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Propagation, cultivation and breeding of terrestrial temperate orchids, with focus on *Cypripedium* spp.

Cypripedium spp. Bild tagen från Malmgren (2006) med tillstånd.

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Abstract

Temperate terrestrial orchids have received increased horticultural attention as the new exclusive perennials for the garden. Temperate terrestrial orchid seeds have been found to germinate and develop readily asymbiotically on suitable media *in vitro*. To achieve a successful germination the water impermeable seed coat must circumvented; either by sterilization in a hypochlorite solution or culture of immature seeds. Further the culture must be kept in darkness until the first leaves appear. Germination and proliferation media for terrestrial orchids should have a low concentration of mineral salts, where the nitrogen is provided in organic form. Soluble sugars as sucrose are also required. Growth promoting effects has been seen with the vitamin B complex and various organic liquids, especially pineapple juice. Kinetin has been found to improve germination and growth in some *Cypripedium* spp. The optimum temperature for seedling growth *in vitro* is usually around 20°C. The protocorms developed after germination should be transplanted to new media regularly. Periods of lower temperatures should occur to induce dormancy periods resembling natural conditions. Later plantlets of sufficient size could be planted in soil, and acclimatized to outdoor conditions. Vegetative propagation could be done by dividing protocorms or underground parts. Micropropagation of young tissues is another way, which has not yet been fully explored. The genus *Cypripedium* consists of 46 species half of which have been explored in breeding new cultivars. Closely related species rather easy produces vigorous hybrids, but there are indications that all species could be crosses. The distribution of the genus is circumboreal, with a wide span of habitats, which provides good breeding material for hardy and adaptable cultivars. *Cypripedium* cultivars should be planted shallow in a partly shaded site, moist, but yet well drained. The soil should ideally have a high content of organic matter, and be in the slightly acidic pH range.

Acknowledgements

I would like to thank; my supervisor Associate Professor Björn Salomon for guidance and feed back; my examiner Associate Professor Li-Hua Zhu for expert knowledge in micropropagation; terrestrial orchidist Svante Malmgren for valuable comments and last but not least my family for great support and endless patience.

Sammanfattning

Marklevande orkidéer från tempererade områden har på senare tid fått ökad hortikulturell uppmärksamhet. Förbättringar i förökning av frön och vegetativa vävnader har lett till snabbare uppförökning av hortikulturellt intressanta kloner och sortmateriel. Med framgångsrika förädlingsprogram kan fler sorter komma att registreras.

Fröskalet hos marklevande orkidéer från tempererade områden är i princip ogenomträngligt för vatten, vilket försvårar vattenupptag och därmed groning. Detta kringgås bäst genom att skörda och kultivera omogna frön, innan fröskalet har utvecklats. Alternativt kan fröskalet förstöras genom sterilisering under förlängd tid i hypokloritlösning. Dessutom krävs ofta mörker för groningen. Frön av marklevande orkidéer från tempererade områden har konstaterats gro och utvecklas bra asymbiotiskt på lämpliga substrat *in vitro*. I naturen behöver orkidéplantan efter groning sockerenergi och mineralnäringsämnen för att överleva och utvecklas, vilket tillhandahålls av en kompatibel mykorrhizasvamp.

Gronings- och tillväxtsubstrat för marklevande orkidéer bör generellt ha en låg koncentration av mineralsalter. Kvävet föredras oftast och ges i sin organiska form som aminosyror. För utvecklingen krävs också lösliga sockerarter, vilka ofta tillsätts som sackaros i koncentrationer av 10-20 g l^{-1}. Ett substrat-pH kring 5,5 rekommenderas för många arter. Tillsatts av cytokininer i form av kinetin har visats förbättra groning och tillväxt i några *Cypripedium* spp. Vitamin B komplexet har setts ha en viktig effekt på utvecklingen hos små orkidéplantor. Komplexa organiska tillsatser, som ananassaft, kokosmjölk eller potatis extrakt, har tillväxtökande verkan och tillsätts därför frekvent till substrat.

Den optimala temperaturen för tillväxt av orkidéplantor *in vitro* är oftast kring 20-25°C. Perioder av lägre temperaturer bör inträffa för att inducera viloperioder liknande de under naturliga förhållanden.

Efter sådd av *Cypripedium* ska kulturen förvaras mörkt tills bladanlag börjar synas. Groning av *Cypripedium* spp. varierar från några veckor till flera månader. Protokormen bör planteras om till nytt substrat regelbundet. Rhizom- och rotutveckling tar fart efter flera månader, och först därefter uppträder bladen. En kulturtemperatur på 15-18°C är lämplig. Efter några månader bör en köldbehandling påbörjas, först vid 8-10°C, och senare vid strax över 0°C. Efter ytterligare en tid kan plantor av tillräcklig storlek efter tillvänjning planteras i ett jordsubstrat. Acklimatisering av plantorna i krukor utomhus kan göras under det andra året. Det tredje året i krukkultur är plantorna tillräckligt stora för att överleva utplanterade på en fuktig och skuggig växtplats. Många arter och hybrider av *Cypripedium* blommar efter 4-5 år.

Det vanligaste sättet som specialplantskolor förökar *Cypripedium* spp. på är genom *in vitro* sådd av frön. Förökning kan även ske vegetativt genom delning av protokorm *in vitro*, eller delning av rhizomklumpar. Ett ökat skottbrytande hos rhizomet kan stimuleras genom grunda skärsår.

Hittills har inget publicerats om mikroförökning *in vitro* av vävnader från *Cypripedium* spp., förutom frön och protokormer. Rotspetsar, skottspetsar, rhizomspetsar eller –segment, meristem, delade protokormer och bladdelar (baser, spetsar) är några vävnader som kan utforskas.

Släktet *Cypripedium*, av vilket huvudkaraktären är den toffellika läppen hos blomman, består av 46 arter. Hälften av dem har använts i förädlingen av nya sorter. Fram tills nu har över 90 sorter registrerats av the Royal Horticultural Society.

Släktets utbredning är circumboreal med ett brett spektrum av habitat i ljus och skugga, i torrt och fuktigt, och under sura och basiska förhållanden. Detta ger en genpool med stor anpassningsbarhet, vilket är en tillgång i förädlingen. Det är viktigt att ta hänsyn till proveniensen och använda plantmaterial från olika regioner för att kunna ta fram härdiga sorter som kan växa och frodas på flera sorters växtplatser och klimat. Både positiva och negativa hortikulturella egenskaper måste beaktas när förädlingsmaterialet utvärderas för selektering i korsningsarbetet.

Cypripedium har visat sig ha en låg genetisk variation inom släktet, vilket indikerar att alla *Cypripedium* spp. skulle kunna korsas. Närbesläktade arter kan relativt lätt korsas till livskraftiga hybrider.

En växtplats i halvskugga föredras av många sorter. Jorden bör vara väldränerad men ändå fuktighetshållande. Dock är sorter, jämfört med rena arter, vanligtvis lättodlade under en rad växtplatsförhållanden. Innehållet av organiskt material i jorden ska vara högt för att förse plantorna med en långsam men tillräcklig näringstillförsel. Jord från skogar eller orkidéhabitat kan med fördel blandas i substratet på växtplatsen. Mykorrhizabildande svampar kan därmed introduceras. *Cypripedium* spp., som de flesta marklevande orkidéer från tempererade områden, trivs med en lätt sur jordreaktion.

Cypripedium spp. bör för bästa resultat planteras under sin viloperiod. Rhizomet ska planteras ytligt med skottspetsarna precis under jordytan. Rhizomet kräver en konstant, oavbruten, viloperiod och kan då tåla svår frost. Om viloperioden avbryts riskeras frostskador. I områden med milda vintrar kan vinterväta vara förödande. Under vintern bör plantorna skyddas mot varma perioder och väta med någon form av täckning.

Ekonomiska aspekter av *Cypripedium* spp. kultur diskuteras vidare i uppsatsen. Laboratoriedelen av kulturen är både arbetskrafts- och materialintensiv. Den efterföljande jordkulturen är mindre besvärlig. Plantskolor tjänar på att specialisera sig på antingen *in vitro*-kultur eller jordkultur.

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1. Introduction

Temperate terrestrial orchids, especially *Cypripedium* spp., have received increased horticultural interest during recent years. A steadily increasing demand for exclusive perennials for the garden provides a great potential for orchids.

Amateur orchid hobbyists and specialized nurseries have experimented with species and genera crossings as well as asymbiotic seed culture for several decades. Inexpensive methods and devoted time have resulted in beautiful, easily cultivated cultivars. The experiments have also yielded greater knowledge of these often rare plants, and plants have in addition been raised for conservation purposes (Malmgren, 1994).

Future scientific experiments could further contribute to the development of cost efficient production schemes to lower the retail price and make the plants more available to everyone. If successful micropropagation protocols are developed many problems could perhaps be solved and give a rapid increase in plant numbers. Terrestrial orchids provide for an exciting future for horticulturists interested in the subject.

1.1 Aim

The aim of this Bachelor thesis is to provide an overview of important concepts in seed propagation of temperate terrestrial orchids. Emphasis is put on the horticultural important genus *Cypripedium*, where breeding and cultivation issues are also discussed.

2. Materials and Methods

This thesis will address asymbiotic propagation, since this has gained the most progress during the years of amateur and scientific orchid propagation. Symbiotic propagation would need more research in order to be successful. In addition, orchid mycorrhiza is a large science area, and will not be dealt with in detail in this thesis.

Taxonomy for *Cypripedium* spp. follows Cribb's (1997) description, and synonyms have been adjusted according to a synonyms list by Cribb (1997). Taxonomy for other species follows the nomenclature of the cited article.

2.1 Literature

Information on temperate terrestrial orchid seed propagation from breeders and nurseries is readily available on the Internet, but most details remain corporate secrets. Useful information is also published in the large number of amateur papers. Scientific research has during earlier years mainly focused on tropical orchids, but in recent years more articles have been published on temperate orchids, too. Several reviewing books on the topic have been

published, of which Rasmussen (1995) and Cribb (1997) have been used in this thesis. New results are furthermore printed in conference proceedings and symposiums from scientific meetings and amateur societies. Many more existing articles would have been interesting to study, if the time had not been limited.

2.2 Glossary

Endogenous; something that originates from within an organism
Exogenous; something that originates from outside of an organism
Imbibition; the uptake of water, or other liquids, by the seed thus enabling germination.
Introgression; the crossing of two species producing a fertile hybrid, which backcrosses for several generations with one of the parent species. In this way, genetic material and characteristics is transferred from one species to another.
Lipolysis; the breakdown of storage lipids (e.g. fats and oils) in living organisms.
Resupinate; inverted, upside-down. In *Cypripedium*, the flowers are twisted 180 degrees, so that the position of the upper and lower petals is reversed.
Sterilant; a substance used for sterilization of seeds, tools etc.

3. Biology and Ecology of temperate terrestrial orchids

3.1 Pollination, seed set, development and dispersal

When an orchid flower is pollinated, the actual fertilization takes place several days later. The ovules will not fully develop until the pollen tube reaches them (Fredrikson, 1991). In most temperate species fertilization takes place from 1 to 2 weeks after pollination, but in *Cypripedium* spp. it may take several weeks longer (Rasmussen, 1995, table 2.1). In most species embryogenesis lasts about 2 weeks (Veyret, 1974), but in *Cypripedium* spp. this can differ (Light & MacConaill, 1990; Rasmussen, 1995). Based on these facts, and on experience, the optimum time for excising immature seeds for culture, in relation to time of fertilization and seed maturity, have been calculated (Rasmussen, 1995, table 2.1).

