CONSERVATION OF SELECT SOUTH AFRICAN *DISA* BERG. SPECIES (ORCHIDACEAE) THROUGH *IN VITRO* SEED GERMINATION

DAVID IAN THOMPSON

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"Disas were named after a beautiful woman. However, like all women you may never fully understand them".

RON MAUNDER (1999)

PREFACE

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology at the University of Natal, Pietermaritzburg from April 1998 to March 2003, under the supervision of Professor T.J. EDWARDS (School of Botany and Zoology, University of Natal, Pietermaritzburg) and Professor J. VAN STADEN (Research Centre for Plant Growth and Development, University of Natal, Pietermaritzburg).

This thesis, submitted for the degree of Doctor of Philosophy in the Faculty of Science and Agriculture of the University of Natal, Pietermaritzburg, represents original work by the author, except where the work of others is duly acknowledged in the text. These studies have not otherwise been submitted in any form for any other degree or diploma.

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D.I. THOMPSON

September 2003

We certify that the above statement is correct

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Professor T.J. EDWARDS

Supervisor

.....

Professor J. VAN STADEN

Co-supervisor

CONFERENCE CONTRIBUTIONS FROM THIS RESEARCH

JANUARY 1999

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Paper: Conservation of the southern African terrestrial orchid genus *Disa* Berg.
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Poster: *In vitro* asymbiotic seed germination of *Disa nervosa* Lindl.
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Poster: Preliminary results regarding the *in vitro* seed germination of *Disa* section *Emarginatae*.
(THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J.)

Poster: Geographic shifts in the germination of *Disa* in southern Africa.
(THOMPSON, D.I; EDWARDS, T.J; CROUS, H. & VAN STADEN, J.)

AUGUST 2000

50th AETFAT Conference, Brussels, BELGIUM.

Paper: Explaining patterns of germination in the South African *Disa*: where to look?

(THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J.)

Poster: *Disa* seed testa: a function of habitat and a determinant of germinability.

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THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. 2002. The *in vitro* asymbiotic seed germination of *Disa nervosa* Lindl. In: Proceedings of the 16th World Orchid Conference., (eds.) CLARK, J; ELLIOTT, W.M; TINGLEY, G. & BIRO, J. Vancouver Orchid Society, Vancouver.

THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. 2002. Conquering coat-imposed seed dormancy in the South African *Disa* (Orchidaceae): old ideas & new techniques. (Conference abstracts). *South African Journal of Botany* 68 (2): 242-281.

THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. 2002. Deciduous *Disa* species *in vitro*: Seasonal rhythms, seed dormancy and sleepless nights. (Conference abstracts). *South African Journal of Botany* 68 (2): 242-281.

THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. 2003. Assessing a newly formulated dual-phase technique in orchid seed culture: conserving the South African *Disa in vitro*. (Conference abstracts). *South African Journal of Botany* 69 (2): 224 – 268.

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THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. (**in prep.**) Polymorphic seeds within and between species of *Disa* (Orchidaceae): Taxonomic and ecological links and their influence on germination.

THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. (**in prep.**) Nutrient reserves and patterns of seed germinability in the South African *Disa* (Orchidaceae).

THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. (**in prep.**) Assessing seed viability in previously ungerminated South African *Disa* species (Orchidaceae): Problems linked to an impermeable testa.

THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. (**in prep.**) Seed dormancy patterns in the genus *Disa* (Orchidaceae): Requirements for germination *in vitro*.

THOMPSON, D.I; EDWARDS, T.J. & VAN STADEN, J. (**in prep.**) *In vitro* germination of previously ungerminated summer-rainfall *Disa* species (Orchidaceae): Assessing a newly formulated dual-phase technique.

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ABSTRACT

Disa comprises 163 species, 131 of which occur in South Africa (SA). The genus is distributed across winter- and summer-rainfall areas, but few species transverse both climatic regions. Species are therefore regarded as winter-rainfall or summer-rainfall endemics – yet release their seeds in autumn, irrespective of provenance. *Disa* contributes 40 % of threatened Orchidaceae in SA, with half of the local species requiring conservation initiatives. *In vitro* seed germination is a potential conservation tool for producing large numbers of genetically diverse plants in relatively short periods. However, only 11 winter-rainfall *Disa* species are easily germinated *ex situ*. Studies were therefore undertaken on summer-rainfall taxa, which are ungerminated *in vitro*, in an effort to define their germination parameters. This thesis describes mechanisms that control germination in *Disa* and establishes practical propagation methods for seed culture.

Two seed types occur in *Disa*; i) comparatively large, pale and pyriform seeds in members of the *D. uniflora* sub-clade, which populate streamside habitats under conditions of winter-rainfall maxima, and ii) smaller, variously brown and fusiform seeds in the remainder of the genus. Seed morphometrics distinguished seed types, although embryo dimensions were similar. Testa continuity, which is disrupted in the large seeds, also supported separation. Typically, small seeds are ungerminated *in vitro*, whilst large seeds germinated readily. Increased seed size did not necessarily impart increased germinability, as several germinable, small-seeded species exist – being winter-rainfall species

Attempts to establish *in vitro* germinability revealed that increased water availability and charcoal supplementation promoted germination in intractable species. The control of germination was therefore proposed as a trade-off between water availability and the presence of phyto-inhibitors – two features typical of seeds exhibiting water-impermeable dormancy. Three germinability categories were recognized; i) easily germinable species, ii) poorly germinable species through media manipulation, and iii) ungerminated species. Germination

of immature seed in the absence of media modification was comparable to mature seed germination under modified conditions, providing evidence of the role of an impermeable seed testa in regulating germination.

Testa impermeability in mature, small-seeded species was demonstrated using aqueous EVANS' blue dye and was linked to i) testa integrity and ii) increased levels of leachable phenolics (LPC) – which are hydrophobic and phytotoxic. In addition, this research revealed an impervious and elaborate embryo carapace in small seeds. Large-seeded species were highly permeable at dehiscence, with perforated testae and negligible LPC. Germinability was ultimately defined by a significant regression with LPC. Phenolic deposition increased exponentially with increasing seed maturity and reflected decreased permeability and the development of testa colouration. The testa precludes the use of viability stains such as TTC and FDA, unless rendered permeable through scarification. This was achieved using NaOCl. Viability and germinability percentages did not correlate well for the small-seeded *Disa* species, indicating that i) the methods used to break dormancy are inadequate, ii) additional factors may be acting in concert with the testa to regulate germination and iii) that the determination of mature *Disa* seed viability is ineffective. As an alternative, the germination potential of immature seed was estimated as the ratio between the proportion of embryos stained with TTC and the proportion of seeds permeable to EVANS' blue.

Attempts to relieve water-impermeable dormancy in *Disa* resulted in the formulation of a dual-phase protocol – with the specific aim of increasing water availability to the embryo. Dual-phase cultures comprised a solid, charcoal-rich medium overlaid with a reduced strength, liquid medium fraction of the same type. The solid fraction negated the influence of leached phenols and allowed protocorms to establish polarity, whilst the fluid fraction increased water availability. The dual-phase protocol allowed germination of nine summer-rainfall *Disa* species, usually in percentages that approximated their estimated germination potential.

For the remaining species, germination is controlled by more complex factors. Large seeds are atypical in containing starch, the hydrolysis of which facilitated their rapid, autonomous germination. Small-seeded *Disa* species stored lipids and proteins and germinable species accumulated starch post-germination. The embryo protoplasts of all species contained appreciable amounts of soluble sugars, irrespective of germinability. However, decreased sucrose and increased fructose correlated significantly with decreased seed germinability. This study provides evidence of the nutritional value of mycotrophy, with endophytes liberating soluble carbohydrate and non-carbohydrate compounds upon lysis. However, few species were germinated symbiotically, suggesting that endophytes isolated from adult roots do not necessarily support germination in the same species. Similar endophytic fungi occur in Australian and Holarctic orchids.

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CHAPTER ONE

INTRODUCTION TO THE GENUS *DISA* BERG.

1.1 *DISA* - AN OVERVIEW

Southern Africa, including South Africa, Namibia, Botswana, Lesotho and Swaziland, supports 466 orchid species (COMPTON, 1976; LACROIX & CRIBB, 1995; LACROIX & LACROIX, 1997; LINDER & KURZWEIL, 1999). The tribe Diseae (subfamily Orchidoideae) is exceptionally well represented south of the Limpopo and Kunene Rivers – the northern borders of the subcontinent (LINDER & KURZWEIL, 1999), with 250 species in 11 genera. The majority of these (70 %) are southern African endemics, with over 50 % (137 species) placed within the sub-tribe Disinae.

Disa Berg. is widespread in eastern Africa south of the Sahara, including Madagascar and Reunion. The genus has its center of diversity in southern Africa, with 131 of the 163 species occurring in South Africa (LINDER & KURZWEIL, 1999). The vast majority of the species are geophytes and produce aerial parts for only a few months of each year. The subterranean organs are annual root tubers which, to some extent, alternate with the aerial growth and are replaced at the end of each growing season. A handful of species within *Disa* are evergreen perennials (LINDER, 1981; WODRICH, 1997; LINDER & KURZWEIL, 1999). Vegetative stolon formation occurs in these evergreen species, which is atypical for the genus (VOGELPOEL, 1993). Primarily roots are horizontal and penetrate shallowly into humus layers. Other species have descending roots that penetrate deeper into mineral rich soils.

Three types of leaf arrangement (viz. radical, basal and cauline) are recognized which, in addition to leaf morphology, provide good characters for classification (LINDER, 1981; STEWART, LINDER, SCHELPE & HALL, 1982; LINDER & KURZWEIL, 1999). The production of separate sterile (leaf-bearing) and fertile (flower-bearing) shoots is consistent in two sections (*Micranthae* Lindl. and *Ovalifoliae* H.P. Linder) of *Disa* and occasional in a third (*Hircicornes* Kraenzl.). Both shoots arise from the same tuber and are presented alongside each other. In the remainder of the genus, leaves cluster basally – to a greater or lesser degree, around the flowering stalk (LINDER, 1981).

The inflorescence is essentially a raceme, although the formation of corymbs occurs in three sections (*Coryphaea* Lindl., *Disa* and *Stenocarpa* Lindl.). In the latter the corymbs are commonly dense, giving the appearance of a capitate inflorescence. Racemose inflorescences may be dense or lax.

In the majority of species the flowers are resupinate, but in species with single flowers and corymbs resupination is absent. In several cases this is achieved by twisting the ovary through 360°. Flowering generally occurs in late spring or summer (October – February; VOGELPOEL, 1993; LINDER & KURZWEIL, 1999). The dorsal sepal (galea) is variously concave and usually vertical, so that the flower entrance faces horizontally to the exterior. Alternatively, the galea is angled forward so that the entrance faces downwards at approximately 45°. This usually results in an ascending spur.

Sepal spurs are widespread within the genus, but are absent in some species from the sections *Amphigena* Bolus, *Disa* and *Phlebidia* Lindl. (LINDER, 1981; LINDER & KURZWEIL, 1999). The orientation of the spur depends on the angle of origin at the base (galea extension) and its subsequent curvature. Commonly it is horizontal at the base and is either gradually or sharply decurved. However, in a few groups the spur is pendent at the base. Lateral sepals and petals are highly variable from lanceolate to almost orbicular. Orientation of these

lateral floral appendages is extremely variable and has proved useful in sectional delimitation (LINDER & KURZWEIL, 1999). Petal apices are usually acute, whilst sepal apices are navicular (concave).

The lip in *Disa* is usually a simple lanceolate structure that is smaller than the sepals. Rarely, it may be enlarged and ovate or spathulate in structure. The rostellum is essentially tri-lobed with lateral extensions that support the viscidia. Two extremes exist; either the lobes are canaliculate and deeply emarginate apically, or they are shallowly concave or almost flat and square apically. Rostellum shape correlates well with sectional taxonomy (LINDER & KURZWEIL, 1999). Although some variation is found, the anther is typically reflexed in the Disinae sub-tribe. In most species the anther is horizontal, but may be pendent or rarely ascending. Anther shape becomes increasingly globular with decreasing flower size. Viscidia are correlated to the form of the rostellum, with canaliculate lobes housing globular viscidia, while in square lobes viscidia are typically flat.

In *Disa* the stigma is essentially tri-pulvinate and sessile at the apex of the ovary or the base of the rostellum. The stigmatic surface may be flat, concave or very rarely convex. Generally the two front lobes are of equal size and the rear lobe is reduced.

1.2 PHYTOGEOGRAPHY

The diversity of orchids on the African continent is relatively poor when compared to the tropical areas of the Americas or Asia. Estimates indicate that more than 1500 orchid species occur in sub-Saharan Africa (STEWART *ET AL.*, 1982). A comparison of the orchid floras of the four major geographical regions in Africa reveals the southern African sub-continent, although containing only 52 genera and the lowest of any African geographical region, as being relatively speciose. The tribe Diseae is exceptionally well represented in southern Africa, with most species being endemic to the sub-continent (LINDER & KURZWEIL, 1999).

