4000 seed sown symbiotically each month). When seeds from the same source were stored at different temperatures and humidities, the drop in germination capacity only occurred in seeds stored at room temperature, not at +6, −20 and −80 (C. Stæger 1990). Similarly, *Cattleya aurantiaca* showed a sudden loss in viability under certain storage conditions (Seaton & Hailes 1989). However, orthodox seeds do not usually loose viability suddenly after a long storage period. This suggests that orchid seed samples may loose germinability for other reasons than viability loss. Manipulating the atmosphere in the germination dishes showed that the viability of our sample was better than indicated by our standard germination test. A sort of dormancy had apparently developed in the seeds, which could be released by an ethylen enriched atmosphere (Fig. 5).

Obviously, if changes in dormancy pattern or germination requirements can take place during storage, a standardized germination procedure does not give an objective estimate of the changes in viability of a seed sample during storage.

Dormancy in orchid seeds is difficult to distinguish from loss of viability, because an independent viability test is so difficult to perform. Contrary to the findings of Pritchard (1985) we had great difficulties in getting reproducible results of repeated viability tests with flourocein diacetate. Vital staining properties seem to vary with the germination capacity (Waes 1984): increased pretreatments of the seeds with calcium hypochlorite – which raised the germination percentage – could also increase the "viability" expressed in staining tests with triphenyl tetrazolium chloride.

Dormancy due to inappropriate physical factors is furthermore difficult to distinguish from germination failure due to a lack of an appropriate fungus, because we do not know the precise nature of seed-fungus signals, nor of the effects the two organisms may have on their common microenvironment. Complicated dormancy patterns, perhaps unrelated to the symbiosis, could be a common phenomenon among terrestrial orchids, and it could be the reason why many of these species do not germinate readily. One fact in support of this is that immature seeds are often easier to raise than those from open capsules (Withner 1943; Borris & Albrecht 1969).

Recently we induced symbiosis and development of immature embryos of *Listera ovata* (Rasmussen et al. 1991). This technique may be a generally useful method for germination and cultivation of those species where dormancy is induced late in the seed development, and for assessing orchid-fungus compatibility detached from problems of seed dormancy.

Storage and survival

Although seeds from several orchid species tolerate temperatures down to −196°C in liquid nitrogen, all species tested so far has only been exposed to this low temperature for a very short time (Pritchard 1984). At the correct moisture content orchid seeds can be stored at subzero temperatures without noticeable loss of viability (Pritchard 1984; Seaton & Hailes 1989). *Odontoglossum laeve* even showed a considerable increase in germinability after a short exposure to −80°C (Jørgensen 1990). In contract, the seeds of *Dendrobium linguella* lost viability very fast and the samples showed very low germination percentages after 180 days (Fig. 7),both at room temperature and at sub zero temperatures. In this case, a storage over sodium iodide at 6°C was optimal with no change in viability after 360 days of storage, provided that the germination test was prolonged: 46 days of incubation after 360 days of storage, compared with 28 days for the initial germination test. We interpret this as a loss of vigour expressed as slower germination. The same effect was to a minor extent observed in the samples stored at subzero temperatures (Jørgensen 1990). A standardized test procedure used throughout a storage period will therefore tend to underestimate the viability of stored seeds. A deceleration of germination after storage is not uncommon in seeds generally (Heydecker 1972).

Conclusion

Many plants besides orchids are more or less dependent on symbiosis with microorganisms, and several mycorrhizal fungi are equally dependent on their host. A similar mutual dependency often exists between a plant and its pollinator. Every time ex situ conservation of a new plant species is considered, the associated organisms should also be taken into account. Extinction of any of these could effectively preclude a succesful reintroduction of the conserved plant species. The ex situ conserved germ plasm would then constitute a living museum specimen which could, however, still be useful in horticultural and agricultural breeding programs.

Although orchids are probably more complex than other plant groups it is evident that seed banking makes little sense without an intensive supporting research. Disregarding this necessity may result in an accumulation of seeds with high demands for technical installations and labour but with little returns in terms of actual ex situ conservation.

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