The seed quality depends on a number of factors. The source and quality of the pollen is important. Self-pollination often results in reduced quality (Rasmussen, 1995). The number of viable seeds depends on the effective pollination, which in turn is influenced by the age of the flower at pollination time (Ballard, 1987).

After fertilization the ovule develops into the ovoid embryo, and the integument develops into the water impermeable seed coat, the testa. The suspensor end of the embryo

differentiates to form mycotrophic tissue and the chalazal end forms meristematic tissue (Figure 1) (Burgeff, 1936; Rasmussen, 1995).

The seed dispersal can only be effective with a large seed set, since the probability of successful symbiotic germination then increases. The mature capsule splits in dry weather, releasing the seeds equipped for wind dispersal with an inflated air-filled testa. The seeds can commonly disperse over distances of 5-10 km (Rasmussen, 1995).

The minute amount of nutrients in the seeds is stored in the embryo cells in lipid and protein bodies, rarely in starch grains or glucoprotein bodies, and orchids have thus no external nutrient tissues such as an endosperm (Rasmussen 1990; Richardson *et al*., 1992). After germination, the seedling often immediately needs infection from a compatible fungus to aid in nutrient mobilization and to transfer mycotrophic nutrition for further development. Observations suggest that some orchid species have evolved mechanisms preventing the seeds from germinating in the absence of a suitable fungus (Rasmussen, 1995).

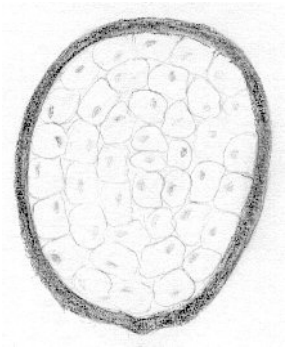

Figure 1. The structure of an orchid seed. The cellular structure of the ovoid embryo of a *Dactylorhiza* spp. with the testa surrounding it. The upper part is the suspensor end which will form mycotrophic tissues. The lower part shows the chalazal end which subsequently will form meristematic tissues. With inspiration from Rasmussen (1995). © Rännbäck, 2006

3.2 Orchid Mycorrhiza

3.2.1. Orchid nutrition and nutritional systems

The orchid seedling needs energy and mineral nutrients to survive and proliferate, which are provided by a fungal partner mycorrhiza. Differing from other types of mycorrhizal systems where the green plant provides the fungus with sugars as a source of energy, and the fungus gives minerals in exchange; orchid mycorrhiza supplies the plant with both minerals and sugars, giving the orchid a net gain of the symbiosis (Rasmussen, 1995). The use of a fungus as an energy source is called mycotrophy. When the seedling has developed leaves, and phototrophy via photosynthesis can begin, the dependence of fungal energy is partially lost. However, during the early life stages, and sometimes during later adult dormant phases, the orchid can rely entirely on mycotrophy for nutrition. *Neottia nidus-avis* and *Corallorhiza*

trifida are examples of chlorophyll-deficient species relying entirely on mycotrophy for survival. Mycotrophy will enable orchids to colonize and tolerate extreme habitats such as deep shade, summer droughts, poor soils, and soils with low accessibility of minerals because of extreme pH such as acid bogs and highly calcareous soils (Rasmussen, 1995).

The orchid can thus live as a heterotrophic organism utilizing both mycotrophic and phototrophic nutrition, either supplementing each other or one alternating or replacing the other. (Rasmussen, 1995) The relative importance of the nutritional systems of mycotrophy and phototrophy as a carbon source in adult green orchids has not been fully investigated (Whigham, 1984; Alexander & Hadley, 1985; Rasmussen, 1995).

3.2.2. Compatible fungal species

Orchid endophyte fungi are difficult to trace in the soil and to identify as potential symbionts unless they are found in orchid tissues. (Rasmussen, 1995) The fungus should then be extracted from root tissues, preferably protocorms, in seedlings or young plants as mature plants may house a variety of fungi (Harvais & Hadley, 1967; Hadley, 1970; Rasmussen, 1995).

A range of fungi have been found as endophytes in orchid roots. The largest group belongs to the imperfect, polyphyletic, genus *Rhizoctonia*, many of which have proved to be compatible with seeds *in vitro*. Since sexual sporulation is not present, the species must be distinguished on hyphal characters. By comparison of hyphal ultrastructures together with RFLP analysis, imperfect taxa (anamorphic) can be matched with perfect taxa (teleomorphic) (Andersen, 1990a; 1990b; Andersen & Stalpers, 1994).

Occasionally formation of reproductive organs is induced in vitro, and *Rhizoctonia* strains can then be referred to teleomorphic genera (Rasmussen, 1995). Those associated with orchids belong most commonly to *Tulasnella*, *Sebacina*, *Ceratobasidium* and *Thanatephorus* (Currah & Zelmer, 1992). *Tulasnella calospora* may be a universal orchid symbiont, since it is a commonly found root and seedling endophyte (Andersen, 1990b; Rasmussen, 1995). Strains of this species have proved to be compatible with several temperate orchids *in vitro* (Warcup, 1985, Williamson & Hadley, 1970; Rasmussen, 1995). Several fungal species can be present at the same time in orchid tissues as symbionts. (Rasmussen, 1995)

If orchid endophytes are kept in pure cultures for an extended period of time it often leads to a loss of characters. *Rhizoctonia* spp. have been found to reduce or loose the capacity to establish orchid mycorrhiza, or even become virulent. (Alexander & Hadley, 1983)

Most orchid compatible fungi subsist on organic debris in the soil and typical orchid soils often have high humus content. Mycotrophy depends on the provision of organic matter and high activity and development of saprophytic fungi in temperate areas coincide with leaf fall and moist weather in autumn. Heterotrophic orchids may have the advantage of a longer growing season compared to phototrophic plants, often extending into early winter (Masuhara *et al*., 1988; Alexander & Alexander, 1984; Rasmussen, 1995).

The fungal activity is restricted by dry spells and low temperatures, conditions often coinciding with orchid dormancy (Rasmussen, 1995). In areas with high precipitation and mild winters, orchid mycorrhiza could be active most of the year (Masuhara *et al*., 1988; Alexander & Alexander, 1984). The root growth occurs mainly during autumn, after the shoots have died out in summergreen species, suggesting that orchids by mycotrophic nutrition could continue to grow if the fungi are active (Rasmussen, 1995).

3.2.3. *Cypripedium* mycorrhiza

Shefferson *et al*. (2005) tried to identify primary mycorrhizal symbionts for seven *Cypripedium* spp. at different life stages from several populations in Europe and North America. The result revealed that the great majority of mycorrhizal fungi associated with the genus are members of narrow clades within the fungal family Tulasnellaceae. The specificity was quite high in all *Cypripedium* spp. investigated, except for *C. californicum*, which is assumed to be because of its sparse range of habitats and narrow distribution. These findings support that high specificity is not limited to non-photosynthetic orchids. High specificity could partially explain why *Cypripedium* spp. are so rare.

In contrary to previous findings (Vinogradova & Andronova, 2002) where mycorrhizal infection could only be found in protocorms and young plants, digested pelotons were found in roots of adult plants (Shefferson *et al*., 2005).

3.2.4 Fungal infection and mycolysis

The fungi infect orchid root organs and the hyphae aggregates loosely and form hyphal coils, pelotons, in the infected cells. The pelotons subsequently undergo lysis and release the nutrients to the orchid. Mycotrophic tissues are mainly found in the cortex of protocorms, roots and rhizomes (Rasmussen, 1995).

3.3 Seed Germination

3.3.1. Imbibition and germination

The testa softens and ruptures after being processed in soil or after *in vitro* sterilization. This process enables the embryo to imbibe. The embryo swells and mitotic activity starts in the meristematic cells. The meristem extends the leafless seedling forming a protocorm. After some time the meristem differentiates to a shoot meristem and a root meristem (Rasmussen, 1995).

During embryo imbibition the protein bodies are hydrolysed and the nutrient products mobilized (Rasmussen, 1990; Rasmussen, 1995). Lipolysis requires a higher level of metabolic activity, and *in vitro* this process requires external energy in the form of e.g. sucrose for some species. Lipolysis is regarded a crucial step in germination of orchid seeds (Manning & Van Staden, 1987). Later, starch is accumulating in the embryo cells, particularly in asymbiotic cultures (Purves & Hadley, 1976; Rasmussen, 1995).

Since several species have been found to germinate well on water agar, it is suggested that fungi are not required in germination for all species (Vermeulen, 1947; Downie, 1941; Stoutamire, 1974; Rasmussen, 1995 table 4.3). Some species could in fact subsist on their own reserves while waiting for a compatible infection (Vermeulen, 1947; Rasmussen, 1995). Orchid species vary in the extent to which they depend on fungi, and it is possible that the role of the fungi is to aid in rapid nutrient reserve metabolization (Harrison, 1977; Rasmussen, 1995). Germination *in vitro* has shown that some species require soluble sugars, or even mineral salts in order to germinate, and that the fungi aid in the supply of these compounds in one way or another (Harvais, 1982; Nakamura, 1962; Rasmussen 1995, table 4.3).

3.3.2. Germination time in nature

In nature most temperate terrestrial orchid seeds germinate in spring, or even late autumn (Rasmussen, 1995, table 3.1). An explanation could be that fungal activity is intense during autumn with its input of dead biomass, which would provide ideal conditions for the seeds to germinate (Rasmussen, 1995).

3.3.3. Seed dormancy

Some mature temperate orchid seeds are troublesome to culture *in vitro* due to seed dormancy, which can be caused by high endogenous levels of abscisic acid (van der Kinderen, 1987), or due to physical and chemical properties of the testa and by illumination. Dormancy could be broken by certain temperature regimes, lengthy imbibition, chemical breakdown of the testa, signals from fungi or germination in darkness (Rasmussen, 1995).

3.4 Life history and phenology

The life history of early underground stages of young seedlings is largely unknown, and the stages observed *in vitro* could differ from those in nature (Rasmussen, 1995).

The protocorm grows monopodially into a mycorhizome, which later develops into other underground structures such as rhizomes or tubers. The duration of the underground phase in nature varies according to several factors such as germination depth in soil, soil porosity, humus content, climatic factors, and individual genetic variance (Rasmussen, 1995).

The protocorm stage could be defined as the stage from germination until the seedling has developed a shoot tip but no roots, and the mycorhizome stage being initiated when first roots develop. In *Cypripedium*, however, the roots develop before the first shoot emerge (Figure 2). The mycorhizome is the earliest and most heavily infected part of the rhizome, and as the rhizome develops, the mycotrophic function is transferred from the rhizome to the roots. *Cypripedium* spp. produces a rhizome system (Figure 2), but other genera could form cormous rhizomes or roots modified into tubers (Rasmussen, 1995).