Disa is distributed from the western Cape of South Africa up the eastern third of the African continent, mainly in montane grasslands. One species (*D. pulchella* A. Rich.) extends into the Arabian Peninsula. Only two species of *Disa* (*D. welwitschii* Rchb. f. and *D. hircicornis* Rchb. f.) have a pan-African distribution, whilst several of the *Disa* species from the western Cape, which houses South Africa's largest species number (83), are localized on single mountains (REBELO, 1992; LINDER & KURZWEIL, 1999). Six *Disa* species have single locality records (Table 1.1), although these may constitute atypical forms of more widely distributed species.

Table 1.1. Southern African *Disa* species with single locality records.

D. brevipetala H.P. Linder	*D. forcipata* Schltr.
D. cedarbergensis H.P. Linder	*D. nubigena* H.P. Linder
D. ecalcarata (Lewis) H.P. Linder	*D. subtenuicornis* H.P. Linder

Adapted from LINDER & KURZWEIL (1999).

In South Africa, *Disa* extends from the Western Cape province north eastwards up the coast into the Eastern Cape and KwaZulu-Natal, and finally into the Mpumalanga and Limpopo provinces (Figure 1.1). In South Africa the genus spans three geological systems (MOUNTAIN, 1968), viz. Postbushveld-prekarroo, Karroo and Basement complex. Pedological characters and the distribution of each within the subcontinent can be found in MOUNTAIN (1968), COMPTON (1976), LACROIX & CRIBB (1995) and LINDER & KURZWEIL (1999). Geological ages were presented by ARDUINI & TERUZZI (1982).

Soils in South Africa which support *Disa* are primarily of sandstone origin and are oligotrophic (nutrient poor), coarse grained and acidic (COWLING, 1992). Such a restriction makes sense in terms of *Disa* mycotrophy (HADLEY & PEGG, 1989). In areas where shale strata or granite intrusions occur, soils tend to be more alkaline, of finer grain and generally more fertile (ARDUINI & TERUZZI, 1982).

Disa occupies a range of microhabitats across its distribution (LINDER & KURZWEIL, 1999) with some, such as *D. maculata* Harv. ex Lindl., *D. rosea* Lindl. and *D. longicornu* L. f., being restricted to moss-covered cliffs and rock seepages. *Disa nervosa* Lindl., *D. pulchra* Sond. and many other KwaZulu-Natal species, occur on the deeper soils of the Drakensberg foothills. Marsh or wetland species include *D. pillansii* L. Bolus and *D. bivalvata* (L. f.) T. Durand & Schinz, whilst *D. uniflora* Berg., *D. cardinalis* H.P. Linder and *D. tripetaloides* (L. f.) N.E. Br. are streamside plants that are commonly submerged during heavy rains. *Disa sagittalis* (L. f.) Sw. is the only litho- or epiphytic species. Flat, sandy plains in the western Cape support populations of *D. pygmaea* Bolus and *D. conferta* Bolus. *Disa bracteata* Sw., *D. chrysostachya* Sw. and *D. woodii* Schltr. colonize disturbed sites such as road verges and are regarded by some (STEWART *ET AL.*, 1982; LINDER & KURZWEIL, 1999) as "weedy".

Apart from geology, the most important factor in controlling southern African vegetation distribution and species composition is rainfall (RUTHERFORD & WESTFALL, 1986, 1994; LINDER & KURZWEIL, 1999). The interior and western quarter of southern Africa are relatively dry (< 250 mm rainfall per annum) whereas the south western, southern and eastern areas receive larger amounts of rain (> 250 mm rainfall), with the eastern coastal regions receiving in excess of 1000 mm rainfall per annum. Two major regions with different rainfall regimes can be distinguished; a region east of 26° E longitude where rain falls predominantly in summer (summer-rainfall region) and a region west of 22° E longitude where rainfall maxima occur in the winter months (winter-rainfall region). Between these areas is a transitional zone where rain falls throughout the year (non-seasonal rainfall; Figure 1.1; LOW & REBELO, 1996; LINDER & KURZWEIL, 1999).

Within South Africa, most of the flora comprises either winter-rainfall or summer-rainfall species and comparatively few species occur under both rainfall regimes (COWLING & RICHARDSON, 1995; LINDER & KURZWEIL, 1999).

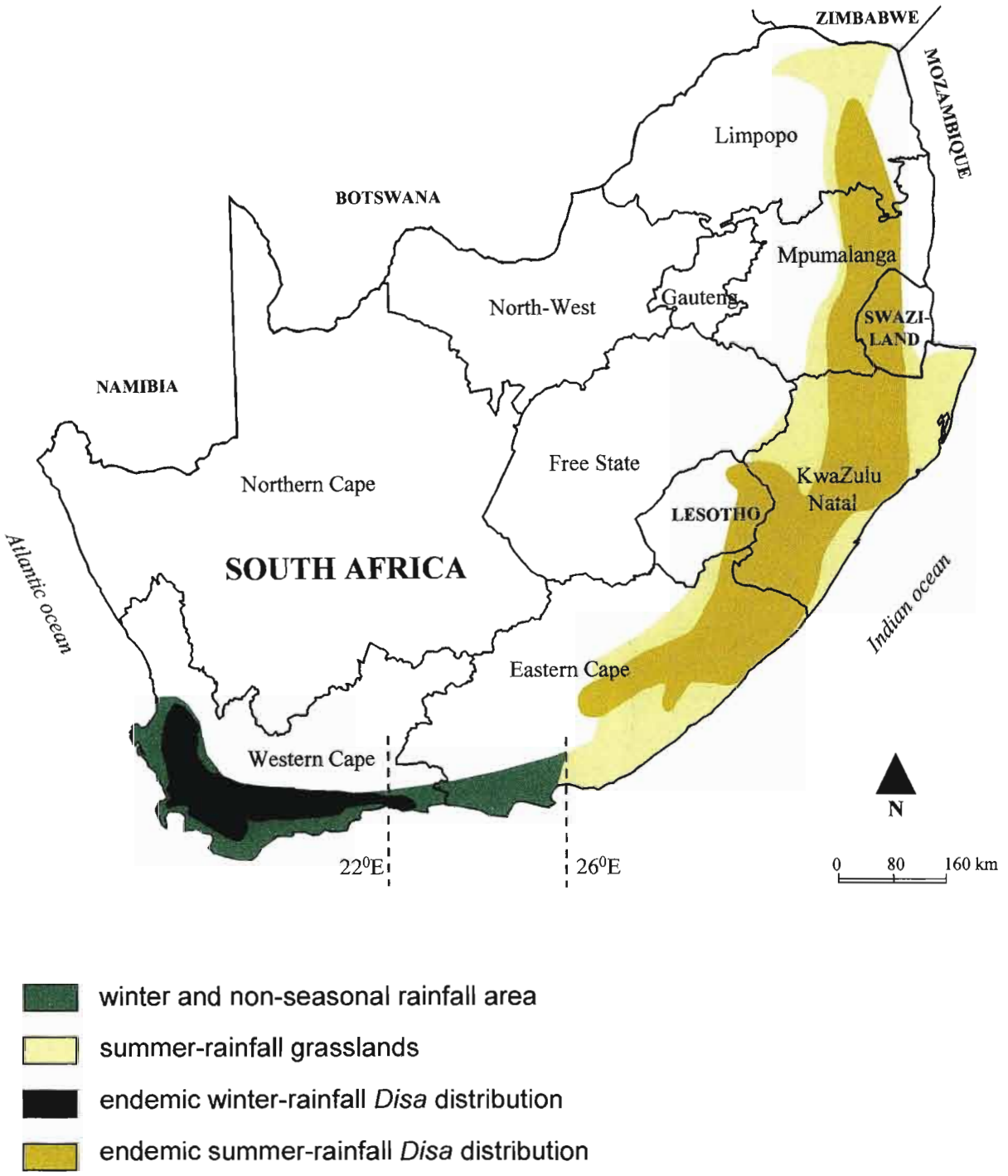


Figure1.1. Distribution of *Disa* in South Africa. Dominant rainfall is indicated, with winter maxima occurring west of 22° E and summer maxima east of 26° E longitude. Between these longitudes rainfall is non-seasonal. Neighbouring country and South African provincial names are included.

Phytogeographic evaluation reveals that *Disa* displays rainfall-mediated endemism. Eighty-five species (winter-rainfall endemics) are limited to winter or non-seasonal rainfall areas whilst others are restricted to the summer-rainfall grasslands of the eastern seaboard. These summer-rainfall grasslands support a suite of 43 endemic *Disa* species (summer-rainfall endemics). Only three species (viz. *D. tripetaloides*, *D. cornuta* (L.) Sw. and *D. sagittalis*) occur in both winter and summer rainfall areas (Figure 1.1).

The combination of varied geology and climate produced diverse vegetation in southern Africa (RUTHERFORD & WESTFALL, 1986, 1994), resulting in the recognition of seven vegetation biomes. In South Africa *Disa* is distributed across three biomes, viz. savanna, grassland and fynbos. Typically, the former two experience summer rainfall and cold, dry winters. Fynbos, with the exception of anomalous vegetation pockets occurring at high altitude throughout eastern Africa, experiences winter rainfall and hot, dry summers. Characteristic vegetation, endemism, primary threats and defining features such as burning frequency and the occurrence of frost are discussed by GOLDBLATT (1978), OLIVER, LINDER & ROURKE (1983), MOLL, CAMPBELL, COWLING, BOSSI, JARMAN & BOUCHER (1984), LUBKE, EVERARD & JACKSON (1986), RUTHERFORD & WESTFALL (1986, 1994), REBELO (1992) and LINDER & KURZWEIL (1999).

Disa is therefore distributed across a wide range of physical environments. Points of commonality include primary restriction to acidic, oligotrophic sandstone soils and an annual rainfall in excess of 250 mm. Rainfall maxima may occur in winter (June – August) or summer (December – February) and *Disa* distribution reflects this dichotomy.

1.3 LIFE HISTORIES

In the family Orchidaceae dust seeds are present and forms of anomalous nutrition are found involving symbiosis, saprophytism or parasitism

(HARPER, LOVELL & MOORE, 1970; HADLEY & PEGG, 1989). Such forms of nutrition are necessary because most of the weight of the seed consists of the undifferentiated embryonic tissue (HADLEY, 1982). Reserves are minimal and only sustain initial germination (CLEMENTS, 1982; CLEMENTS, MUIR & CRIBB, 1986). It has been speculated that many small-seeded species have insufficient reserves to germinate (ARDITTI, 1967, 1982; ARDITTI & ERNST, 1984; RASMUSSEN, 1995).

Post-germination development for *Disa*, such as the length of the subterranean phase, is unknown from seeds germinated *in situ*. No aerial growth is visible in many orchid species for several years after germination (WELLS, 1981). Where *in vitro* germination of *Disa* has been achieved (ARDITTI, 1982; VOGELPOEL, 1987,1993; WODRICH, 1997; CROUS, 1999, PERS. COMM) spherical, achlorophyllous mono-embryonic protocorms are produced. Further development results in the formation of an apical leafy shoot that produces adventitious roots (Figure 1.2).

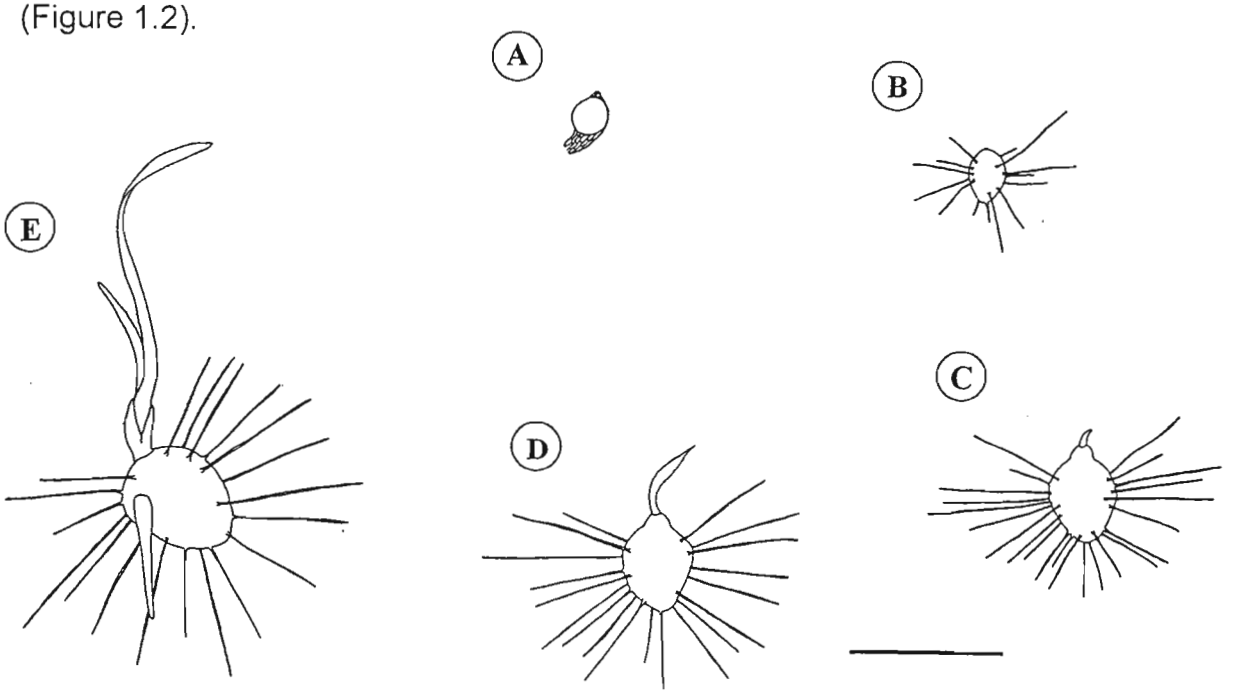


Figure 1.2. Post-germination development of *Disa* seed. Germination of seed with rupture of testa (A); Production of rhizoids (B); Production of leaf primordium (C); Production of first chlorophyllous tissue (D); Production of root initial (E). Scale bar for A: 1 mm; B – E: 2 mm. Adapted from CLEMENTS *ET AL.* (1986).