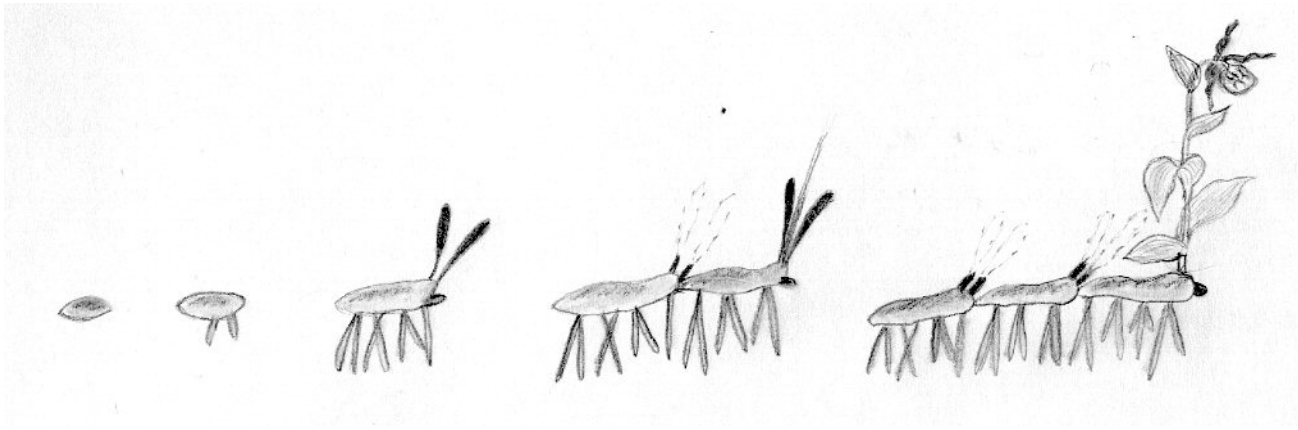

Figure 2. Development of underground structures in *Cypripedium* spp. From left to right; **1.** Protocorm; **2.** Protocorm with first roots; **3.** Protocorm developed into mycorhizome with first aerial shoots; **4.** Creeping rhizome with long, slender segments each ended by a shoot and buds from which growth continues; **5.** Rhizome continues as a sympodium with several annual growths, shoots and buds. The plant have reached blooming size. Note that the flowering shoot is not in the same scale as the underground parts. With inspiration from Rasmussen (1995). © Rännbäck, 2006

Transition from the protocorm stage is determined by seedling size and the duration of the cold period of the first winter after germination. The combination of the two factors determines when the dormancy in the leaf bud is broken. Some individuals may require more than one winter (Rasmussen, 1995). Seedlings of summergreen species in *in vitro* cultures require a cold period as well, and neither transfer to a fresh medium nor a long photoperiod suffices to break bud dormancy (Borris, 1969). However, in some summergreen North American swamp species and wintergreen Mediterranean species, some internal rhythm exists,

which allows bud break to occur without external stimuli *in vitro* under constant conditions (Myers & Ascher, 1982; Mitchell, 1988), but shoot development could be stimulated by a decrease in temperature to 12-14°C (Stephan, 1988; Rasmussen, 1995).

The roots of terrestrial orchids are generally thick, fleshy, brittle and usually unbranched (Brundrett & Kendrick, 1988; Rasmussen, 1995). The first roots develop exogenous close to the top of the protocorm, but later roots develop endogenously from uninfected tissues. The later roots will thus be colonized by secondary infection from the soil rather than that the original fungi are passed on (Hadley, 1982). The early mycotrophic roots develop a large proportion of phloem compared to xylem. The phloem is responsible for the translocation of organic and inorganic material to other parts of the plant. The material transported through the phloem of the mycotrophic roots are thus of fungal origin (Fuchs & Ziegenspeck, 1925; Rasmussen, 1995).

The first green leaflets appeared often do not have a functional photosynthesis, and mycotrophy is vital at the early seedling stage (Rasmussen, 1995).

4. In vitro asymbiotic germination and development of temperate terrestrial orchids

4.1 Breaking seed dormancy in vitro and sterilization

Germination is impeded or prevented by illumination in several orchid species and thus darkness is often required for germination *in vitro* (Fast 1978; Ballard 1987; Rasmussen *et al.*, 1990; Rasmussen & Rasmussen, 1991).

Scarification and sterilization *in vitro* by using hypochlorites, is used to break dormancy by rupturing the water-repellent testa in orchid seeds. Calcium hypochlorite ($Ca(OCl)_2$) is usually preferred, since it often yields better germination results after sterilization than sodium hypochlorite (NaOCl) (Rasmussen, 1995). Hypochlorites are strongly alkaline in solution and oxidizing agents. Other oxidants, such as alcohols, do not promote germination. However, other strong bases, such as sodium hydroxide (NaOH) (Eiberg, 1970), promote germination, suggesting that it is the alkalinity of the sterilant that improves germination (Rasmussen, 1995).

NaOCl is used in concentrations ranging from 0.2% to 5% (Purves & Hadley, 1976; Lucke, 1978b; Frosch, 1982), and $Ca(OCl)_2$ in approximately the same ranges (Stoutamire, 1963; van Waes, 1984; Malmgren, 1988). Solutions of hypochlorites are volatile, and the sterilizing effect may be reduced if the solution is allowed to stand for a long time. The effect of the solution (the alkalinity) will also decrease if a large number of seeds are treated in a

small volume of sterilant (Harvais, 1980; Arditti, 1982). Germination usually increases with increasing duration of sterilization. (Lindén, 1980; Rasmussen, 1992).

Addition of a detergent to the sterilizing solution will improve the contact between water and lipoid surface (seed coat), since it contains both hydrophilous and lipophilous groups. Tween 80 has been widely used with good results in sterilizing solution for orchid seeds (Rasmussen, 1995). The scarification of *Cypripedium calceolus* seeds is increased by pretreating the seeds in 0.5-2% sulphuric acid (H_2SO_4) before sterilizing in hypochlorite solution (Malmgren, 1993). Lightly sterilized seeds could be treated in a solution of testa degrading enzymes for up to two weeks before sowing, which has been successful in some *Cypripedium* sp (Lindén, 1992).

Liquid media could improve germination in some species, but the medium should then not be stirred (Curtis, 1936; Miyoshi & Mii, 1987; Rasmussen, 1995).

The best germination percentage for many species is usually achieved between 22 and 25°C (Eiberg, 1970), but some germinate best below 20°C, such as *Cypripedium* spp. (Stoutamire, 1974). Species that are difficult to germinate may require a cold stratification to break dormancy (Rasmussen, 1995). The physiological effect of cold stratification is either a decrease in the level of endogenous abscisic acid or an increase of content of endogenous cytokinin, both of which can stimulate germination (van Staden *et al*., 1972). Application of exogenous cytokinin could wholly or partially replace cold stratification as a germination stimulatory procedure (Webb & Wareing, 1972).

4.2 Culture of immature seeds

Immature seeds have been found to germinate better *in vitro* than ripe seeds. Embryo culture has become important for a number of horticultural important temperate terrestrial species (Fast, 1982; Malmgren, 1989; Rasmussen, 1995).

If orchid seeds are excised before the water-repellent testa has fully developed, imbibition will be rapid (Rasmussen, 1995). If the embryo cells are still hydrated and mitotically active, nutrient reserves will be mobilized (Kermode, 1990).

4.3 Media for germination and proliferation

Orchids of temperate terrestrial origin have until recently been quite troublesome to propagate *in vitro* (Arditti & Ernst, 1992; Rasmussen, 1995). The problems have mainly been failed germination, low germination rate and high death rate among plantlets (Malmgren, 1988). During the 1980 several breakthroughs were achieved mainly by amateur orchid hobbyists

(Fast, 1978; Lindén, 1980; Frosch 1982; Malmgren, 1988; Rasmussen, 1995). Many of their methods have been further developed and refined.

Many medium recipes for temperate terrestrial orchids have been developed through modifications of salt compositions for tropical orchids. The mineral salt concentration has then been reduced and organic compounds increased. Additions of complex organic compounds containing vitamins, growth regulators and amino acids are suggested to some extent mimic the conditions provided by the fungus (Rasmussen, 1995).

The media developed are convenient for horticultural plant production purposes since they are composed to promote both germination and subsequent seedling development (Rasmussen, 1995). A list of important nutrient compositions, and their recipes, are given by Rasmussen (1995).

The demand for mineral nutrients, sugars, etc. during germination and seedling growth varies between genera and species, and sometimes need careful adjustments (Malmgren, 1988; 1989; Rasmussen, 1995).

The problems associated with asymbiotic seed propagation are mainly the degree of maturity of the seeds, and the choice of growing media (Malmgren, 1988). *Dactylorhiza* spp. have been found to be easily propagated, while *Orchis* spp. are more troublesome (Malmgren, 1989).

4.3.1 Carbohydrates

For germination *in vitro*, some terrestrial orchid species can germinate on pure water agar (Vermeulen, 1947; Rasmussen, 1995), but most species require soluble sugars. In other species the need for exogenous sugars arises after germination (Rasmussen, 1995, table 4.3). Soluble sugars in asymbiotic media are usually added in concentrations between 10 and 20 g l^{-1} (van Waes, 1984; Malmgren,1989; Rasmussen, 1995) in the form of glucose, fructose or sucrose (Knudson, 1943; Rasmussen, 1995).

For media of symbiotic germination, non-soluble carbohydrates, such as starch or cellulose, are used, which are metabolized by the fungus (Rasmussen, 1995). The fungal sugar trehalose is also suitable in asymbiotic cultures (Smith, 1973). When it is necessary to distinguish between nutritive and osmotic effects of sugars, mannitol can be used since most species do not utilize it (Smith, 1973; van Waes, 1984; Rasmussen, 1995).

4.3.2 Ion concentration and medium type

Traditional tissue culture media have been found to be too concentrated for temperate orchids and should be diluted 2-10 times (Harvais, 1974; Fast 1980; van Waes, 1984; Rasmussen,

1995). Fast (1978) recommended the use of growth media with less than 0.25 g l^{-1} of inorganic salts. The actual germination might benefit from low osmolarity and the complete absence of mineral salts (Rasmussen, 1995).

Mechanical disturbance, e.g. from a shaker, will inhibit germination. Liquid media with agitation are thus disadvantageous, but low agar content is a compromise which will both give a high water potential and ensures stability (Lindquist, 1960; Galunder, 1984).

4.3.3 Nitrogen compounds

Several macro- and micronutrients affect the growth and development of orchids, but the largest single effect is seen with nitrogen (Rasmussen, 1995). Orchid soils are naturally poor in readily absorbable nitrogen, but the humus content of the soil can be as high as 30% (Möller, 1985), which gives a high concentration of organic nitrogen accessible to fungi.

Nitrogen occurs in the oxidized form as nitrate (NO_3^-), or in the reduced form as ammonium (NH_4^+), bound in inorganic salts or in organic compounds (Rasmussen, 1995). High concentrations of inorganic nitrogen affect the germination negatively (van Waes, 1984; Malmgren, 1989; Rasmussen, 1995), especially NO_3^- (Dijk, 1988). Many species show a preference for nitrogen in reduced form as NH_4^+ (Ichihashi & Yamashita, 1977).

Asymbiotic germination *in vitro* often yields a better result with organic nitrogen, where the nitrogen is less readily absorbed (Fast, 1980; van Waes, 1984; Malmgren, 1989; Rasmussen, 1995). Organic nitrogen could be applied by yeast extracts, peptone, plant juices or pure amino acids, such as glutamine (Rasmussen, 1995).

4.3.4 Acidity

Many media are acidic, but neutral or slightly alkaline pH might better correspond to natural habitats for some terrestrial species (Rasmussen, 1995). Fast (1980) considered pH values of 4.8-5.5 to be most suitable for protocorm development in *in vitro* cultures. For calciphilous, species the pH is advisably adjusted to 7.0 (Rasmussen, 1995).

After medium preparation and pH adjustment, the pH could be changed by autoclaving and during the culture. The absorption of ions from the medium affects the pH (Vacin & Went, 1949; Rasmussen, 1995). When the tissue absorbs cations such as NH_4^+ it gives off H^+, which lowers the pH. Absorption of anions such as NO_3^-instead raises the pH of the medium. Thus the initial proportion of ions in the medium and the capacity of the tissue to utilize them affect the direction and extent of changes in the pH (Rasmussen, 1995). Changes in pH could be reduced by buffering with e.g., mixture of basic and acidic phosphates (Malmgren 1989; Vöth,

1976; Anderson, 1990; Rasmussen, 1995). Addition of activated charcoal could stabilize the pH to some extent (Fast, 1980; Rasmussen, 1995).

4.3.5 Plant Growth Regulators

The addition of cytokinins in the form of benzyladenine (BA) or kinetin has been found to improve germination in troublesome species, for instance *Cypripedium calceolus* and *C. reginae* (Harvais, 1982; Malmgren 1989; Rasmussen, 1995).

Some species in the genera *Cypripedium* could need a cold stratification, and the cytokinins would then induce responses resembling a cold treatment (Rasmussen, 1995). Cytokinins could counteract the effects of the germination inhibitor abscisic acid (Hartmann & Kester, 1983), and it could also accelerate the transition from the protocorm stage to the leafy stage in some species (Ueda & Torikata, 1970; Rasmussen, 1995).

Addition of gibberellins (GAs) have not yielded any positive results in germination (Rasmussen, 1995), but GA_4 applied at 5 mg l^{-1} can promote the differentiation of shoot and roots in *Cypripedium calceolus* (Borris, 1969, Malmgren, 1993). Auxins do not either have any pronounced effect on germination in most terrestrial temperate species (van Waes, 1984; Rasmussen, 1995).

4.3.6 Other additives

The vitamin B complex has been shown to have an important effect in orchid seedling development, particularly thiamine, nicotinic acid and biotine (Fast, 1978; 1983; Rasmussen, 1995). Yeast extract and peptone contain vitamins from the B complex, particularly nicotinic acid. Yeast also contains amino acids and phosphorus (Arditti, 1982). Malmgren (1988) uses a complex vitamin B additive (Solu-Vit) in *Dactylorhiza* culture with growth promoting results.

Organic liquids, such as fruit juices, are often added to orchid media with good results. Unfortunately, such undefined organic substances have a variable composition which makes it impossible to exactly reproduce a medium in which they are included (Rasmussen, 1995). They are often acidic and contain soluble sugars, amino acids, vitamins and plant growth regulators. Pineapple juice, coconut milk and potato extract are frequently used (Lucke, 1977; Ramin, 1983; Rasmussen, 1995).

Pineapple juice has proven particularly suitable for propagating European orchids (Malmgren, 1989). It is strongly reducing and will thus lower the production of phenolic exudates in the cultures (Rasmussen, 1995). The pine apple juice has also been proven to

promote root differentiation and growth in several temperate terrestrial species (Malmgren, 1988; 1993).

Coconut milk added to orchid cultures is reported to stimulate the development of tubers and roots (McIntyre *et al*., 1974; Rasmussen, 1995).

Accumulation of phenolics could also be prevented by addition of activated charcoal (van Waes, 1987; Rasmussen, 1995) or anti-oxidants (van Waes, 1984; Rasmussen, 1995).

4.4 Abiotic factors affecting development

4.4.1 Temperature

The optimum temperature for seedling growth *in vitro* is usually around 20-25°C (Rasmussen, 1995), but some species develop better at cooler temperatures. Periods of lower temperatures resembling natural conditions should occur to induce dormancy periods (Malmgren,1989; 1993). Thermoperiodicity with chilling temperatures should also be utilized *in vitro* for breaking bud dormancy (Riley, 1983; Malmgren, 1988; Anderson 1990; Rasmussen, 1995). Most investigators suggest vernalization for 3 months at 5°C. For *Dactylorhiza majalis* bud dormancy can be broken in 8 weeks if the seedlings are sufficiently large (longer than 5-8 mm) when the cold treatment is begun (Rasmussen, 1995).

In seedlings of *Cypripedium*, vernalization could lead to development of a new segment of the rhizome instead of leaf development, which suggests that *Cypripedium* can remain underground for more than one season. If the seedling has reached a sufficient size a single cool period (10-12 weeks at 4-5°C) will induce shoots (Stewart & Mitchell, 1991; Rasmussen, 1995).

4.4.2 Irradiation

Etiolation in orchid seedling culture has not been reported as a problem, since the heterotrophic seedling has no requirement for light at early growth stages (Rasmussen, 1995). Darkness stimulates the development of the protocorm and mycorhizome, but inhibits shoot and root formation, thus prolonging the non-photosynthetic stage (Fast, 1982; Rasmussen, 1995).

5. The genus *Cypripedium*

5.1 Botany and distribution of Cypripedium *spp.*

5.1.1 *Cypripedium* taxonomy

The genus *Cypripedium* belongs to, although debated, the subfamily Cypripedioideae within the family Orchidaceae (Cribb, 1997). According to Cribb (1997), the genus is further divided into 11 sections, 2 subsections, 46 species and 4 varieties (Table 1). Of these 50 taxa, 25 have yet been explored in *Cypripedium* breeding (Table 2). Several botanists have suggested further divisions of some variable species complexes into subspecies, varieties and forma (Cribb, 1997).

5.1.2 *Cypripedium* morphology

Most species have a short rhizome, consisting of a chain of annual growth buried shallow in the soil. New growth is added at the anterior end, and dying at the posterior end. The leaf-bearing stem emerges from the terminal bud of the rhizome (Figure 2). The stem, leaf and flower morphologies vary within the genus (Cribb, 1997).

The inflorescences are racemose or almost spicate, and the bracts are leaf-like but smaller than the leaves. Flower number varies from one to twelve (*C. californicum*) according to species, but most common is one to three per inflorescence. The flowers are resupinate with a slipper-shaped lip. The outer floral whorl consists of three sepals, of which the two lower are fused. The inner floral whorl consists of three petals of which one is shaped as a deeply pouched lip. The column in the middle comprises a terminal staminode, two stalked lateral anthers and a stalked tripartite stigma (Figure 3). The ovary is trilocular, which is either almost sessile or borne on a short pedicel. The seeds have the structure of typical orchid seeds (Cribb, 1997).

5.1.3 Biogeography

The genus *Cypripedium* is terrestrial, and mainly temperate and circumboreal distributed throughout the Northern Hemisphere, as far south as the Himalayas in Asia and Guatemala and Honduras in America, and as far north as Alaska and Siberia. The centre of diversity is China, where 30 of the species have been found. North America is the second species richest area (Cribb, 1997).

Fig. 3. A *Cypripedium* flower with a leaf-like bract and a true leaf. The outer floral whorl consists of three sepals, of which the two lower are fused. The inner floral whorl consists of three petals of which one is shaped as a deeply pouched lip. The column in the middle consists of staminode, anthers and stigma. © Rännbäck, 2006

Some species are common in the distribution, such as, *C. calceolus* and *C. parviflorum* var. *parviflorum*, but several species have a very limited distribution (Cribb, 1997). Distribution maps are given by Cribb (1997).

The *Cypripedium* spp. are generally colony forming, and the species often have rather specific habitat requirements. Their habitats range from the sea level into high altitude mountains, and comprise meadows, coniferous forests, mixed deciduous woodlands, bogs, fens, grasslands and prairies. They thrive in light to deep shade, in dry and moist conditions, and in both acidic and calcareous substrates (Cribb, 1997).

Table 1. *Cypripedium* taxonomy based on Cribb (1997) table 5 and Perner (1999). Frequency in parentage (F) is counted as the number of times a species has been used in artificial hybridizations to produce cultivars. For example, in the cultivar 'Gisela', *parviflorum* and *macranthos* are included which each will have a frequency of 1 in the species list. The frequencies will then add up to a total frequency number for that species when used in cultivars. Species within cultivars included in artificial hybrids will not be counted. For example, the artificial hybrid 'Lisbeth' including the cultivar 'Gisela' and the species *calceolus*, will have a species frequency of 1 compared to the normal 2. The natural hybrid x *ventricosum* used in cultivars is also not counted.

Section	Subsection	Species	Variety	F
Subtropica S.C. Chen & K.Y. Lang		*C. subtropicum* S.C. Chen & K.Y. Lang *C. wardii* Rolfe		
Irapeana Cribb		*C. irapeanum* La Llave & Lex. *C. molle* Lindl. *C. dickinsonianum* Hágsater *C. californicum* A. Gray		
Obtusipetala (Pfitzer) Cribb		*C. flavum* P.F. Hunt & Summerh. *C. reginae* Walt. *C. passerinum* Richardson		2 8
Cypripedium	***Cypripedium***	*C. calceolus* L. *C. henryi* Rolfe *C. segawai* Masam. *C. shanxiense* S.C. Chen *C. cordigerum* D. Don *C. fasciolatum* Franch. *C. farreri* W.W. Sm. *C. parviflorum* Salisb. *C. kentuckiense* C.F. Reed *C. montanum* Douglas ex Lindl. *C. candidum* H.L. Mühl. ex Willd.	var. *parviflorum* Sheviak var. *pubescens* (Willd.) O.W. Knight	16 9 3 2 5 12 3 16 15 15 3 6
Cypripedium	***Macrantha*** (Kränzl.) Cribb	*C. macranthos* Sw. *C. yunnanense* Franch. *C. ludlowii* Cribb *C. tibeticum* King ex Rolfe *C. smithii* Schltr. *C. franchetii* E.H. Wilson *C. himalaicum* Rolfe *C. froschii* Perner		25 1 10 2 3 5
Enantiopedilum Pfitzer		*C. fasciculatum* Kellogg ex S. Watson		
Arietinum C. Morren		*C. arietinum* R. Br. *C. plectrochilum* Franch.		 1
Flabellinervia (Pfitzer) Hennessy ex Cribb		*C. japonicum* Thunb. *C. formosanum* Hayata		 3
Acaulia (Lindl.) C. Morren		*C. acaule* Aiton		2
Bifolia (Lindl.) C. Morren		*C. guttatum* Sw. *C. yatabeanum* Makino		 2
Retinervia (Pfitzer) S.C. Chen		*C. palangshanense* T. Tang & F.T. Wang *C. elegans* Rchb. f. *C. debile* Rchb. f.		
Trigonopedia Franch.		*C. bardolphianum* W.W. Sm. & Farrer *C. micranthum* Franch. *C. margaritaceum* Franch. *C. lichiangense* S.C. Chen & Cribb *C. forrestii* Cribb *C. fargesii* Cribb *C. wumengense* S.C. Chen	var. *bardolphianum* var. *zhongdianense* S.C. Chen	 2

5.2 Breeding of Cypripedium *spp.*

The initial achievements on orchid breeding have been made by the German Werner Frosch and the American Carson E. Whitlow, who have 39 and 11 hybrids registered, respectively (Table 2). During recent years, however, several new breeders have entered the *Cypripedium* industry. Large actors are Pinkepank with 12 hybrids, Corkhill 6, Keisling 5 and Robinson 5 (Table 2; Whitlow, 2006). The Swede Svante Malmgren only has 2 hybrids registered (Table 2), but the 'Ulla Silkens' is a widely spread success. Other successful, beautiful and easily cultivated cultivars, which are most often seen and offered by nurseries, are 'Gisela' and 'Philip' (Malmgren, 2005).