A single tuber forms from one of the leaf axils on the rhizome, but not necessarily from the first inter-node (CROUS, 1999, PERS. COMM). CLEMENTS (1982), DIXON (1987) and DIXON & SIVASITHAMPARAM (1991) observed the same sequence of organ development in *ex vitro* and *in vitro* Australian terrestrial orchid species.

Disa perennates by means of a root-stem tuber. Other members of this assemblage are characterized by a short-lived protocorm and the inconspicuous development of the rhizome (RASMUSSEN, 1995). RASMUSSEN (1995) comments that it is remarkable that species in this group can produce the beginnings of a leafy shoot without having roots to supply it with water – even if only for a short while. This sequence of organ development implies that the transpiration of the shoot must initially be accommodated by the protocorm and / or its hyphal connections (HADLEY, 1982). Consequently this is only observed where germination occurs in habitats where water is readily available. In seasonal environments, where water availability may fluctuate, the timing of seed dispersal and germination may prove critical. Leaves, rhizomes and roots die each year and are replaced annually each spring. Only a newly produced tuber survives the unfavorable season (LINDER, 1981). The evergreen *Disa* species, which display year-long aerial growth and stolon rather than tuber formation (LINDER, 1981; VOGELPOEL, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997), are anomalous with respect to RASMUSSEN's (1995) categorization.

Time taken to first flowering is also not known for *Disa in situ*, but under certain environmental conditions plants may remain vegetative for several years. Under *in vitro* conditions the evergreen *Disa* species can be flowered two years from sowing (VOGELPOEL, 1987, 1993; WODRICH, 1997; LINDER & KURZWEIL, 1999; ORCHARD, 1999, PERS. COMM.). During the prolonged period between seed dispersal and flowering, each plant faces a wide range of environmental challenges (drought, waterlogging, predation, mechanical damage; PRITCHARD, 1989; JOHANSEN & RASMUSSEN, 1992; RASMUSSEN, 1995). During this period

daughter plants of the deciduous *Disa* species may occasionally arise through vegetative propagation. However, asexual reproduction is irregular and does not account for a significant percentage of population recruitment (HUTCHINGS, 1989). Alternatively, the evergreen *Disa* species form large clonal mats as a result of stolon formation (LINDER, 1981; VOGELPOEL, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997).

Flowering generally occurs in late spring or summer (October – February), with capsule dehiscence in late summer or autumn (January – May), irrespective of phytogeographical distribution (SCHELPE, 1966; LINDER, 1981; STEWART *ET AL.*, 1982; LINDER & KURZWEIL, 1999). Winter-rainfall endemic *Disa* species therefore release seeds just before and at the onset of the rainy season. Alternatively, species in the summer rainfall areas release their seeds prior to the dry season.

Three *Disa* species do not display rainfall-based endemism and experience winter, summer and non-seasonal rainfall across their distribution ranges. These species can be found flowering for seven months (LINDER, 1981; LINDER & KURZWEIL, 1999) of the year depending on provenance, indicating a significant four to five month shift in the flowering time between winter and summer rainfall areas. Consequently seed is always released at the onset of the rainy season in these species.

Descriptions of *Disa* phenolgy are scarce in the literature (LINDER, 1981; LINDER & KURZWEIL, 1999). Regulation of developmental events such as the termination of the protocorm stage, the unfolding of the leafy shoot, flowering or the development of a tuber must, in part, rely on environmental conditions (ARDITTI, 1982). However, germination and progression in the life history must be induced jointly by environmental conditions and endogenous factors such as seed characteristics, chronological age, size and past history. Investigations of environmental stimuli (often seasonal) on seeds and post-germinative *Disa* plants have not been undertaken.

Terrestrial orchids are considered more dependent on mycorhizal fungi than epiphytic species, since terrestrial seedlings can remain underground (and thus mycotrophic) for months or even years (RASMUSSEN, 1995). Mycorrhizal symbiosis in southern African orchid taxa has been neglected and consequently no endophytes have been isolated or identified for *Disa*. Little is known about the competitive advantages of orchid mycorrhiza in the Orchidaceae as a rule, or about the significance of fungi in habitat preference, geographical distribution and speciation (HADLEY, 1982; HADLEY & PEGG, 1989). Some life stages of orchids rely entirely on mycotrophy for nutrition (HADLEY & PEGG, 1989; RASMUSSEN & WHIGHAM, 1998), while others make use of both mycotrophy and phototrophic nutrition (ARDITTI, 1982). The great diversity of orchid niches, which include habitats where paucity of soil or light precludes most plant life, are best understood in context of mycotrophy (HADLEY, 1982; HADLEY & PEGG, 1989; HÁGASTER & DUMONT, 1996; RASMUSSEN & WHIGHAM, 1998; BATTY, DIXON, BRUNDRETT & SIVASITHAMPARAM, 2001a, b).

1.4 TAXONOMY

The subtribe Disinae comprises well over half of the tribe Diseae. Attempts to establish a phylogeny for the subtribe based on morphological data have had limited success (LINDER, 1986; LINDER & KURZWEIL, 1990). Recent morphological work (LINDER & KURZWEIL, 1999) has reduced the subtribe to two genera; *Shizodium* Lindl. and *Disa*.

Contemporary taxonomy (LINDER, 1981; KURZWEIL, LINDER, STERN & PRIDGEON, 1995; DOUZERY, PRIDGEON, KORES, LINDER, KURZWEIL & CHASE, 1999; LINDER & KURZWEIL, 1999) has *Disa* split into 16 sections, within five subgenera (Table 1.2). Although these taxa seem natural it is difficult to isolate any one suite of characters that unambiguously distinguish one taxon from the others.

Phytogeographically and ecologically the taxa proposed by LINDER (1981)

and LINDER & KURZWEIL (1999) form fairly homogenous entities. Members of 10 sections are restricted to a particular rainfall regime. An additional two sections can be included here if species that occur under both summer and winter rainfall regimes are ignored. Ultimately, only four sections in the genus have members that span both rainfall regimes. Evergreen species only occur in sections *Disa* and *Phlebidia* and, apart from *D. tripetaloides*, are restricted to winter rainfall areas. Deciduous species are limited to winter or summer rainfall areas.

Table 1.2. Classification of *Disa*, with reference to distribution and rainfall in South Africa (SA). Cladograms showing the presumed relationships within those sections highlighted by means of an asterisk are included in Appendix One.

Subgenus	Section	Distribution and rainfall-based endemism
Disa	*Disa**	• 22 species in SA. 21 in winter rainfall areas and 1 species under both regimes
	Phlebidia	• 3 species in SA. Winter rainfall areas
Falcipetalum H.P. Linder	*Disella** Lindl.	• 13 species in SA. Winter rainfall areas
	Intermediae H.P. Linder	• 2 species in SA. Summer rainfall areas
	*Repandra** Lindl.	• 3 species in SA. 1 in summer rainfall and 1 in winter rainfall areas. 1 species under both regimes
	*Aconitoideae** Kraenzl.	• 8 species; 2 in SA. Summer rainfall areas
Hircicornes (Kraenzl.) H.P. Linder	*Hircicornes** Kraenzl.	• 16 species; 12 in SA. Summer rainfall areas
	Monadenia (Lindl.) Bolus	• 18 species in SA. 17 in winter rainfall and 1 in summer rainfall areas
	Ovalifoliae	• 1 species in SA. Winter rainfall areas

Table 1.2. (continued)

Subgenus	Section	Distribution and rainfall-based endemism
Micranthae (Lindl.) H.P. Linder	*Micranthae**	• 23 species; 6 in SA. Summer rainfall areas
Stenocarpa (Lindl.) H.P. Linder	*Amphigena*	• 4 species in SA. Winter rainfall areas
	*Coryphaea**	• 9 species; 8 in SA. 7 in winter rainfall areas and 1 species under both regimes
	Stenocarpa	• 16 species in SA. 5 in winter rainfall and 11 in summer rainfall areas
	*Herschelianthe** (Rauschert) H.P. Linder	• 16 species; 14 in SA. 13 in winter rainfall and 1 in summer rainfall areas
	Emarginatae H.P. Linder	• 6 species; 5 in SA. Summer rainfall areas
	Austroalpinae H.P. Linder	• 3 species; 2 in S.A. Summer rainfall areas

Adapted from LINDER & KURZWEIL (1999).

The relationships between sections and subgenera within *Disa* were not highlighted by LINDER & KURZWEIL (1999), apart from the link between sections *Aconitiodeae* and *Repandra*. Additionally, only seven cladograms representing eight sections are proposed (Table 1.2; Appendix One), which include over 65 % (110 species) of the genus. Consequently the morphological review of LINDER & KURZWEIL (1999) stands as the most comprehensive attempt at establishing a phylogeny. However, the relationships between the eight sections were not included (Table 1.2).

Research carried out by BELLSTEDT, LINDER & HARLEY (2001) using non-coding *trnL-trnF* chloroplast genome sequences represents the most recent phylogeny for the subtribe and genus (Appendix Two). Despite concentrating on members of the section *Racemosae* (Table 1.2), representatives of most other

sections were included (following the classification of LINDER & KURZWEIL, 1999) – a total of 41 species (25 % of the genus).

Monophyly of the Disinae is strongly supported in the strict consensus tree (parsimony analysis) presented by BELLSTEDT *ET AL.* (2001), with a bootstrap (BS) value of 95 % and DI = 5. This corroborates the findings of DOUZERY *ET AL.* (1999). The first of three emergent clades (BS 95 %; DI = 5) includes two species originally included in section *Coryphaea* (LINDER, 1981). However, subsequent morphological analyses (LINDER & KURZWEIL, 1999) indicated that these species might be isolated or misplaced. The findings of BELLSTEDT *ET AL.* (2001) corroborate this.

The second clade (BS 82 %; DI = 4) includes 14 species, supporting several subclades. The first segregate includes a single species of the section *Monadenia*. The remaining 13 species form a weakly supported clade (BS < 50 %; DI = 2) with almost no resolution of the relationships among the species of the clade. This second division separates section *Disa* from the rest of the genus. However, the patterns within section *Disa* are not congruent with the morphological groupings of LINDER & KURZWEIL (1999). Notably, the molecular phylogeny failed to detect the morphological clade that contains the members of the series *Racemosae*. This clade is based largely on the presence of stolons and an evergreen habit, rather than floral characters (LINDER, 1981).

The third clade in the Disinae contains the remaining 25 of the 41 *Disa* species studied by BELLSTEDT *ET AL.* (2001) and is less well supported (BS 64 %; DI = 2). Within this clade a number of subclades appear, but with weak support (BS often < 50 %) and a loss of resolution in the consensus tree. Well-supported subclades include representatives of sections *Phlebidia* (BS 95 %; DI = 3) and *Emarginatae* (BS 100 %; DI = 8). The placement of section *Phlebidia* within this clade contradicts the monophyly of the subgenus *Disa*, as delimited by LINDER

(1981).

Sections *Stenocarpa* and *Herschelianthe* group together weakly on the strict consensus tree (BELLSTEDT *ET AL.*, 2001) but are regarded as closely related (KURZWEIL *ET AL.*, 1995). Evidence for monophyly of section *Disella* is present, but weak. A further weakly supported clade contains members of sections *Stoloniferae*, *Micranthae* and *Hircicornes*. The nodes within these clades are generally well supported, but may indicate poly- or paraphyletic origin. With comparatively few representatives of these sections sampled, these results may reflect sampling bias. Additionally, the placement of at least six species remains unresolved.

Several discrepancies therefore exist between the molecular phylogeny (BELLSTEDT *ET AL.*, 2001) and the phylogeny based on morphological characters (LINDER, 1981; LINDER & KURZWEIL, 1999), since most morphologically delimited sections are not monophyletic or contain misplaced elements. The morphological classification proposed by LINDER & KURZWEIL (1999) is the more comprehensive review of *Disa*, whilst the molecular phylogeny generated by BELLSTEDT *ET AL.* (2001) represents the more recent work. Both studies are comparatively informative, but neither establishes complete phylogenetic relationships for the genus *Disa*.