There now exists a range of nurseries specialized in *Cypripedium* spp. worldwide. Some of them have also participated in the breeding process, e.g. Raising Rarities (owned by Robinson), and have during the years successfully delivered new cultivars to the market.

5.2.1 Intrageneric relationship within *Cypripedium*

Closely related species can be relatively easy to produce vigorous hybrids. *Cypripedium* species within the same subsection have frequently been used in crossing programs (Whitlow, 2006; Table 1, Table 2), and many hybrids have been registered (Table 2), as well as many natural hybrids recorded (Cribb, 1997; Table 3).

Genome variability and phylogenetic relationship, an indication for intrageneric hybridization within the *Cypripedium*, have been investigated at molecular level by Cox *et al*. (1997), Case (1994), Cox *et al*. (1998), and are also extensively discussed by Cribb (1997).

C. irapeanum from Mexico and Guatemala has been shown to form a sister clade to all other northern hemisphere temperate *Cypripedium* spp. in cladistic studies (Cox *et al*, 1997). *C. arienatum* has also been under debate, but the genetic divergence from, e.g., *C. calceolus*, where not larger than that between *C. aciculae* and *C. reginae* (Case, 1994)

Cypripedium has showed to have low genetic intrageneric variability (Case, 1994), suggesting that all *Cypripedium* spp. could in fact be crossed. The *C. calceolus* complex, on the contrary, shows a greater genetic variation than other species (Case, 1994; Cribb, 1997).

Several other ecological and evolutionary studies conducted with *Cypripedium* spp. could be viewed for breeding purposes. Flower fragrances of *C. calceolus* and *C. parviflorum* var. *pubescens* have been studied by Bergström *et al*. (1992), and Barkman *et al*. (1997) analyzed 9 taxa. Bergström *et al*. (1992) found that the Eurasian taxon of *C. calceolus* had a distinctive fragrance spectrum from its North American relative of *C. parviflorum*. Sheviak (1996a; 1996b) gives some notes on this horticultural trait.

Table 2. Hybrids registered by the Royal Horticultural Society (Perner, 1997; Whitlow, 2006). Taxonomy is adjusted according to Cribb (1997). In the parentage, the pod parent is stated first then the pollen parent. 1= Hybridization within section, 2 = Hybridization within section *Cypripedium*, 3 = Hybridization within subsection *Cypripedium*. For convenience C. *parviflorum* var. *parviflorum* is noted as parviflorum and C. *parviflorum* var. *pubescens* as pubescens. Species, subspecies, varieties, forma, hybrids, etc not noted by Cribb (1997) are treated as synonyms according to an index in Cribb (1997). For horticultural purposes colour forma is however noted. Some of the forma ranks has no taxonomic validity. *C. macranthos* f. *speciosum* has pink flowers; f. *rebunense* has pale yellow flowers; f. *hotei-atsmorianum* has large flowers with white ribbed lips; f. *albiflorum* has white flowers; f. *taiwanianum* has small flowers. Furthermore, *C. calceolus* f. *flavum* has flowers with a yellow perianth instead of the normal chocolate. *C. parviflorum* var. *pubescens* f. *planipetalum* has yellow-green sepals and petals with the petals not being twisted, compared to the normal form (Cribb, 1997). A white form of x *ventricosum* is by Whitlow (2006) noted as ventricosum {manschuricum} nothovar virescens ['Alba'], but for convenience noted as x *ventricosum* f. *albiflorum*. For details see Whitlow (2006) and Cribb (1997), or contact the RHS.

Cultivar name	Pod parent	Pollen parent	1	2	3	Registrant	Year
Genesis	reginae	pubescens				Whitlow	1987
Promises	formosanum	acaule				Whitlow	1988
Karl Heinz	calceolus	cordigerum	yes	yes	yes	Frosch	1990
Ingrid	parviflorum	cordigerum	yes	yes	yes	Frosch	1990
Rascal	kentuckiense	parviflorum	yes	yes	yes	Whitlow	1990
Carolin	parviflorum	macranthos	yes	yes		Frosch	1991
Maria	parviflorum	macranthos	yes	yes		Frosch	1991
Otto	calceolus	pubescens	yes	yes	yes	Frosch	1991
Gisela	parviflorum	macranthos	yes	yes		Frosch	1991
Hank Small	parviflorum	henryi	yes	yes	yes	Whitlow	1991
Carson	parviflorum	formosanum				Frosch	1992
Fantasy	reginae	lichiangense				Whitlow	1992
Kathleen Anne Green	kentuckiense	henryi	yes	yes	yes	Whitlow	1992
Emil	calceolus	parviflorum	yes	yes	yes	Frosch	1993
Chauncey	parviflorum	segawai	yes	yes	yes	Whitlow	1993
Gidget	candidum	henryi	yes	yes	yes	Whitlow	1993
Werner	candidum	yatabeanum				Whitlow	1993
Ulli	pubescens	cordigerum	yes	yes	yes	Frosch	1994
Princess	reginae	lichiangense				Whitlow	1995
Favillianum	pubescens	candidum	yes	yes	yes	Whitlow	1994
Memoria Gerd Kohls	calceolus	henryi	yes	yes	yes	Lowland-Biotech	1995
Philipp	macranthos	kentuckiense	yes	yes		Frosch	1996
Aki	pubescens	macranthos	yes	yes		Frosch	1996
Ulla Silkens	flavum	reginae	yes			Malmgren	1996
Hedi	Ingrid	macranthos	yes	yes		Frosch	1997
Heike	formosanum	segawai				Frosch	1998
Michael	macranthos	henryi	yes	yes		Frosch	1998
Sebastian	parviflorum	montanum	yes	yes	yes	Frosch	1998
Axel	parviflorum	tibeticum	yes	yes		Malmgren	1998
Lucy Pinkepank	kentuckiense	tibeticum	yes	yes		Pinkepank	1998
Patrick Pinkepank	pubescens f. taiwanianum	tibeticum	yes	yes		Pinkepank	1998
Tanya Pinkepank	macranthos f. rebunese	calceolus f. flavum	yes	yes		Pinkepank	1998
Robin Lee	farreri	kentuckiense	yes	yes	yes	Keisling	2000
Bernd	macranthos	segawai	yes	yes		Frosch	2001
Hideki Okuyama	fasciolatum	(x ventricosum)	yes	yes		Frosch	2001
Francis	kentuckiense	macranthos f. speciosum	yes	yes		Robinson	2001
Hilda	kentuckiense	(x ventricosum)	yes	yes		Robinson	2001
Annette	macranthos	candidum	yes	yes		Frosch	2002
Sabine	fasciolatum	macranthos	yes	yes		Frosch	2002
Pixi	calceolus	tibeticum	yes	yes		Corkhill	2003

Achim	pubescens	froschii	yes	yes		Frosch	2003
Dietrich	calceolus	kentuckiense	yes	yes	yes	Frosch	2003
Gabriela	fasciolatum	kentuckiense	yes	yes	yes	Frosch	2003
Inge	parviflorum	fasciolatum	yes	yes	yes	Frosch	2003
Erika	calceolus	candidum	yes	yes	yes	Frosch	2003
Ursel	fasciolatum	henryi	yes	yes	yes	Frosch	2003
Kristi Lyn	pubescens	henryi	yes	yes	yes	Keisling	2003
Tower Hill	pubescens	macranthos f. speciosum	yes	yes		Keisling	2003
Werner Frosch	henryi	macranthos f. hotei-atsmorianum	yes	yes		Manthey	2003
Johanna	kentuckiense	tibeticum	yes	yes		Robinson	2003
Mike	kentuckiense	(x ventricosum f. albiflorum)	yes	yes		Robinson	2003
Lois	kentuckiense	macranthos f. taiwanianum	yes	yes		Robinson	2003
Lisbeth	Gisela	calceolus	yes	yes		Döpper	2004
Bill	pubescens	tibeticum	yes	yes		Frosch	2004
Hans Erni	franchetii	calceolus	yes	yes		Frosch	2004
Sunny	fasciolatum	calceolus	yes	yes	yes	Frosch	2004
Uta	fasciolatum	froschii	yes	yes		Frosch	2004
Julie Ann	kentuckiense	smithii	yes	yes		Keisling	2004
Prof. Karl Robatsch	reginae	acaule				Raschun	2004
Boots grex Christopher	calceolus	montanum	yes	yes	yes	Corkhill	2005
Joseph Henry	parviflorum	smithii	yes	yes		Corkhill	2005
Lizzy Ann	parviflorum	froschii	yes	yes		Corkhill	2005
Victoria	pubescens	fasciolatum	yes	yes	yes	Corkhill	2005
Hildegard	candidum	cordigerum	yes	yes	yes	Frosch	2005
Gerhard	franchetii	farreri	yes	yes		Frosch	2005
Holger	henryi	macranthos f. speciosum	yes	yes		Frosch	2005
Katrin	Philipp	pubescens	yes	yes		Frosch	2005
Pluto	fasciolatum	franchetii	yes	yes		Frosch	2005
Tilman	tibeticum	fasciolatum	yes	yes		Frosch	2005
Wladiwo	(x ventricosum)	calceolus f. flavum	yes	yes		Frosch	2005
Eurasia	macranthos	tibeticum	yes	yes		Keiichi Nakamura	2005
Carol Ilene	pubescens	macranthos f. hotei-atsmorianum	yes	yes		Keisling	2005
Yezo	macranthos	yatabeanum				Nakamura	2005
Barbara Imfeld-Pinkepank	macranthos f. albiflorum	farreri	yes	yes		Pinkepank	2005
Boots	calceolus f. flavum	montanum	yes	yes	yes	Pinkepank	2005
Hans Arpagaus	froschii	macranthos	yes	yes		Pinkepank	2005
Henning Pinkepank	shanxiense	macranthos	yes	yes		Pinkepank	2005
Karel Polivka	(x ventricosum)	macranthos	yes	yes		Pinkepank	2005
Lothar Pinkepank	pubescens	kentuckiense	yes	yes	yes	Pinkepank	2005
Oma Alli	Tanya Pinkepank	(x ventricosum)	yes	yes		Pinkepank	2005
Siggi	froschii	calceolus	yes	yes		Pinkepank	2005
Til Eulenspiegel	shanxiense	tibeticum	yes	yes		Pinkepank	2005
Roland	pubescens	tibeticum	yes	yes		Raschun	2005
Camiel	parviflorum	plectrochilum				von Rad	2005
Vicky's Delight	Ulla Silkens	flavum	yes			Corkhill	2006
Irene	kentuckiense	reginae				Frosch	2006
Jimmy	fasciolatum	macranthos f. rebunense	yes	yes		Frosch	2006
John	parviflorum	yunnanense	yes	yes		Frosch	2006
Birgit	macranthos	cordigerum	yes	yes		In Vitro	2006
Günter	calceolus	reginae				In Vitro	2006
Wouter Peeters	reginae	fasciolatum				Peeters	2006
FREQUENCY (x of 91)			**78**	**77**	**26**		

Table 3. Natural hybrids of *Cypripedium* (Cribb, 1997).

Name	Parentage	Hybridization within sect./subsec.	Within subsect. Cypr.
C. **x** *alaskanum* P.M. Br.	*C. guttatum* **x** *C. yatabeanum*	yes/-	no
C. **x** *andrewsii* A.M. Fuller	*C. candidum* **x** *C. parviflorum* var. *parviflorum*	yes/yes	yes
C. **x** *colombianum* C.J. Sheviak	*C. montanum* **x** *C. parviflorum* var. *pubescens*	yes/yes	yes
C. **x** *ventricosum* Sw.	*C. calceolus* **x** *C. macranthos*	yes/no	no

Hybridization and introgression have been studied by Klier *et al*. (1991) in *C. candidum* and *C. parviflorum* var. *pubescens* based on morphological characters and allozyme loci. The species have shown to exchange genetic information in some habitats, yielding adaptable ecotypes.

The chromosome number for most *Cypripedium* spp. has been found to be 2n = 20 (Tanaka & Kamemoto, 1974, 1984; Cribb, 1997; Cox *et al*, 1998), although triploid clones are occasionally found (Karasawa & Aoyama, 1986; Cribb, 1997).

5.2.2 Breeding programmes and horticultural traits

Breeding of *Cypripedium* cultivars has been inspired by naturally occurring hybrids (Table 3) and intraspecific variation, which have been exploited in crossings.

It is important to consider the provenance and thus to breed plants from different regions to develop hardy cultivars that can grow successfully in a variety of conditions, for example to incorporate C. *parviflorum* var. *pubescens* from both dry and wet areas into a breeding programme (Klier *et al*., 1991; Robinson, 2002).

The desirable horticultural traits, to be considered when selecting clones for further crossings, include large flowers, a high number of flowers per inflorescence, long flowering, delicate colouring (spotted lip, coloured sepals and petals compared to the lip; concolorous or bicoloured) and lovely fragrance, high winter hardiness, tolerance to extreme growing site requirements and better resistance to diseases and pests.

Cypripedium macranthos plants have a rose-like fragrance (Sheviak, 1996a). The scents of *C. calceolus* plants vary within the complex, they are either intensely sweet or fruity, and in some cases they resemble the smell of sweet peaches (Sheviak, 1996a; 1996b). The *C. parviflorum* species have a varied smell according to variety. The var. *parviflorum* and the var. *pubescens* have the rose-like scent like *C. macranthos* (Sheviak, 1996b). Sheviak (1994) also recognized a var. *makasin*, which has an intensively sweet scent similar to that of *C. calceolus* (Sheviak, 1996b). The flowers of *C. shanxiense* are however faintly, but unpleasantly scented (Sheviak, 1996a). The fragrance of *C. californicum* is reported to resemble that of *Convallaria majalis* (Cribb, 1997).

In highly variable species, such as, *C. calceolus* and *C. macranthos*, flower shape and colour could vary a lot. *C. macranthos* could vary from pink to deep plum over to pure white, with or without spots or stripes (Sheviak, 1996b; Cribb 1997). Some of the variations could be traced to introgression from *C. calceolus* occupying the same habitats, which is suggested to have resulted in the *C. parviflorum* (Sheviak, 1996b). These variations could obviously be utilized in breeding and for improving *Cyperipedium* cultivars.

5.2.3. Rules of trading and artificial propagation of orchids

The trade of endangered species is forbidden and regulated in the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). All orchids, including *Cypripedium* spp., are listed by CITES. At present, a large part of the countries in the world have signed CITES (Anonymous, 2006a).

For artificial propagation and culture of *Cypripedium* spp. a permission is needed which is an exception from the main CITES rules. In Sweden, permissions and certificates are granted by the Swedish Board of Agriculture (Anonymous, 2006b).

5.2.4 Registration of cultivars

Registration of new *Cypripedium* cultivars is done by the Royal Horticultural Society in United Kingdom, and is regularly published in Sanders' List of Orchid hybrid. A frequently updated list is also provided by Whitlow (2006).

5.3 Propagation of Cypripedium *spp.*

5.3.1 Asymbiotic seed propagation *in vitro*

Arditti & Ernst (1992) describes micropropagation from seeds of several terrestrial temperate genera, but gives only a few records on *Cypripedium* spp. In the recent years, the development however has proceeded.

The most common way by which *Cypripedium* spp. are propagated by specialized nurseries is *in vitro* sowing of seeds. (Weber, 1997; May and May, 2002)

Stratification treatments and medium compositions differ with species and researcher. It is difficult to give general recommendations. Rasmussen (1995) gives an overview of common medium recipes. Newer studies are often presented at conferences, in amateur papers and from specialized nurseries on the internet.

In sterilization and rupture of the testa of mature seeds, a lengthy treatment in a weak solution of hypochlorite is generally recommended by most researchers (Malmgren, 1989;

Rasmussen, 1995). However, sowing of immature seeds is often more successful (Malmgren, 1989).

Cold stratification has been tested in several species, often with unsuccessful results (Rasmussen, 1995). However, incubation and imbibition at higher temperatures followed by a cold treatment for more than 2 months at 5°C have been successful in *Cypripedium reginae*, resulting in about 90% germination (Stoutamire, 1974; Ballard, 1987; Rasmussen, 1995). In *C. acaule*, treatment for 3-5 months at 5°C and then transfer to 25°C gave a 70% germination rate (Coke, 1990). Coke (1990) obtained 50% germination in *C. calceolus* when incubating at 5°C for 3-5 months.

Modifications of culture media have provided good results for germination and subsequent growth of several *Cypripedium* spp.

From *C. calceolus*, Malmgren (1989, 1993) has found that the species benefit from culture on low osmotic media with a content of moderate levels of organic nitrogen (amino acids). In addition, growth is promoted by vitamins from the B-complex, cytokinins (kinetin) (Malmgren 1993) and unsweetened pineapple juice (Malmgren, 1989; 1993) and coconut milk (Anderson, 1990). From these studies the SM-spec media have been developed (Appendix). It would probably work well for several *Cypripedium* spp., since many of their requirements are fulfilled with the media. A modification was subsequently developed on which several *Cypripedium* spp. proliferate if 5 mg kinetin is added (Malmgren, 1993; Appendix). Later more modifications have been done, and Malmgren (pers com, 2006) nowadays uses the same simple base recipe for all temperate terrestrials, with modifications for different genera and species (Appendix).

Malmgren (1989) has also used the commercially available TGZ-N (from Manfred Meyer) (Appendix) with addition of mainly 2,5% unsweetened pineapple juice for several temperate terrestrial species. In the first developmental stages the plantlets are particularly sensitive to the medium composition (Malmgren, 1989), but could in later stages proliferate on a number of media.

The germination and proliferation of *Cypripedium* spp. have been enhanced with the modified SM-spec medium (Malmgren, 1993) since initial trials (Malmgren, 1989). Spontaneous death of plantlets could be decreased by lowering the culture temperature (Malmgren, 1989). The chemical requirements of the medium for *C. reginae* have been investigated by Harvais (1973; 1982). The species has responded well to additions of sugars, kinetin and amino acids, and germinates readily on a number of media (Rasmussen, 1995).

Weber (1997) has obtained successful growth results of *C. reginae* on SM-spec (Appendix) compared to two other media tested.

Pauw & Remphrey (1993) studied the optimum time of seed collection and the suitability of various media for *in vitro* germination for *C. candidum*, *C. reginae* and *C. parviflorum* var. *parviflorum*. *C. reginae* had the best overall germination. Seeds collected approximately 8 weeks after pollination germinated best. Germination was not significantly different between the media Harvais, Van Waes, Debergh and modified Norstog. Subsequent protocorm development was however superior on the modified Norstog.

A similar investigation was made by Wagner & Hansel (1994) where germination rates of immature *C. calceolus* seeds at different embryogenic developmental stages were measured. Seeds collected 40 days after pollination had the highest germination percentage. Germination was further enhanced by vacuum infiltration with a nutrient solution.

5.3.1.2 Culture of immature seeds of *Cypripedium* spp.

The most efficient way of improving the germination of *Cypripedium* spp. is probably the culture of excised immature seeds (Rasmussen, 1995). The time interval between pollination and suitable harvest period varies from species to species, and between growing sites, different years, etc (Malmgren, 1989).

According to several studies, optimum germination of *Cypripedium* spp. can be obtained when seeds are excised immature, before the testa is fully developed, between 42 and 60 days after pollination, corresponding to the interval of between 14 and 32 days after fertilization. It is assumed that fertilization occurs roughly 4 weeks after pollination in the genus. Maturation occurs about 85-110 days after pollination (Rasmussen, 1995, table 2.1; Cribb, 1997).

The optimum excision times, in days after pollination, for several *Cypripedium* spp. are (Rasmussen, 1995, table 2.1);

C. acaule 60 days (Withner, 1953),

C. calceolus 42 (Lucke, 1982) or 49-53 (Malmgren 1989), 40 days (Wagner & Hansel, 1994)

C. candidum 42 days (Pauw & Remphrey, 1993),

C. parviflorum* var. *parviflorum 56 days (Pauw & Remphrey, 1993),

C. parviflorum* var. *pubescens 49 (Light, 1989) or 60 days (Withner, 1959),

C. reginae 42 (Lucke, 1978a; Frosch 1985) or 56 (Pauw & Remphrey, 1993) or 60 days (Withner, 1959).

5.3.2. *In vitro* development of seedlings and subsequent *in vivo* planting

Malmgren (1989) gives some guidelines for culture of *C. calceolus*. The development is presumably the same in several *Cypripedium* spp.

After sowing, the culture should be kept in darkness until leaf primordia appear. Germination of *C. calceolus* usually occurs within 2-4 weeks (Malmgren, 1989), but for other species this time could be considerable longer. Transplanting of the protocorms to new media should be done every 2-4 months (Frosch, 1985; Malmgren 1989); otherwise growth could be restricted by e.g., nutrient depletion. Rhizome and roots start to develop at the earliest after 7 months, and thereafter small leaves. From this time the culture should be transferred to light, and the temperature held at 15-18°C. Several months later, dormancy treatment should be initiated, first at 8-10°C, and later at just above freezing. Root development *in vitro* of *Cypripedium calceolus* is stimulated by the decrease in temperature to 10°C, suggesting an active growth phase during the autumn (Malmgren, 1989).

The protocorms could successfully be vegetatively propagated by division during *in vitro* culture to form more plants. (Malmgren, 1989).

For successful planting in soil, root length should be sufficient, preferably over 1 cm (Malmgren, 1988). Root development of plantlets could be stimulated by a period of cool temperature (10°C) (Malmgren, 1989), exposure to light, reduction of the ion concentration in the medium (Rasmussen, 1995) or transfer to sterile compost (Mitchell, 1989; Rasmussen, 1995).

In late spring, plantlets of sufficient size and root differentiation could be planted in soil and slowly weaned to *ex vitro* conditions (Malmgren, 1989; 1993). When removed from *in vitro* conditions, the seedlings are very sensitive to desiccation, thus it is best if shoots are smaller than the roots (Rasmussen, 1995).

The soil used could be collected from natural orchid habitats, preferably from the humus layer of mixed forests (Malmgren, 1993; 1994). The plants are best kept in cool conditions indoors during the first year in soil (Malmgren, 2005). The first winter, the pots should be placed in a light, frost-free room for dormancy treatment at 0-3°C (Malmgren, 1988).

Acclimatization to outdoor conditions could be done in clay pots during the second year. The orchid plants should initially be acclimatized in shade, and should not be allowed to dry out. The plants should be protected from severe frosts and the burning spring sun (Malmgren, 1988; 2005).

After three years in pot culture, the plants are large enough to survive in the ground soil at a moist and shady site (Malmgren, 1994). Older plants of blooming size do not thrive in pot

culture (Malmgren, 2005). Many species and hybrids will bloom in 4-5 years (May & May, 2002; Malmgren, 2005).