BELLSTEDT *ET AL.* (2001) recommend expanded genetic sampling of at least several representatives of each section before prescribing the re-evaluation of morphological characters and a revision of the morphologically based phylogeny (*sensu* LINDER & KURZWEIL, 1999). Consequently, species relationships within sections (where proposed; Appendix One) and the nomenclature of subgeneric and sectional taxa referred to in this study follow LINDER & KURZWEIL (1999). This is done bearing in mind the molecular evidence of BELLSTEDT *ET AL.* (2001). Relationships between sections are taken from the latter.

1.5 CONSERVATION STATUS AND FUTURE PROSPECTS

The popularity of orchids dates back to the 18th century and was initially restricted to the aristocracy of Europe. Demand for specimen plants increased dramatically early in the 1900's since changes in living standards brought amateur orchid cultivation within reach of a much larger proportion of people (LINDER, 1996; LACROIX & LACROIX, 1997). Despite this, the number of plants collected for private purposes was relatively restricted and the commercial exploitation of only select species has occurred on a large scale. Commercial collecting, combined with habitat destruction, has resulted in the extirpation of many species over the last few decades (MCDONALD & DUCKWORTH, 1994).

In addition to negative effects of plant collecting, the primary factor responsible for the loss of orchid diversity is the anthropogenic destruction of natural habitats. With continued destruction and exploitation of habitats, which support orchids worldwide, many orchid species are suffering severe decline and the number of taxa facing extinction is rapidly increasing (HÁGASTER & DUMONT, 1996; KOOPOWITZ, 2001).

In South Africa, 177 orchid taxa (species and infra-specific taxa) are listed as variously threatened with extinction (HILTON-TAYLOR, 1996a, b, 1997; VICTOR, 2002). Using the IUCN Red data categories (PRITCHARD, 1989; GOLDING, 2002), six species are presumed extinct, four critically endangered, 14 endangered, 33 vulnerable, 112 low risk and eight 'insufficiently known' or data deficient, which are possibly threatened but lack precise distribution and population demography data (LINDER, 1996; LINDER & KURZWEIL, 1999).

Disa therefore contributes 40 % of threatened orchid taxa in South Africa (Table 1.3), including half of the extinct and all of the critically endangered species. Over 50 % of the genus *Disa* are contained in the following list and

therefore require urgent attention regarding conservation. Field observations over the last several years have highlighted the rarity of many other *Disa* species, indicating that the values presented here may be underestimates. Perhaps the most disturbing fact regarding *Disa* conservation is that only 11 threatened species (Table 3.2) are germinated *ex situ* (VOGELPOEL, 1980, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997; CROUS, 1999, PERS. COMM). Of these, only three low-risk species (*D. aurata* (Bolus) Parker & Koopowitz, *D. cardinalis* and *D. tripetaloides*) are consistently germinable.

Table 1.3. *Disa* species considered to be threatened in South Africa. Conservation status assigned according to the IUCN Red Data List: EX = extinct; CR = critically endangered; EN = endangered; V = vulnerable; LR = low risk; DD = data deficient.

Species	Status	Species	Status
D. amoena H.P. Linder	V	*D. neglecta* Sond.	V
D. arida Vlok	V	*D. nervosa*	LR
D. aurata	LR	*D. newdigateae* L. Bolus	V
D. barbata (L. f.) Sw.	V	*D. nubigena*	V
D. basutorum Schltr.	LR	*D. obtusa* Lindl. subsp. *obtusa*	LR
D. begleyi L. Bolus	LR	*D. ocellata* Bolus	LR
D. bodkini Bolus	LR	*D. oreophila* Bolus subsp. *erecta* H.P. Linder	LR
D. brachyceras Lindl.	LR	*D. ovalifolia* Sond.	LR
D. brevipetala	EX	*D. pillansii*	LR
D. caffra Bolus	LR	*D. physodes* Sw.	V
D. cardinalis	LR	*D. procera* H.P. Linder	V
D. cedarbergensis	V	*D. pulchra*	LR
D. cernua (Thunb.) Sw.	LR	*D. pygmaea*	DD

Table 1.3. (continued)

Species	Status	Species	Status
D. cephalotes Rchb. f. subsp. *frigida* (Schltr.) H.P. Linder	LR	*D. rhodantha* Schltr.	LR
D. clavicornis H.P. Linder	CR	*D. sabulosa* Bolus	EN
D. cochlearis Johnson & Liltved	V	*D. salteri* Lewis	LR
D. draconis	V	*D. sanguinea* Sond.	DD
D. ecalcarata	EX	*D. sankeyi* Rolfe	LR
D. extinctoria Rchb. f.	LR	*D. schlechteriana* Bolus	V
D. forcipata	EX	*D. scullyi* Bolus	CR
D. forficaria Bolus	LR	*D. spathulata* (L. f.) Sw. subsp. *spathulata*	LR
D. galpinii Rolfe	DD	*D. spathulata* (L. f.) Sw. subsp. *tripartita* (Lindl.) H.P. Linder	EN
D. hallackii Rolfe	CR	*D. stachyoides* Rchb. f.	LR
D. introsa Kurzweil, Liltved & H.P. Linder	V	*D. subtenuicornis*	V
D. longifolia Lindl.	LR	*D. tenella* (L. f.) Sw. subsp. *tenella*	V
D. lugens Bolus subsp. *lugens*	LR	*D. tenuicornis* Bolus	LR
D. lugens Bolus subsp. *nigrescens* H.P. Linder	V	*D. tenuis* Lindl.	LR
D. macrostachya (Lindl.) Bolus	V	*D. thodei* Schltr. ex Kraenzl.	LR

Table 1.3. (continued)

Species	Status	Species	Status
D. maculomarronina McMurtry	V	*D. tripetaloides*	LR
D. marlothii Bolus	LR	*D. tysonii* Bolus	LR
D. micropetala Schltr.	LR	*D. venusta* Bolus	LR
D. minor (Sond.) Rchb. f.	LR	*D. welwitschii*	LR
D. montana Sond.	LR	*D. woodii Schltr.*	LR
D. multifida Lindl.	LR	*D. zuluensis Rolfe*	LR

Adapted from PRITCHARD (1989), HILTON-TAYLOR (1996a, b, 1997) and GOLDING (2002).

Disa is not evenly distributed across southern Africa. Centres of diversity are the western Cape, the Drakensberg mountains in KwaZulu-Natal and Lesotho, and the escarpment of Mpumalanga (LINDER, 1996). Fortunately, many *Disa* species occur in mountainous areas that are not suitable for agricultural or forestry development. However, two important areas of high diversity are severely threatened. The western Cape fynbos / grasslands (Figure 1.1) are rapidly disappearing under agriculture, urban sprawl and alien vegetation. Secondly, the montane grasslands of KwaZulu-Natal (Figure 1.1) are increasingly destroyed by afforestation (LINDER, 1996; LINDER & KURZWEIL, 1999).

A few ruderal species occur within *Disa*; *D. bracteata* is frequently found in disturbed sites and extensive colonies of *D. chrysostachya* and *D. woodii* can be found on road verges. Nevertheless, the majority of *Disa* species occur only in natural areas and will only survive if their habitat – including associated pollinators and mycorrhizal symbionts, is preserved (HÁGASTER & DUMONT, 1996).

'Conservation through cultivation' or *ex situ* conservation aims to bulk up individual numbers of threatened species, with the aim of reintroducing plants into areas of natural habitat, preferably protected (PRITCHARD, 1989). Another *ex situ*

conservation approach is genebanking or cryopreservation, where seed material is stored indefinitely (HÁGASTER & DUMONT, 1996). Growing all threatened orchid species in cultivation or storing them in genebanks is not a feasible long-term solution to current conservation pressures (WELLS, 1981). Not only is it impractical to grow and store all species, there is the added problem of selecting only a small portion of the gene pool available (JOHANSEN & RASMUSSEN, 1992). Problems also arise where long-term seed viability is not known or in specialized species where pollinator mutualisms exist. Once disrupted, species reintroduction would be rendered pointless.

If plants already housed in collections could be used to produce seed, which could be made available for long-term storage and to commercial growers and researchers alike, then hopefully some level of protection may be afforded to the remaining few stands of naturally occurring plants. As regards conservation, there is little point in preserving seed banks and genetic material of terrestrial orchid species unless living cultures of compatible fungi are also preserved (JOHANSEN & RASMUSSEN, 1992). The chance of establishing viable populations by reintroducing propagated plants depends on the ability of the introduced specimens to give rise to spontaneous seedlings, and this may require the presence of suitable fungi. Orchid endophytes are difficult to trace in the soil and difficult to identify as potential symbionts unless they are actually extracted from orchid tissue and this will become less feasible as orchid populations decline in size (WELLS, 1981).

Terrestrial orchid species have the reputation of being difficult to raise from seed (ARDITTI, 1982; RASMUSSEN, 1995). In some taxa germination obstacles have been overcome by using asymbiotic or symbiotic methods. However, it is clear that terrestrial species do not form a homogenous group with respect to germination and seedling requirements. An understanding of the variation in requirements of species and the varying degrees of fungal compatibility and dependency is essential for improving effective methods of propagation. Such

research concerning *Disa* seed and the ability of seeds to be germinated *in vitro* using asymbiotic and symbiotic techniques has been neglected. If the conservation of *Disa* is to be successful, then these issues need to be addressed.

CHAPTER TWO

POLYMORPHIC SEEDS WITHIN AND BETWEEN SPECIES OF *DISA*: TAXONOMIC AND ECOLOGICAL LINKS

The seed morphology of 24 *Disa* species was investigated by means of scanning electron and light microscopy. Empirical data is provided in support of two previously recognized seed types. *Satyrium*–type seeds, which are synapomorphic for the subfamily, were significantly shorter and narrower than their *D. uniflora*-type counterparts. Seed morphometrics were not significantly different within types, making their distinction unambiguous. Testa continuity, which is disrupted only in the large seeds, and to a lesser degree colouration, also supported seed separation. These data confirmed that the *D. uniflora* – type condition is derived, an adaptation to hydrochory. Testa colour morphs occurred convergently as a germination regulating factor under variable environmental conditions. Embryo dimensions were similar between large and small-seeded *Disa* species, resulting in a substantially larger percentage air space in the former. The advantages of a buoyant seed with a permeable testa are discussed with reference to hydrochorous dispersal.

2.1 INTRODUCTION

Orchid seeds are typified by an exaggerated reduction in size (WITHNER, 1974), but data on exact seed morphometry is absent from the literature (HARPER *ET AL.*, 1970; BARTHLOTT, 1976; DRESSLER, 1981; ARDITTI, MICHAUD & HEALEY, 1979, 1980; HEALEY, MICHAUD & ARDITTI, 1980; RASMUSSEN, 1995). DAHLGREN & CLIFFORD (1982) and DAHLGREN, CLIFFORD & YEO (1985) report that seed morphology is highly variable in the Orchidaceae, with seed size and shape ranging from almost filiform where the testa is extended at each end (reaching a maximum length of 5 mm) to minute, oblong to subglobose and less than 0.1 mm in length.

Early works by ARDITTI (1967), CLIFFORD & SMITH (1969) and BARTHLOTT (1976) conclude that the morphological diversity of orchid seeds is a useful tool for subtribal and tribal taxonomic delimitation. However, these works lack the details necessary for comparative studies.

Terrestrial species produce seeds that measure 0.11 – 1.97 x 0.07 – 0.4 mm, inclusive of the testa (HARVAIS, 1974; ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; RASMUSSEN, 1995), epiphytic species tend to be even smaller (STOUTAMIRE, 1983; RASMUSSEN, 1995; ARDITTI & GHANI, 2000). Seeds of tropical, mostly epiphytic species often weigh less than 1 μg, whilst those of Holarctic or temperate, mostly terrestrial species, commonly range from 1 – 8 μg (ARDITTI & GHANI, 2000). *Galeola septentrionalis* Rchb. f., an Asian terrestrial species, is anomalous with unusually heavy seeds (22 μg) that are correlated with animal dispersal (NAKAMURA, 1982).

Orchid seeds commonly display a large volume to weight ratio due to their inflated testae (RASMUSSEN, 1995). Orchid embryos tend to be relatively uniform in size within genera, whereas the dimensions of the testa are more variable. The ratio between seed and embryo volumes determines the percentage air space within a seed, which may be as high as 96 % (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980). Seed buoyancy has been neglected in orchid research (BARTHLOTT, 1976; HEALEY *ET AL.*, 1980), but a high percentage air space is correlated to an increased floatation capacity of the seed in air (ARDITTI *ET AL.*, 1979, 1980) – a critical factor in aerial dispersal. Similar seed physiognomy characterizes wind dispersal in several other families, although their seeds are usually much larger and heavier than those of the orchids (HARPER *ET AL.*, 1970; BEWLEY & BLACK, 1994; RASMUSSEN, 1995). Orchids are able to migrate further than any other wind-dispersed flowering plant (RIDLEY, 1930), with colonization distances of 5 – 2000 km being recorded (DOCKRILL, 1969; CRACKLES, 1975; CLOSE, MOAR, TOMLINSON & LOWE, 1978; ARDITTI *ET AL.*, 1979, 1980; WILLEMS, 1982; RASMUSSEN,

1995). Dispersal over 5 – 10 km seems most common (ARDITTI & GHANI, 2000). Specialization to enhance anemochory often involves the seed testa, which may be modified as a thin membrane or wing around the seed margin (DRESSLER, 1981; WILLEMS, 1982; RASMUSSEN, 1995).

Orchid seeds that are not especially modified for wind dispersal are sclerous (hard) with variously brown or black seed coats (WITHNER, 1974; ARDITTI *ET AL.*, 1979, 1980; KURZWEIL, 1993, 2000; BEWLEY & BLACK, 1994; RASMUSSEN, 1995), although DRESSLER (1981) reports on loose, papery seed coats around the embryo. However, the testa is commonly translucent and the seed opaque (STOUTAMIRE, 1974; WITHNER, 1974; ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; DRESSLER, 1981; DAHLGREN & CLIFFORD, 1982; DALHGREN *ET AL.*, 1985, RASMUSSEN, 1995). Sclerous seed coats are restricted to essentially terrestrial genera (STOUTAMIRE, 1974; ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; RASMUSSEN, 1995) where they represent a barrier to germination (BEWLEY & BLACK, 1994). GARAY (1960) and DALHGREN *ET AL.* (1985) suggest that such hard-seededness is the primitive condition within the orchid family.

Polymorphisms occur in the colour, size, ornamentation and thickness of the testa. Where polymorphic seeds exist germination is often asynchronous as a result of variable dormancy (VAN WAES & DEBERGH, 1986b; RASMUSSEN, 1995; THOMPSON, EDWARDS & VAN STADEN, 2001). This offers several ecological advantages, especially in seasonal or fluctuating environments (BEWLEY & BLACK, 1994). SENGHAS, EHLER, SCHILL & BARTHLOTT (1974), ARDITTI *ET AL.* (1979, 1980), HEALEY *ET AL.* (1980) and KURZWEIL (1993) all demonstrate functional correlations between seed morphometrics and certain aspects of the reproductive biology (viz. seed dispersal and germination) of terrestrial Orchidaceae.

The size, shape and ornamentation of cells within the testa may also be polymorphic (WITHNER, 1974; ARDITTI *ET AL.*, 1979, 1980; AVERYANOV, 1990; KURZWEIL, 1993, 2000; MOLVRAY, 2002). Cell shape, as well as the ratio of cells

on the transverse and longitudinal seed axes are important in generic separation of several northern hemisphere terrestrials (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; AVERYANOV, 1990). Here the direction of specialization is from seeds with a relatively multicellular testa to extremely simplified seed coats consisting of fewer cells.

Testae cells of terrestrial orchid species are commonly polygonal, grouped in a reticular-foveate arrangement (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; KURZWEIL, 1993) and bound by thickened, lignified anticlinal walls. Anticlinal walls in the testa are closely associated at the wall adhesion zone and are without intercellular spaces (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; MOLVRAY, 2002). As the seed matures, cells of the single layered testa lose their cytoplasm and the periclinal walls fuse with one another medially. The surface structure or ornamentation of the periclinal walls of the testa cells is highly variable.

Testa ornamentation may be longitudinal, transverse, concentric or have reticulate trabeculae, which are variously branched (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; DRESSLER, 1981; TOHDA 1985, 1986; AVERYANOV 1990; RASMUSSEN, 1995). Some species have prominent, subcircular holes at irregular intervals between the anticlinal walls of adjacent cells (KURZWEIL, 1993). Anticlinal walls may also be irregularly thickened or beaded (CLIFFORD & SMITH, 1969; MOLVRAY, 2002). In some of the vandoid orchid species anticlinal walls are hook-like (CHASE & PIPPEN, 1988). Occasionally, the marginal parts of the periclinal walls exhibit minute papillate bulges or linear concavities (KURZWEIL, LINDER & CHESSELET, 1991; KURZWEIL, 1993). They may also be covered with wax deposits as in some representatives from the Epidendreae (DRESSLER, 1981).

Variation in testa anatomy may be used to distinguish genera and species. However, seed characters may change as a result of environmental and ontogenetic influences (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; BEWLEY &

BLACK, 1994). Accordingly, the use of seed morphometry in taxon delimitation must be selective since multiple seed morphs may occur within a single taxon. Despite this, seed ultrastructure is used abundantly in orchid taxonomy (BARTHLOTT, 1976; ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; TOHDA, 1983, 1985, 1986; CHASE & PIPPEN, 1988, 1990; KURZWEIL *ET AL.*, 1991; PETTERSSON, 1991; MOLVRAY & KORES, 1995; KURZWEIL, 2000; MOLVRAY, 2002).

Sculpturing or ornamentation may be inconsistent within genera or species. For example, AVERYANOV (1990) revealed that the morphology of northern hemisphere terrestrial orchid seeds correlates with their geographic origin. The genus *Dactylorhiza* Neck. can be separated into four groups based on the presence and form of periclinal wall trabeculae, with seed groupings reflecting latitudinal, longitudinal and continental geographic separation. However, the exact adaptive value of the ornamentation was not identified.

KURZWEIL (1993, 2000) reports on the value of seed morphology as a taxonomic tool, but only in the context of the Orchidoideae (Orchideae and Diseae; *sensu* DRESSLER, 1981). The literature carries no reference dedicated strictly to reviewing terrestrial or more specifically *Disa* seed morphology. Among the 36 *Disa* species investigated (Table 2.1), KURZWEIL (1993) recognized two distinct seed types based on a combination of seed size and shape, testa colour and testa cell arrangement.

Most southern African Orchidoideae possess synapomorphic *Satyrium* Sw. - type seeds (Table 2.2; KURZWEIL, 1993). These seeds are fusiform and have testa cells with collapsed and concave periclinal walls, similar to other Orchidoideae (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; KURZWEIL *ET AL.*, 1991; TOHDA, 1983). The southern African members of the sub-family produce slightly smaller seeds than their northern hemisphere counterparts.

The brown seeds found in most species are apparently primitive in the Orchidoideae (GARAY, 1960; KURZWEIL, 1993, 2000). Intensely brown seeds occur sporadically and are assumed to have evolved convergently. In *Disa*, dark seeds are also sporadic (KURZWEIL, 1993). Unfortunately no comment is made on polymorphic seed colouration within species.

Table 2.1. South African *Disa* species for which morphological seed data is available.

D. aconitoides Sond.	*D. filicornis* (L. f.) Thunb.	*D. sagittalis*
D. atricapilla	*D. fragrans* Schltr.	*D. sanguinea* Sond.
(Har. ex Lindl.) Bolus		
D. bivalvata	*D. longicornu*	*D. stachyoides*
(L. f.) T. Durand & Schinz		Rchb. f.
D. bodkinii Bolus	*D. lutea* H.P. Linder	*D. stricta* Sond.
D. caffra Bolus	*D. nervosa*	*D. telipogonis*
		Rchb. f.
D. cardinalis	*D. obtusa* Lindl.	*D. tenuicornis* Bolus
D. caulescens Lindl.	*D. ovalifolia* Sond.	*D. tenuis* Lindl.
D. chrysostachya	*D. patula* Sond.	*D. tripetaloides*
D. cooperi Rchb. f.	*D. pillansii* L. Bolus	*D. uncinata* Bolus
D. cornuta	*D. racemosa* L. f.	*D. uniflora*
D. elegans	*D. richardiana*	*D. vaginata*
Sond. ex Rchb. f.	Lehm. ex Bolus	Harv. ex Lindl.
D. ferruginea	*D. rosea* Lindl.	*D. vasselotii*
(Thunb.) Sw.		Bolus ex Schltr.

Adapted from Kurzweil (1993).

Four *Disa* species produce a markedly different type of seed. The *D. uniflora* – type seed (Table 2.2), a derived state, is relatively large and pyriform with smooth, convex testa cells (KURZWEIL, 1993). Seed colouration in these species is always pale brown or tan. The remainder of the subfamily, including

Disa, produces *Satyrium* – type seeds.

Table 2.2. Morphological seed data for South African members of the Orchidoideae. *Disa* seed and embryo morphometrics are included where available.

Seed Type	
1 – *Satyrium* - type	
• found in all members of the southern African Orchidoideae, including 32 of the 36 *Disa* species investigated	
Shape	Variously fusiform (2.1 – 3.1 length : width ratio); rarely shortly fusiform to globose (1.4 – 2.0 length : width) or elongate fusiform to filiform (4.5 – 6.5 length : width).
Size	Commonly 0.3 – 0.4 mm in length, globose or filiform seeds are shorter (0.27 mm) or longer (0.8 mm) respectively. ***Disa*: 0.32 ± 0.06 x 0.14 ± 0.03 mm (mean ± SD)**
Colour	Variously brown, rarely blackish brown or black. Testa translucent, embryo darker.
Embryo	Visible through the testa, situated centrally, subglobular to globose. Rarely elongate. Commonly occupies almost the full width of the seed. ***Disa*: 0.14 x 0.09 mm**
2 – *Disa uniflora* - type	
• found in only 4 *Disa* species	
Shape	Balloon-like (sub-globular to pyriform).
Size	1.09 x 0.52 mm (± 2 length : width ratio).
Colour	Light brown, not translucent.
Embryo	Not visible, but situated centrally.

After KURZWEIL (1993). All measurements are mean values.

2.2 AIMS

- To illustrate and supplement the seed morphology data presented by KURZWEIL (1993).
- To correlate seed morphology, germinability, taxonomy and species phytogeography for 24 South African *Disa* species.

2.3 MATERIALS AND METHODS

Unless acknowledged to either DR. L. VOGELPOEL or H. CROUS, all seed was collected from wild populations of *Disa* occurring within the summer-rainfall grasslands of South Africa (Table 2.3). Seed was collected into brown paper envelopes from mature, undehisced capsules and stored at ± 4 °C at 0 % relative humidity. Seeds from DR. VOGELPOEL were supplied dry (in paper envelopes) whilst those from H. CROUS were stored and transported in FAA (ethanol : H_20 : acetic acid : formalin; 10:8:1:1). However, in both cases the origin and method of seed collection were unknown.

Seed from 24 *Disa* species (Table 2.3) was prepared for macro-morphological examination. For scanning electron microscopy (SEM) studies seeds were subjected to critical point desiccation (CPD). Desiccated seeds were mounted onto brass stubs and coated with gold-palladium using a Polaron Coating Unit E – 5100. Coated stubs were stored at 0 % relative humidity over silica gel before viewing using a Hitachi S – 570 scanning electron microscope. A minimum of 25 seeds were observed for each species.

Table 2.3. South African *Disa* species in which basic seed morphology was investigated. Species repeated from KURZWEIL (1993) are indicated by means of an asterisk.

D. brevicornis (Lindl.) Bolus	*D. fragrans**	*D. saxicola* Schltr.
*D. cardinalis**[1]	*D. longicornu**[2]	*D. stachyoides**
D. cephalotes Rchb. f. subsp. *cephalotes*	*D. nervosa**	*D. thodei* Schltr. ex Kraenzl.
*D. chrysostachya**	*D. patula* var. *transvaalensis* Summerh.**	*D. tripetaloides**[1]
*D. cooperi**	*D. polygonoides* Lindl.	*D. versicolor* Rchb. f.
*D. cornuta**	*D. pulchra*	*D. woodii*
D. crassicornis Lindl.	*D. racemosa**[1]	*D. uncinata*[2]
D. draconis[2]	*D. sagittalis**	*D. uniflora**[1]

Seed supplied by DR. L. VOGELPOEL[1], Cape Town, South Africa and H. CROUS[2], Kirstenbosch, South Africa. ** KURZWEIL (1993) reported seed morphology for *D. patula*, but did not specify the variety.

Seeds and embryos were measured under a Wild Heerbrugg M400 photomicroscope (at the longest and widest axes of the seed). In the case of fusiform seeds (*Satyrium* – type), which approximate two cones joined at their bases, seed volumes were calculated using the formula:

$$2\ [\ (w/2)^2.(\tfrac{1}{2}\ l).(1.047)\ ]$$

where w = seed width, $(w/2)^2 = r^2$, l = seed length, ½ l = height of each cone and 1.047 = $\pi/3$ (ARDITTI *ET AL.*, 1979; SEATON & HAILES, 1989). The expression within the brackets can be reduced to $\pi/3r^2h$, which is the formula for calculating the volume of a cone. *Disa uniflora* – type seeds and orchid embryos are pyriform, sub-globular or globose and are roughly elliptical in cross section. The volume of these structures is best approximated using the formulae for a prolate spheroid (ARDITTI *ET AL.*, 1979):

$$^4/_3\ \pi(\tfrac{1}{2}\ l).(\tfrac{1}{2}\ w)^2$$

Assumptions of normality and variance homogeneity were tested with KOLMOGOROV -SMIRNOV and LEVENES' tests respectively. Seed and embryo dimensions between seed types were independently compared using an ANOVA crossed design, where the fixed factor was seed type (*Satyrium* or *D. uniflora* – type). Seed and embryo dimensions within seed types and within species were compared using a MANOVA, where the fixed factors were species and dimension (length or width). All analyses were performed using Statistica (STATSOFT INC., 1998). Ratio and volume data were not analysed statistically. Seed testa colour was described and compared subjectively following visual inspection.