5.3.3. Micropropagation

Various young explants of most tropical orchids have been found to form protocorm-like bodies *in vitro*. Some attempts have also been made in culturing explants from temperate terrestrials *in vitro*. Often, it is the danger of extinction rather than horticultural interest that has promoted the development of tissue culture propagation methods for temperate terrestrial orchids (Arditti & Ernst, 1992).

Shoot tips from tubers of *Orchis maculata* were successfully proliferated on media in a trial by Thomale (1957). Meristem culture of *Anacamptis pyramidalis* succeeded for Morel (1970, 1974). Protoplast isolation was done in *Barlia robertiana* (Pais *et al*., 1982, 1983), but no report on further proliferation was given in Arditti & Ernst (1992). Dormant shoots of *Dactylorchis fuchsia* were successfully cultured by Stokes (1974). Meristems of *Nigritella nigra* proliferated in trials made by Haas (1977a,1977b). Successful attempts have also been reported for *Ophrys* spp. (Arditti & Ernst, 1992).

So far, no publications have been made on micropropagation of tissues other than seeds and protocorms of *Cypripedium* spp. Root tips, rhizome tips or segments, divided protocorms and leaves (bases, tips) are some tissues which could be explored in the future.

5.3.4. *Ex vitro* vegetative propagation

Mycotrophic roots are nutritionally independent of the rest of the plant. Root fragments could then remain alive if detached, which should make vegetative propagation fairly easy in some species. Vegetative reproduction in nature could be common, since the root sprouts easily. Bud production on the roots has been observed in many temperate terrestrials, which would make propagation by division successful (Rasmussen, 1995). In some temperate terrestrial orchid species a root tip meristem can even transform directly into a shoot meristem (Rasmussen, 1986; Rasmussen, 1995).

Rhizome clumps of *Cypripedium* should be divided before the start of the annual root growth, which is immediately after flowering. Each division should have at least 3-4 scars representing not less than 3 years growth, and have 3 cm rhizome before the bud, but the more rootstock mass and buds the better. Bud proliferation could be promoted by making shallow cuts on the rhizomes during the growing season before the intended division. The cuts could be powdered with charcoal to dry it out. The divisions should be instantly transferred to new

compost to minimize the root disturbance (Cribb & Bailes, 1989; Rasmussen, 1995; Perner, 1997; Neptune, 1999).

5.4 Cultivation of Cypripedium *spp.*

The early and presumably unsuccessful cultivation of hardy *Cypripedium* spp. began with wild collected *C. calceolus* and *C. reginae* in the late nineteenth century. The great success of cultivation, however, arose in the 1980s when the first artificially produced *Cypripedium* hybrids were registered (Perner, 1997).

5.4.1. Requirements on growing site

For garden cultivation, the choice could be to either plant in pots or in a specially prepared bed. Robust species and hybrids can grow in ordinary garden soil which is well drained (Perner, 1997). The new artificial hybrids are so easily cultivated that they can be treated as ordinary perennials (Tullock, 2002; May and May, 2002; Andersson, 2005).

The preferred light conditions for most hybrids and species are half shade, and exposure to sun should only occur early or late during the day. Planting is preferably done under shading trees or together with non-invasive companion plants (Pippen, 1997; Perner, 1997, Malmgren, 2005). Average temperature during the day should ideally not reach above 20°C, since dry and hot conditions are injurious. The plants should further be protected against windy conditions to prevent the fragile stems to bend or break (Perner, 1997).

5.4.1.1. Requirements on soil

Recipes of soil substrate mixtures are countless, and every amateur grower and nurseries have their own preferences, but there are some guidelines to follow. Perner (1997) provides some suggestions.

Ideal composts for *Cypripedium* spp. should be well drained but also retain cool water (Perner, 1997). The soil should be rather moist during growth and flowering in early summer, but dry in late summer when the new shoots are formed (Malmgren, 1994).

The soil should further have a high content of organic matter (May and May, 2002) to provide a sufficient but slow mineral release (Perner, 1997). Materials decomposing too fast would give a rapid release of nutrients and compress the compost, which would hold excessive amounts of water, and ultimately support pathogenic fungi and bacteria. Organic materials such as leaf mould, leaf litter and bracken peat are recommended only in small amounts (Perner, 1997). Often included is partly decomposed organic matter such as leaf mulch (Pippen, 1997), pine bark (Tullock, 2002) or pine needles (Perner, 1997).

In contrary to Perner (1997), Pippen (1997) highly recommends a generous addition of chopped leaf litter. A thick layer of 15 cm around the planting site keeps the ground moisture well balanced as well as the substrate aerated. A good microenvironment for beneficial micro-organisms is also created. With time fine humus is built up. Leaves from ideally maple, ash, beech or oak are preferably collected and chopped with a lawn mower at leaf drop, and applied to the bed (Pippen, 1997). This will also insulate and protect the delicate corms from frost heaving and the winter cold (Pippen, 1997; May and May, 2002).

Drainage material should be included to keep the roots aerated (Pippen, 1997; Perner 1997). Pumice gravel and lava gravel have a high porosity, giving both high water holding capacity and well-aerated substrate. The same properties are attributed to burned clay particles, which in addition have a buffer capacity for nutrients. Perlite or vermiculite is also suitable for drainage (Perner, 1997). Some prefer rock gravel or sand as drainage material, but that could in contrary compact the soil even more.

Species from boggy habitats could be grown in *Sphagnum* (Myers & Ascher, 1982; Rasmussen, 1995), or mixes containing it (Riley, 1983; Rasmussen, 1995).

Some prefer to include field or wood soil, or soil from orchid habitats (Pippen, 1997; Malmgren, 1994; Malmgren, 2006). That would naturally give a perfect balanced soil and good structure, or even include important potential orchid symbionts. In deciduous woodland, clay and humus form pellets which give the soil a good crumb structure. Such clay-humic pellets collected in deciduous woods add important properties to the compost (Perner, 1997). The proportions of the mixture agents used depend on the species to be cultivated.

5.4.1.2. Soil reaction

The natural substrate of orchid habitats is often a calcareous basic mineral soil, mixed with acidic organic matter (Rasmussen, 1995). *Cypripedium* spp., as most temperate terrestrials, thrive when the soil pH is in the range of 6,5 to 7,0. It could be adjusted up with palletized lime or crushed oyster or mussel shells, and down with pine compost (Pippen, 1997, Perner, 1997).

5.4.2. Planting

Cypripedium spp. should be purchased in autumn or early spring when still dormant (Malmgren, 2006). Before planting, decaying tissues should be cut away. The rhizome should be planted in a shallow hole with the tips of the buds upright, and then covered by 2-4 cm of compost. The tips of the buds should be just below the surface, and not deeper than 2 cm

(Perner, 1997). After planting, the surface could be mulched with suitable covering for ultimate moisture maintenance. (Perner, 1997; May and May, 2002)

5.4.3. Dormancy and winter survival

The rhizome of *Cypripedium* spp. requires a constant dormancy period of at least three months to maintain healthy growth in the following season (Plummer, 2000). When in full dormancy, the species requires a cold, constant winter and can then tolerate severe frosts. If the dormancy is interrupted by warm spells stimulating growth, the *Cypripedium* spp. might be killed. The plants should be protected from such spells by coverage (Perner, 1997). A 2-3 cm layer of fine gravel (Malmgren, 2005) or organic mulch, e.g., chopped leaf litter, could be applied in the autumn for fertilization and frost protection (Tullock, 2002). In mild regions lacking snow, the coverage protection should also be done against wet conditions during dormancy. Malmgren (2005) uses roofing tiles for this purpose.

5.4.4. Watering.

Tap water is of sufficient quality for irrigation, but species requiring acidic condition should be watered with rainwater. The substrate should ideally never be allowed to fully dry out. Season, weather conditions and species determine the watering frequency. No extra water should be applied when plants enter dormancy; whereas dry spells in the summer can demand watering every day (Perner, 1997; Pippen, 1997; May and May, 2002).

5.4.5. Fertilization.

Fertilization is generally not recommended for terrestrial orchids, but alternatively very weak solutions or organic matter could be applied (Rasmussen, 1995). The strength should typically be ¼ of the full recommended, and applied once a month to every second week (Perner, 1997), starting when the new growth begins to emerge, and then every two weeks until after flowering (Pippen, 1997). Late applications could delay dormancy and thus give frost damages.

5.4.6. Pests and Diseases

If good cultural practices are maintained to keep the plants healthy, pathogens and pests are avoided.

The most common pests are slugs, mites, leaf miners, weevils, thrips, spittlebugs and grasshoppers. Virus can occur, but is extremely rare (Pippen, 1997).

Slugs attack newly emerging tips in the spring, and caterpillars later munch on the flowers.

Rodents are attracted by nutrient rich storage roots, and rabbits and deer can feed on the leaves or roots (Rasmussen, 1995; May and May, 2002).

Rots at the base of the stem occur if the compost is kept too wet. Sometimes the entire root system can rot after attacks by fungi or bacteria, especially if damaged during transplantation. High temperature in combination with high humidity can result in leaf spots, *Cercospora cypripedii*. The fungus causes blackish spots ca. 1 cm across. Another disease is leaf tip die-back, which sometimes occurs (Perner, 1997).

5.5 Cultural recommendations for species and hybrids

For the non-hybrid species, their natural requirements for cultivation must be met. Many *Cypripedium* spp. are woodland edge species, and thrive in half-shade in soils rich in organic matter. Others are moisture tolerant swamp species, which can be grown at the edge of artificial bogs (Perner, 1997). Some originates from harsh habitats, and may require winter coverage for long periods, (Malmgren, 1994). Artificial hybrids are usually easy to maintain in a range of conditions (Perner, 1997).

Species native to moist or wet areas, including *C. reginae* and *C. passerinum*, require watering every few days. An alternative approach is to construct an artificial bog, where drainage and suitable compost should be provided (Perner, 1997; May & May, 2002).
A drier state should be maintained for e.g., *C. montanum* and *C. kentuckiense*, where the surfaces of the beds are allowed to dry out between waterings (Perner, 1997; May & May, 2002).

Most *Cypripedium* spp. prefer a close to neutral soil. *C. calceolus* requires a basic soil reaction, while *C. reginae* thrive in slightly acidic conditions (Malmgren, 2005). *C. acaule* demands a pH as low as 4-4,5 (Malmgren 1994; Perner, 1997).

Perner (1997) gives a good review of the demands of almost all *Cypripedium* spp. Cultivation guidelines are also given by Malmgren (1994, 2005) and Härtl (1996). Durkee (2000) gives very throughout growing descriptions of *Cypripedium acaule* and *C. reginae*. Plummer (2000) gives a good description of windowsill pot culture of *Cypripedium* spp., especially *C. parviflorum*.

5.6 Economical aspects

Materials used in temperate terrestrial orchid propagation are quite inexpensive. The costs involved derive from the prolonged culture with rising energy costs, expenses from labour in sterile work when sowing and replanting, and subsequent soil culture. In order to get economy of horticultural and commercial propagation of temperate terrestrial orchids, the germination

percentage and survival rate among seedlings must be high. Probably the best economy of culturing orchid would be to have it as an additional culture in a nursery business.

With micropropagation or *ex vitro* vegetative propagation, the juvenile stage is shortened and the optimum would be to get blooming size of plants in 3 years. Another approach is to specialize in a part of the growth stages of the plants, e.g. *in vitro* stages, and then sell them further to other growers.

6. Discussion

6.1 Symbiotic and asymbiotic culture

In horticultural production of temperate terrestrial orchids, asymbiotic *in vitro* propagation on media with nutrient forms readily accessible to the embryo has been shown to be more convenient and inexpensive than symbiotic methods. Subsequent planting in soil substrates suggests that adult orchids could in fact survive and thrive without symbiotic fungi. This implies that fungi perhaps are only involved in the germination of the seeds, and are not essential for growing plants. However, adult orchid plants could perhaps benefit from inoculating the soil substrate with compatible fungi, or including soil from natural orchid habitats.

Soil from deciduous woods or orchid habitats is often ideal as a part of substrate for terrestrial orchid cultivation. This could be attributed to soil composition and structure, but the soil could also contain potential orchid symbionts, which would enhance growth of the plants. Trials could be done to sow orchid seeds symbiotic in soil from orchid habitats, or sow in sterilized soil inoculated with a symbiont. Soil inoculation could be initially tested with an universal orchid symbionts, such as *Tulasnella calospora*. If the plant gets the opportunity to use both phototrophic and mycotrophic nutrition, the soil nutrition will be more effectively exploited, and thus the establishment in the garden will be better. Once established, the orchid plant will be a long lived perennial. It could also be tested to inoculate the seeds with fungi, and then sow them in ordinary soils. Inoculation of seeds would also protect against pathogen attacks.

Symbiotic seed germination remains a more advanced, expensive method than asymbiotic culture. The expense derives from the steps of fungus isolation, compatible testing and *in vitro* mass production, before the fungus could be used in germination. *In vitro* pure culture of fungi could be troublesome if the culture prolongs. With symbiotic culture, the demands of the fungi for the medium also need to be met. An interesting article on symbiotic seed germination of *C. macranthos* was recently presented by Shimura & Koda (2005).

It would be interesting to test asymbiotic vs. symbiotic seed propagation, from germination to blooming sized orchid plants, to evaluate which one is more effective and economical in a horticultural production system. Symbiotic seed propagation with mycorrhizal fungi might be more effective for some troublesome genera and species. Thus, from a scientific point of view it is still important to continue the research about orchid mycorrhiza and symbiotic germination.

6.2 Medium compositions

Most researchers have their own improvements of well known media, or have composed their own. One successful medium with a wide use for mass production is the SM-spec. (Appendix), which has been continuously improved by trial and error by Svante Malmgren (Appendix). It would be interesting to test it in vegetative micropropagation experiments.

Medium would be more economical and convenient if it supports both germination and subsequent growth, since the number of transfers is decreased.

Attention should mainly be given to the form and amount of nitrogen, which is regarded by most experimenters to have the greatest effect on growth *in vitro*. Organic nitrogen has been shown to be more beneficial than inorganic forms for temperate terrestrials. Of complex organic substances, pineapple juice has been proven to be of great importance.

Difficulties in germination and proliferation do not mainly lie on medium compositions any longer, instead improvements could be done in culture temperature regimes and vernalization (Malmgren, pers com., 2006). A faster culture time could be obtained if a decreased dormancy treatment is given, with respect to the species internal rhythm and response to annual temperature variation.

Svante Malmgren (pers com, 2006) nowadays uses the same simple base recipe for all temperate terrestrials, but with only small modifications for different genera and species (Appendix).

6.3 Problems and suggestions

The propagation of *Cypripedium* spp. from seeds has been successful during recent years, proven by many specialized nurseries. Unfortunately medium recipes have not always been published, since media and procedures developed for commercial purposes often remain corporate secrets.

Successes have been delivered by amateurs experimenting in the home kitchens, where no pressure of time, economy, or scientific results, or prejudiced about media have been exerted. Their successes are mainly based on trials and errors. It is thus desirable that more scientific

culture experiments of temperate terrestrial orchids, and *Cypripedium* spp., are conducted in the future. At present, orchid propagators stick tight to their secrets at the cost of the development of rational production schemes. Rational production schemes include reducing the length of *in vitro* culture and the number of transfers to new medium. Such improvements demand cooperation between *Cypripedium* breeders.

In general, for in vitro propagation, if immature seeds are used in addition to hypochlorite sterilization and germination in darkness, seed dormancy can be most often overcome. For *Cypripedium* spp., the addition of kinetin is beneficial. However, trials should be done to find out what methods are most efficient and economical to vegetatively propagate a given cultivar. At present, division of protocorms *in vitro* is often made to enhance the number of surviving plantlets. Division of mature rhizomes are done in some cases, and bud proliferation could be further enhanced by severing the rhizomes.

In multiplying a superior clone, it should be self-pollinated if possible (Malmgren, pers com, 2006). Another approach could be to make continuous F_1-sowings from successful parents. The number of plants could then be increased by dividing the protocorms in minute pieces and further culture them. The protocorm-like bodies formed could perhaps also be divided and subcultured.

Successful culture of other organs than seeds and protocorms has not been reported for *Cypripedium* spp. Micropropagation should be tested for young explants, such as, leaves, roots, root tips, stems, root or shoot meristems, etc. Meristem propagation of *Cypripedium* spp. is a desirable goal for many researchers and propagators (Malmgren, pers com, 2006).

6.4 Breeding issues

The most frequently used pod parents and pollen parents occur within the section *Cypripedium* and especially the subsection *Cypripedium*. This is thought to be because they produces vigorous hybrids, are easily seed-propagated and cultivated. Mostly used are the calceolus-complex, the parviflorum-complex, *C. kentuckiense* and *C. cordigerum* in subsection *Cypripedium*; and the macranthos-complex and *C. tibeticum* in subsection *Macrantha*. All of them are large flowered species, often with great intraspecific variance, which together exhibit a large variation in the distribution around the world. A variable habitat range provides wide adaptations for cultivars. However, it is also important to increase the cultivation experience of the more rare species, since they could contribute with valuable traits to the *Cypripedium* breeding.

Short cultivars for balcony and windowsill culture could be a new target, with the use of e.g. *C. fasciculatum* or *C. franchetii*. It is of great importance to have the opportunity to utilize the entire *Cypripedium* gene pool.

Scientific research of e.g. flower scent and orchid mycorrhiza should be utilized to target breeding traits and develop better cultivation *in vivo*.

Challenges in the breeding process include failed seed formation of hybridization and mortality in seed propagation.

Breeding and cultivation of temperate terrestrials, and *Cypripedium* spp., will give a better knowledge of them, which is also positive from a nature conservation point of view.

6.5 Trends and potentials

Easily grown *Cypripedium* hybrids offer new exclusive and exotic products to the horticultural market, which gives them a great potential of success, and thus orchids can be a profit bringing new crop for the entire horticultural sector.

Cypripedium hybrids offer a new tool for landscape architects to create exiting exclusive bogs or plantings. Some hybrids could be viewed as a new companion plant for ericaceous plantings.

Trends in colours and shapes could be considered when developing new cultivars. Black has been a popular colour for many years now, and could be delicately matched with pale pinks in extravagance plantings. Hybrids between *C. shanxiense* and *C. tibeticum* have been shown to result in almost black clones.

Flowering perennials are easy to sell at the garden centres, but blooming sized *Cypripedium* hybrids do not thrive in pot cultures (Malmgren, 2005). That could be a marketing problem, which could be overcome by informative labels with nice pictures delivered together with the plant.

Since the plants do not thrive in pot culture when in blooming size, the cut flower industry could be another potential market for flowering *Cypripedium* hybrids. Single multiflowered stems in suitable colours could be a new exclusive trend for Valentines and other occasions. Or small bouquets of fragrant large flowered varieties such as ‘Ulla Silkens’ could also be offered. The flowers are reported to last up to 1-2 weeks (Malmgren, pers com, 2006).

Future potentials for other lovely temperate terrestrials for the garden include e.g. larger and more fragrant *Platanthera* spp., culture of red *Cephalanthera* spp. and development of easily cultivated dark chocolate vanilla fragrant *Gymnadenia nigra*.

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*) For these papers I have not read the original article but they are quoted in accordance with Rasmussen (1995).

**) For these papers I have not read the original article but they are quoted in accordance with Arditti & Ernst (1992).

***) For these papers I have not read the original article but they are quoted in accordance with Cribb (1997).

Appendix

SM-spec. to 1000 ml deionized water (Malmgren, 1989)

150 mg KNO_3
60 mg $MgSO_4 \cdot 7H_2O$
60 mg KH_2PO_4
125 mg $(NH_4)_2SO_4$
70 mg $Ca_3(PO_4)_2$
2 mg $MnSO_4 \cdot 4H_2O$
7 mg Fe-tartrate

10 g sucrose
6-8 g agar
1 g activated charcoal
1 ampoule Solu-Vit* (vitamin B complex)
5 ml Vamin* 7 g (corresponds to 0,35 g aminoacids)
5 mg kinetin
25 ml unsweetened pineapple juice

pH adjusted to 5,5-5,7

*registered pharmaceuticals

SM-spec. modified to 1000 ml deionized water (Malmgren, 1993)

75 mg $MgSO_4$
75 mg $Ca_3(PO_4)_2$
75 mg KH_2PO_4
10 mg $FeSO_4$

10 g sucrose (or up to 20 g)
7-8 g agar
Vamin* corresponding to 0,5-1 g aminoacids
1 ampoule Solu-Vit* (vitamin B complex)
25 ml unsweetened pineapple juice (pH 5,7)
0,5 g activated charcoal

pH adjusted to 5,5-5,7

*registered pharmaceuticals

5 mg kinetin added for culture of *Cypripedium* spp.

SM-spec. modified to 1000 ml tap water (Malmgren, 2006; Malmgren, pers com, 2006)

50-100 mg $Ca_3(PO_4)_2$
50-100 mg KH_2PO_4
50-100 mg $MgSO_4$

Nitrogen source;
either Vaminolac* (corresponding to 300 mg amino acids)
or 150 mg $NH_4H_2PO_4$ **and** 100 mg NH_4NO_3

10-15 g sucrose
0,5-1 g activated charcoal
4-5 g agar

pH adjusted to 5,5-6,0

*registered pharmaceuticals

A complex organic additive should also be added. The optimal one or combinations depends on the species to be cultured.

For ***Cypripedium*** spp., Malmgren (pers com, 2006) now, in contrary to previous publications, recommends the inorganic nitrogen source found above. As complex organic additive; 2-3-5 % pineapple juice and a 0,5-1 cm^3 potato piece (per flask); are used. The amounts depend on the species/hybrid to be cultured. The pineapple juice should be neutralised with NH_3 in water solution, which further gives additionally 100-150 mg nitrogen.

For ***Dactylorhiza***, ***Gymnadenia***, ***Orchis*** etc Malmgren uses Vaminolac as nitrogen source. As complex organic additive; 2 % pineapple juice and a 0,5-1 cm^3 potato piece (per flask); are used. The pineapple juice should be neutralised with NH_3 in water solution, which further gives additionally 100-150 mg nitrogen.

For ***Ophrys*** and some ***Orchis***, the Vaminolac is used in addition with 1 cm^3 Swedish turnip per flask.

Manfred Meyer's TGZ-N medium (Malmgren, 1989)

Contains per litre solution;

0.5 g inorganic salts,
10 g sucrose (sackaros),
3 g peptone (polypeptides),
10 g agar
1 g activated charcoal

pH adjusted to 5,5.