Due to their minute size, seed mass is commonly established by calculating the mean across a specific seed lot where the combined mass has been measured (STOUTAMIRE, 1964). Such lots, depending on individual seed dimensions, may need to contain hundreds or thousands of seeds before appreciable masses can be recorded (STOUTAMIRE, 1964; RASMUSSEN, 1995). Due to a paucity of suitable material for most species where seed morphometrics were investigated, the masses were not determined. THORNHILL & KOOPOWITZ (1992) recorded the mean mass of a single seed of *D. uniflora* as 1.6×10^{-5} g.

Phylogenetic relationships and convergence in the evolution of *Disa* seed was investigated by firstly plotting seed characters onto morphological cladograms (LINDER & KURZWEIL, 1999; Appendix One) and onto a molecular phylogeny (BELLSTEDT *ET AL.*, 2001; Appendix Two), although neither represents a comprehensive review of *Disa*. The characters plotted were seed and embryo size (seed type), testa ornamentation and testa colouration. In the cases of seed type and testa ornamentation the data plotted reflects a combination of observations from this study together with those made by KURZWEIL (1993). Testa colouration data stems solely from this research. Morphological cladograms, or portions thereof, showing the evolution of seed character states are reproduced indicating parsimonious character placement. Sectional cladograms (section *Herschelinathe*) or clades where no data were available are omitted, as

highlighted by means of an asterisk. Species for which data were unavailable are indicated in grey (Figure 2.3). Seed type data were plotted for 34 species, seed colouration data for 16 species and testa ornamentation data for five species.

2.4 OBSERVATIONS AND DISCUSSION

Satyrium – type seed (Figure 2.1B; *sensu* KURZWEIL, 1993) was recorded in 32 of the 36 *Disa* species examined by KURZWEIL (1993) and in 21 of the 24 species investigated in this study. These data corroborate the findings of KURZWEIL (1993), in addition to recording *Satyrium* - type seed characters for *D. brevicornis*, *D. cephalotes*, *D. crassicornis*, *D. draconis*, *D. polygonoides*, *D. pulchra*, *D. saxicola*, *D. thodei*, *D. versicolor*, *D. woodii and D. uncinata*. *Disa uniflora* – type seeds (Figure 2.1A; *sensu* KURZWEIL, 1993) were recorded for three species, corroborating previous data.

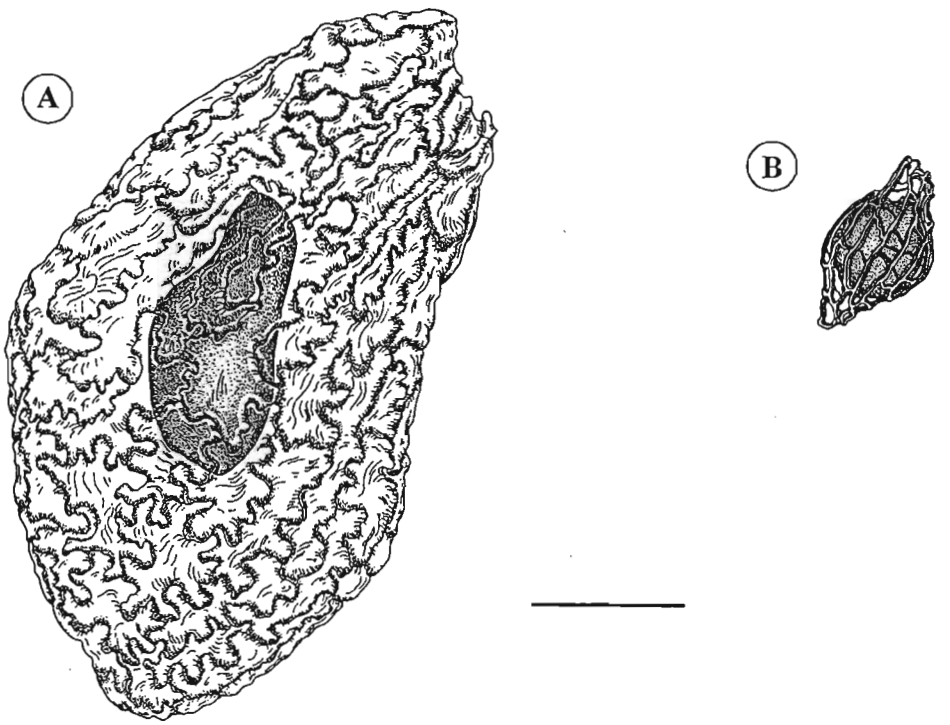


Figure 2.1. Polymorphic seed morphology in the South African *Disa*. Note the position and relative size of the embryo. *Disa uniflora* - type seed (*D. tripetaloides*; A) and *Satyrium* - type seed (*D. cooperi*; B). Scale bar: 0.2 mm. Adapted from KURZWEIL (1993).

For each seed type, seed dimensions are in accordance with those presented previously for the genus (KURZWEIL, 1993; Table 2.2) and with those recorded for the entire family (HARVAIS, 1974; ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980, DAHLGREN & CLIFFORD, 1982; DAHLGREN *ET AL.*, 1985; RASMUSSEN, 1995).

Satyrium – type seeds were minute in all *Disa* species investigated and measured 0.4 ± 0.06 x 0.16 ± 0.04 mm (mean ± SD, n = 105; Table 2.4). Seeds of this type, which dominate in the genus *Disa*, are significantly shorter ($F_{1,148}$ = 2081.14, $P < 0.01$) and narrower ($F_{1,148}$ = 1758.15, $P < 0.01$) than their *D. uniflora* – type counterparts (Figure 2.2). Such data provides strong empirical evidence in support of the two seed types recognized by KURZWEIL (1993). The latter seeds measured 1.13 ± 0.13 x 0.58 ± 0.08 mm (mean ± SD, n = 45; Table 2.4). Calculated seed volumes are 2.68 and 199.12 $mm^3 \times 10^{-3}$ for *Satyrium* and *Disa uniflora* – type seeds respectively (Table 2.4).

Table 2.4. Seed morphometric data collected for 24 South African *Disa* species.

	Seed							
	Length (l; mm)	Width (w; mm)	l/w	# cells		Wall ornamentation		Volume ($mm^3 \times 10^{-3}$)
	mean (SD)	mean (SD)		long axis	short axis	Peri-clinal	Anti-clinal	
***Satyrium* - type seed (n = 105)**	0.50 (0.06)	0.16 (0.04)	2.5	3	4	P	S	2.68
***Disa uniflora* - type seed (n = 45)**	1.13 (0.13)	0.58 (0.08)	1.95	6	9	0	U	199.12

Abbreviations: P – periclinal wall ornamentation; 0 – ornamentation on periclinal walls not detectable; S – anticlinal walls straight; U – anticlinal walls undulate.

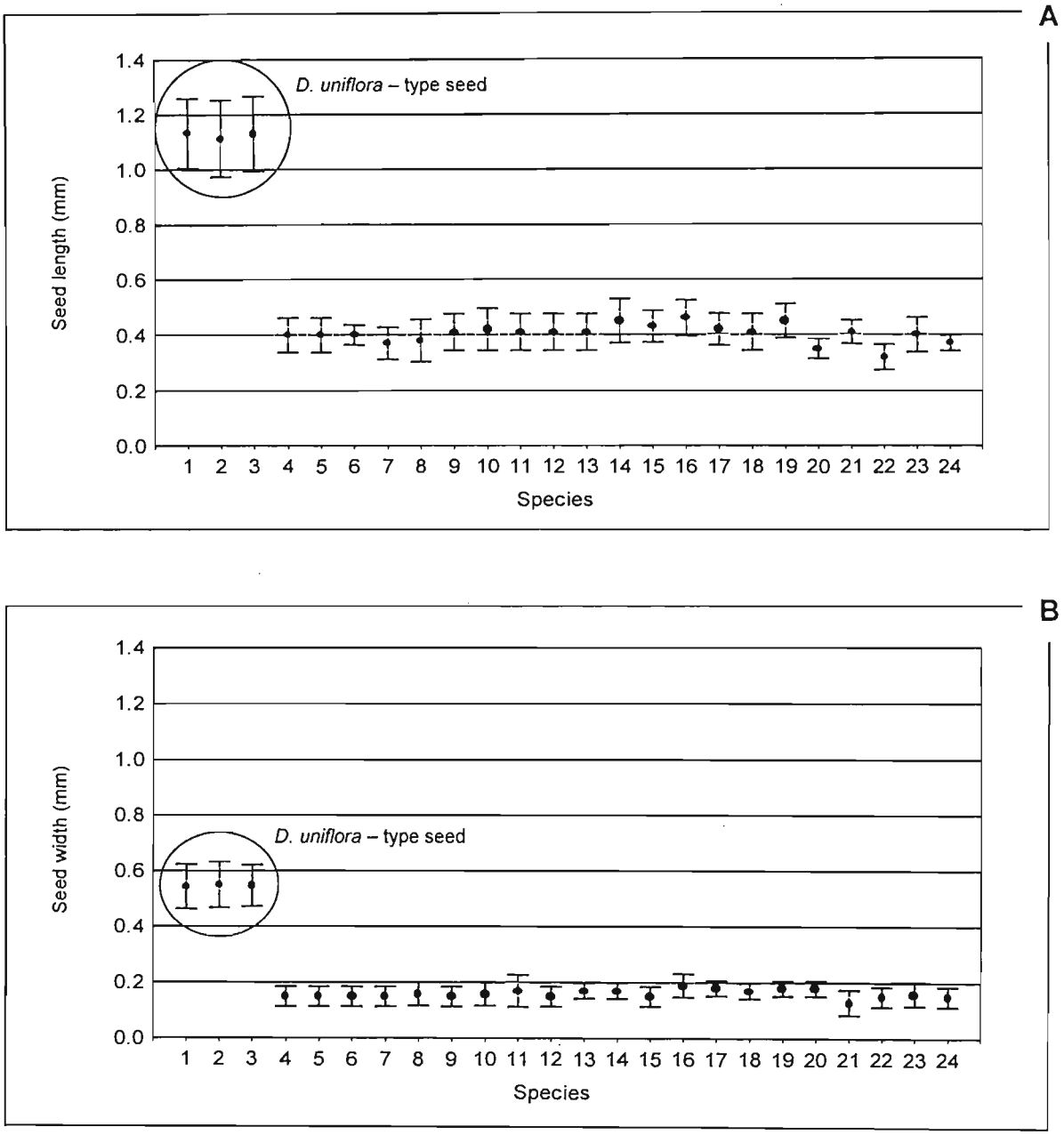


Figure 2.2. **Seed dimensions in *Disa* (mean ± SD, *n* = 15 for *D. uniflora* - and 5 for *Satyrium* – type species). Specific seed lengths** (A) **and specific seed widths** (B). ***Disa uniflora* – type seeds are indicated. All measurements in mm. Species: 1 – *D. cardinalis*; 2 – *D. tripetaloides*; 3 – *D. uniflora*; 4 – *D. brevicornis*; 5 – *D. cephalotes*; 6 – *D. chrysostachya*; 7 – *D. cooperi*; 8 – *D. cornuta*; 9 – *D. crassicornis*; 10 – *D. draconis*; 11 – *D. fragrans*; 12 – *D. longicornu*; 13 – *D. nervosa*; 14 – *D. patula*; 15 – *D. polygonoides*; 16 – *D. pulchra*; 17 – *D. racemosa*; 18 – *D. sagittalis*; 19 – *D. saxicola*; 20 – *D. stachyoides*; 21 – *D. thodei*; 22 – *D. versicolor*; 23 – *D. woodii*; 24 – *D. uncinata*.**

Within type analysis saw no significant morphometric differences in *D. uniflora* – type seeds ($F_{2,84} = 0.035$, $P = 0.966$), indicating relatively constant dimensions within and between the seeds of these three species (viz. *D. cardinalis*, *D. tripetaloides* and *D. uniflora*; Figure 2.2). Seed shape was pyriform to subglobular (Plate 2.1A, B), with a length : width ratio of less than two. Seeds are never globose, since mean seed length is significantly greater than mean seed width ($F_{1,84} = 622.85$, $P < 0.01$).

Satyrium – type *Disa* seeds are variously fusiform in shape (Plate 2.1C – F) and were, on average, two-and-a-half times longer than wide. Mean seed length was significantly larger than mean maximum seed width ($F_{1,168} = 1267.58$, $P < 0.01$). Variation in seed dimensions were pronounced in the *Satyrium* – type seeds varying significantly in both length and width ($F_{20, 168} = 1.688$, $P < 0.05$) between, but not within, individual species (Figure 2.2). Rarely, seeds were shortly fusiform, tending towards globose (*D. cornuta*; Plate 2.1F). Very long seeds (those that are narrowly fusiform, tending towards filiform) are found in very few *Disa* species, culminating in extraordinary length (1.8 – 2 mm) in *D. pillansii* (KURZWEIL, 1993). *Disa pulchra* (Plate 2.1C) displayed the greatest seed length of the *Satyrium* – type species investigated in this study, with a mean length of 0.45 mm.

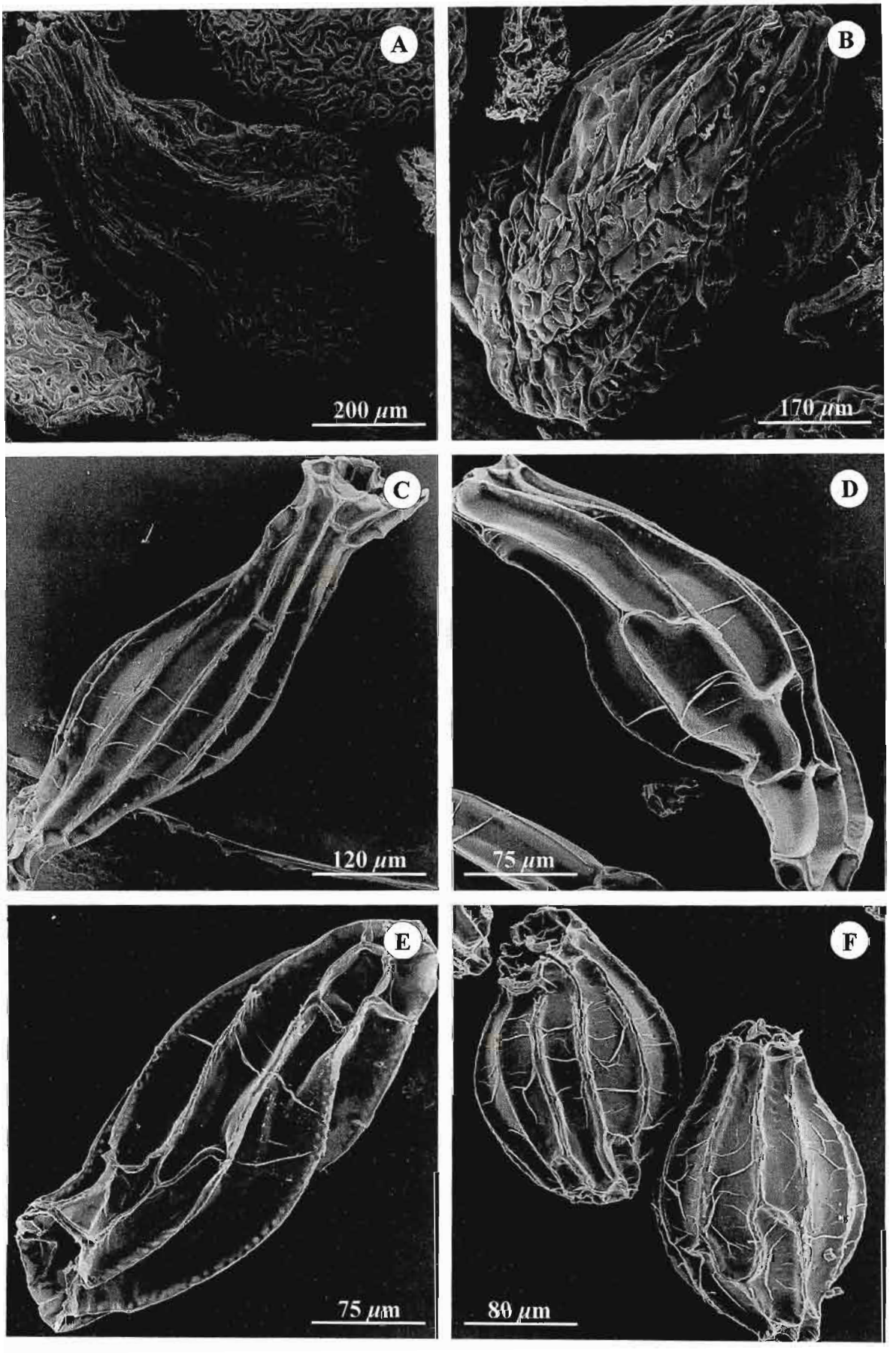
The *Satyrium* - type seeds identified in this study belong to species that, except for *D. uncinata*, experience summer rainfall. Previous studies (KURZWEIL, 1993) have identified both summer- and winter-rainfall representatives as having *Satyrium* – type seeds. Congruent seed morphometrics (Table 2.2 and Table 2.4) between this essentially summer rainfall data set and a combined summer – winter rainfall data set (KURZWEIL, 1993) provide indirect evidence that seed dimensions are not correlated to phytogeographical distribution.

The large seeds of *D. uniflora* (Plate 2.1A) and allied species were

interpreted by KURZWEIL (1993, 2000) as being apomorphic, since *Satyrium* - type fusiform seeds appear to be synapomorphic across the entire subfamily Orchidoideae, and certainly within *Disa* (Figure 2.3). The evolution of the *D. uniflora* – type seed on the morphological cladogram for the section (Figure 2.3B) reveals that the four species are terminal to a single sub-clade (LINDER & KURZWEIL, 1999). *Disa aurata*, which was previously included in *D. tripetaloides* and differs only in flower colour (LINDER & KURZWEIL, 1999), is included as the final member of the *D. uniflora* sub-clade and is presumed to produce large seeds. Molecular data (Appendix Two) presented by BELLSTEDT *ET AL.* (2001) reveals that this lineage may be unnatural, with polyphyletic origin. Under such a scenario the large seed type appears as a convergent, rather than a phylogenetically constrained character.

KURZWEIL (1993, 2000) explained their apomorphic seed characters as an adaptation to hydrochory. The production of the large *D. uniflora* – type seed is restricted to five streamside species (*D. aurata*?, *D. cardinalis*, *D. caulescens*, *D. tripetaloides*; Plate 2.1B and *D. uniflora*; Plate 2.1A) that, for the most part, experience the winter rainfall of the western Cape (LINDER, 1981; VOGELPOEL, 1987; KURZWEIL, 1993; LINDER & KURZWEIL, 1999). *Disa tripetaloides* also occurs sporadically as isolated, streamside populations in the summer rainfall areas of South Africa.

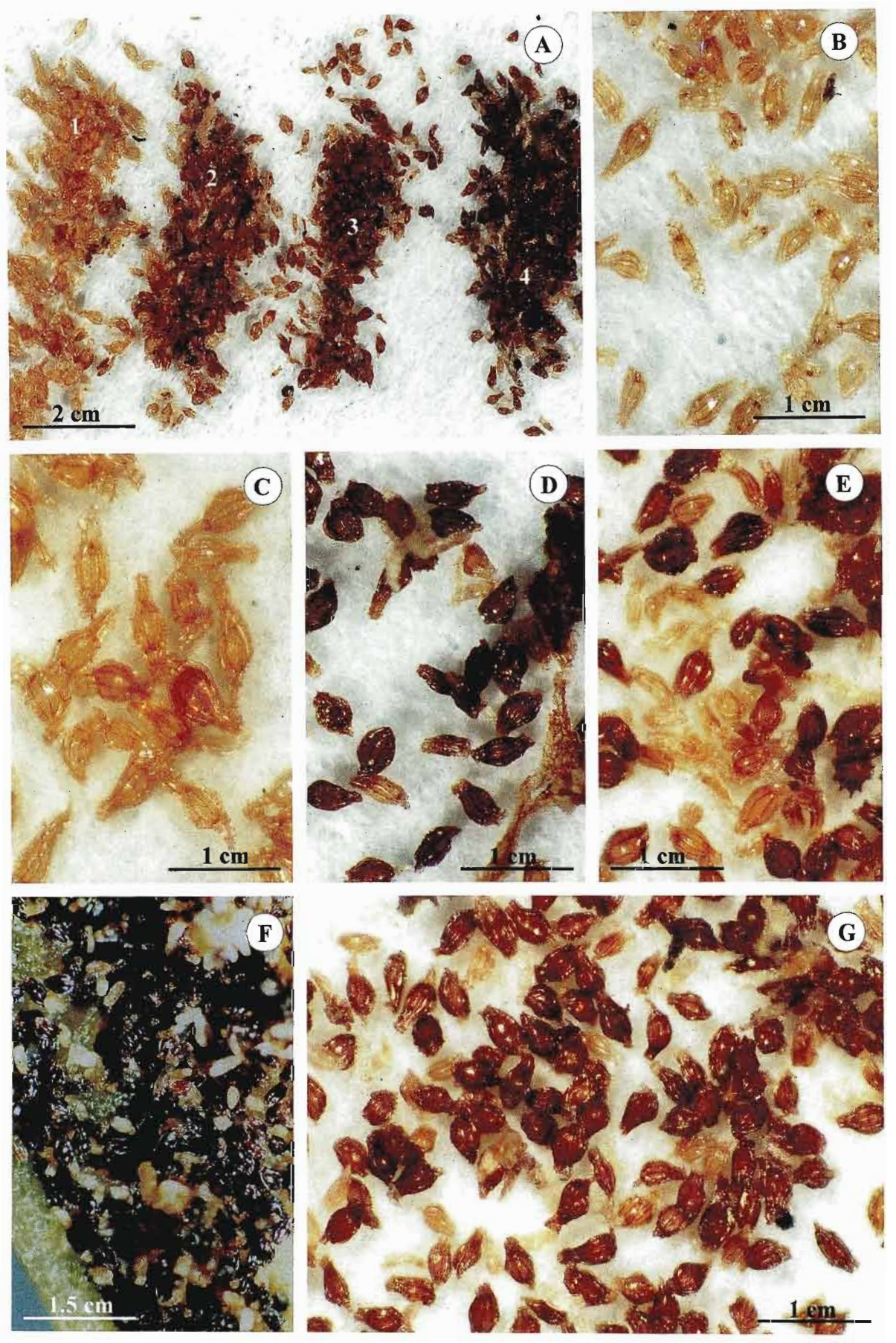
The remaining species of *Disa* possessed small *Satyrium* – type seeds (Figure 2.3). The unusually large *Satyrium* – type seeds of *D. racemosa* and *D. pillansii* are found, not only in unrelated taxa within the section *Disa*, but on separate lineages from the *D. uniflora* – type species (LINDER & KURZWEIL, 1999; Figure 2.3). The molecular phylogeny (BELLSTEDT *ET AL.*, 2001; Appendix Two) placed *D. racemosa* within a largely unresolved clade of six species, including *D. cardinalis* and *D. tripetaloides*. Large seeds and the evolution of the *D. uniflora* – type seed therefore appear to be convergent, rather than the former condition pre-empting the evolution of the latter, as suggested by KURZWEIL (1993).


A
200 μm
B
170 μm
C
120 μm
D
75 μm
E
75 μm
F
80 μm

Increased seed size and volume, in conjunction with rapid *in vitro* germination (ARDITTI, 1982; VOGELPOEL, 1987, 1993; WODRICH, 1997), have lead to speculation over the occurrence of endosperm or rudimentary cotyledons in the *D. uniflora* – type species (KURZWEIL, 1993). However, only 10 members of the Orchidaceae are reported to possess rudimentary cotyledons (NISHIMURA, 1981, 1991) and no reference (other than KURZWEIL, 1993) can be found regarding the occurrence of endosperm in the mature orchid seed. DAHLGREN *ET AL.* (1985) report on endosperm production in orchids, but only in the early developmental (8 or 16 cell proembryo) stages of primitive orchid taxa such as *Vanilla*.

The significantly smaller *Satyrium* – type seeds are, for the most part, ungerminated *in vitro* (THOMPSON *ET AL.*, 2001; THOMPSON, EDWARDS & VAN STADEN, 2002). However, increased seed size does not necessarily impart an increased degree of germinability, since several readily germinated *Satyrium* – type species do exist (ARDITTI, 1982; VOGELPOEL, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997; ORCHARD, 1999, PERS. COMM.).

Mature *Satyrium* – type seeds are variously brown in colour (Plate 2.2A), ranging from tan (*D. pulchra*; Plate 2.2B), golden (*D. crassicornis*; Plate 2.2C), mid-brown (*D. chrysostachya* and *D. cooperi*; Plate 2.2D), reddish (*D. patula*; Plate 2.2E) through to blackish brown (*D. cephalotes* and *D. versicolor*; Plate 2.2F). Seeds are commonly mid-brown, with more (blackish brown) and less (tan or golden) intensely coloured forms occurring sporadically. Since brown seeds represent the primitive condition in *Disa* (KURZWEIL, 1993), alternative colour forms are thought to have evolved independently on multiple occasions. The inclusion of seed testa colour as a character on both the morphological cladograms (LINDER & KURZWEIL, 1999; Appendix One) and the molecular phylogeny (BELLSTEDT *ET AL.*, 2001; Appendix Two) confirms this, with seed colour morphs occurring convergently in multiple clades across all sections represented (Figure 2.3).


A
1
2
3
4
2 cm
B
1 cm
C
1 cm
D
1 cm
E
1 cm
F
1.5 cm
G
1 cm

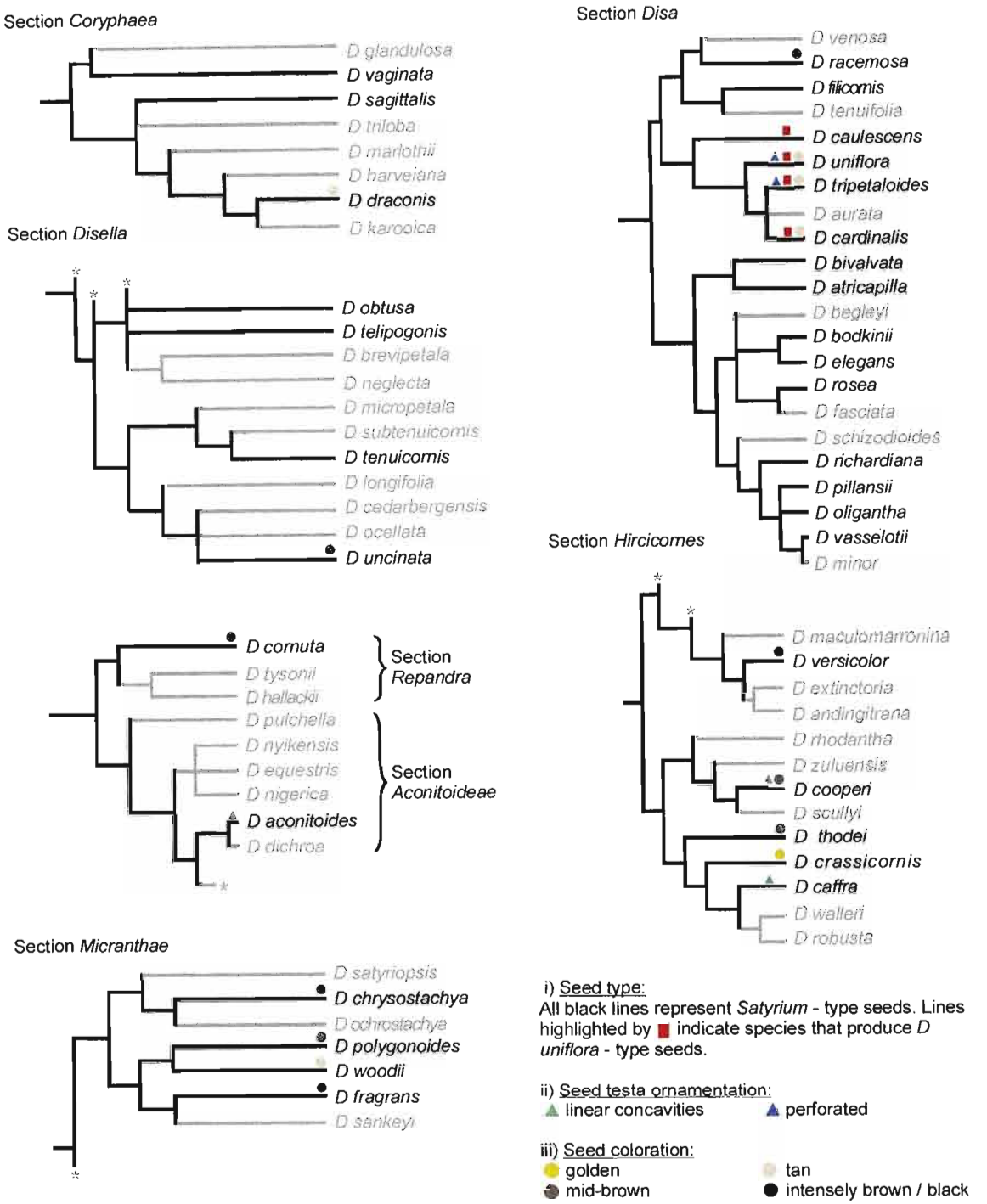


Figure 2.3. Evolution of morphometric seed characters for 34 South African *Disa* species. The characters plotted are i) seed type, ii) testa ornamentation and iii) testa coloration. The first two reflect a combination of data from this study with data from KURZWEIL (1993). Testa coloration data are available for 16 species and stem from this research. Only five species display testa ornamentation. Species for which data are unavailable are indicated in grey. An asterisk highlights where a portion of a cladogram has not been reproduced.

Cladograms are not available for sections *Emarginatae*, *Phlebidia* and *Stenocarpa*, which collectively contain seven species for which seed colour data are available. The former section is re-established as a monophyletic clade in the molecular phylogeny (BELLSTEDT *ET AL.*, 2001), but still contains multiple colour morphs across species *D. nervosa*, *D. patula* and *D. stachyoides*.

VAN WAES & DEBERGH (1986a, b) and RASMUSSEN (1995) proposed that the varying levels of phenolic compounds within the seed testa, which impart varying degrees of colouration, represent seed adaptation to environmental conditions. Such data sheds doubt on using seed colouration as a taxonomic character.

No links were identified between sections or sub-sectional clades and testa colouration except for *D. uniflora* and related species producing seeds of the same type. Although seed colour data is not available for *D. caulescens*, the remaining species have tan testae that are not translucent. However, these species do not form a single clade in the molecular phylogeny, nor is the production of tan seeds restricted to this or any sub-clade within section *Disa*. Tan seeds are distributed without phylogenetic constraint across three sections included (Figure 2.3).

Disa cooperi (section *Hircicornes*) is restricted to permanent marsh or seasonal seepage areas whilst *D. versicolor*, a member of the same section, is one of the most widely distributed *Satyrium* – type members of the genus and experiences highly variable environmental conditions. In either case the ecological advantages of delayed or asynchronous germination are apparent (RASMUSSEN, 1995), as imparted by mid-brown seed colour in the former and intensely brown in the latter species (Figure 2.3). The same is true for *D. woodii* (tan seed colouration) and *D. fragrans* (intensely brown colouration), a moist coastal and a high altitude grassland species respectively. Both species belong to the section *Micranthae* (Figure 2.3)

In species with *Satyrium*-type seeds, two different seed sizes and shapes were observed; i) typically fusiform and variously brown seeds (Plate 2.2A – G) with well developed embryos or rarely ii) filiform, colourless seeds without embryos (Plate 2.3A). In the latter, embryos are assumed to have aborted early during development, as reported for other orchid genera by ARDITTI (1982) and RASMUSSEN (1995). Seed maturity is therefore a controlling factor in phenolic deposition and the development of testa colouration. Where increased levels of phenolics impart a barrier to seed germination (VAN WAES & DEBERGH, 1986b; RASMUSSEN, 1995; THOMPSON *ET AL.*, 2001) it is likely to result in delayed germination.

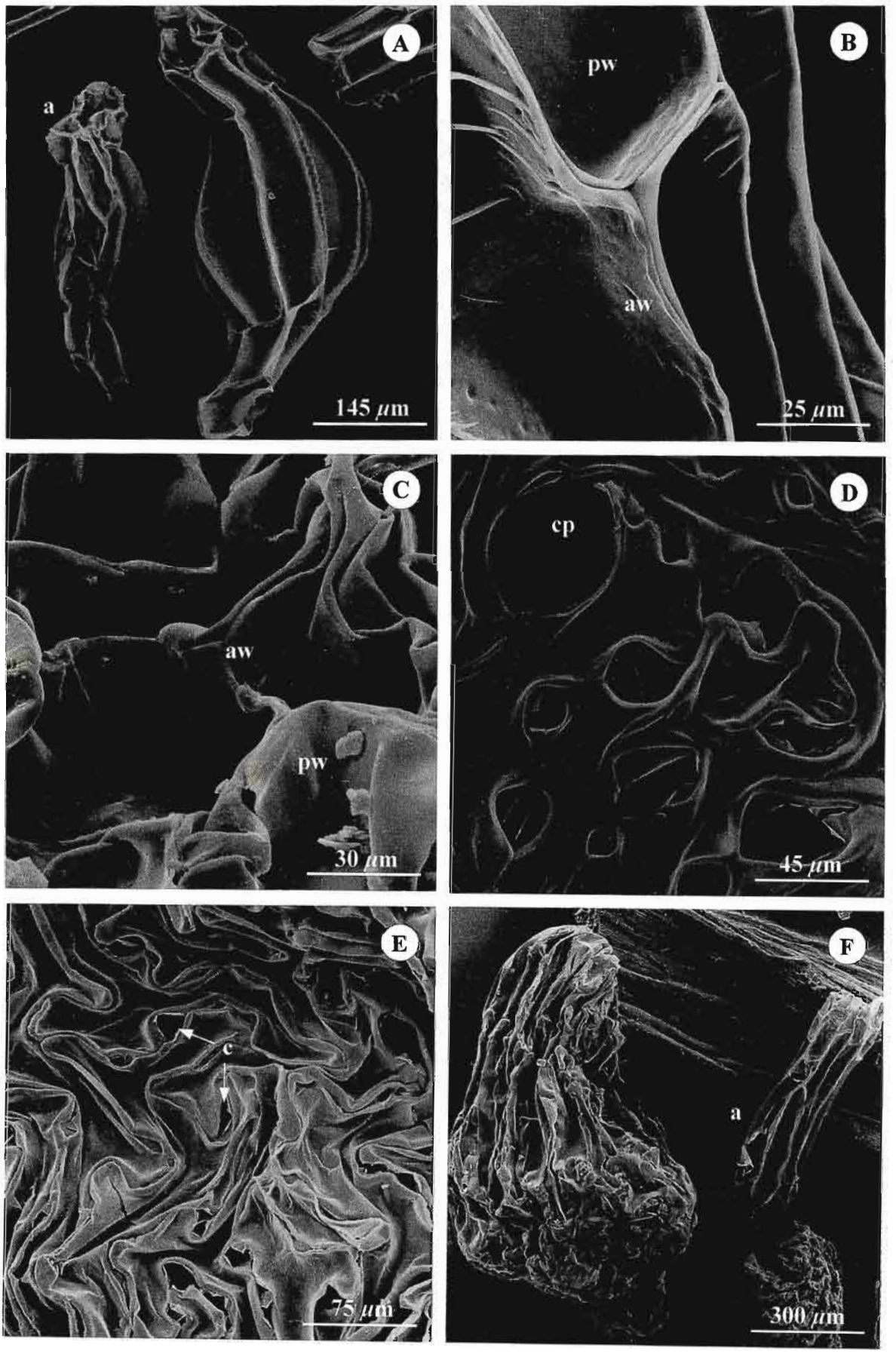
In several *Satyrium* – type species (viz. *D. versicolor*; Plate 2.2F and *D. nervosa*; Plate 2.2G) polymorphic testa colouration was observed. Since phenolic deposition and testa colouration are maturity dependent it can be assumed that within a polymorphic seed batch the pale seeds represent forms in which the testa is underdeveloped. The commonplace seed culture practice of 'green-podding' (ARDITTI, 1982; FAST, 1982; PRITCHARD, 1989; RASMUSSEN, 1995; THOMPSON *ET AL.*, 2001, 2002) is reliant on the fact that such seeds contain embryos capable of germination. Polymorphic testa colouration was not observed in the pale seeded species of either the *Satyrium* – or *D. uniflora* – type.

The coat in the *Satyrium*-type seeds consisted of collapsed testa cells with their outer periclinal walls concave and adpressed to the inner ones (Plate 2.3B). Alternatively, the periclinal testa cell walls of the *Disa uniflora* – type species were convex and smooth, raised above undulating anticlinal cell walls (Plate 2.3C). In *D. uniflora* (Plate 2.3D) and *D. tripetaloides* (Plate 2.3E) circular perforations and large cracks disrupt the integrity of the permeable testa. Perforated seed testae were found only in two of the species that produce the large *D. uniflora* – type seeds (Figure 2.3). According to both the morphological cladograms (LINDER & KURZWEIL, 1999; Appendix One) and the molecular phylogeny (BELLSTEDT *ET AL.*, 2001; Appendix Two), these are not sister species. Testa perforations either

evolved convergently in shared habitat, or evolved in a single clade that now contains mostly members that display a reversed character state (non-perforated seeds). Parsimony suggests the former. The reticular-foveate arrangement of testa cells in the mature *Satyrium* – type seed is also observed in the aborted seeds of the *D. uniflora* - type species, suggesting that both seed types have similar ontogenetic pathways (Plate 2.3F).

Two *Satyrium* – type *Disa* species, *D. cooperi* (Plate 2.4A) and *D. saxicola* (Plate 2.4B) display shallow, linear concavities on the periclinal walls. Similar ornamentation has been reported from *D. caffra* and *D. aconitoides* (KURZWEIL, 1993) – two species with the same seed type and was reported infrequently from other orchid genera (MOLVRAY, 2002). Linear concavities in the testa are restricted to four unrelated summer-rainfall species in sections *Aconitoideae*, *Hircicornes* (Figure 2.3) and *Stenocarpa*. Testa cell walls in the remainder of the *Satyrium* – type and all the *D. uniflora* – type species are without ornamentation. Testa cell reticulation is reported only from the sub-family Monandrae (BARTHLOTT, 1976; ARDITTI *ET AL.*, 1979; HEALEY *ET AL.*, 1980).

Commonly the seed testa of the *Satyrium* – type *Disa* species is made up of three cells in the long axis and four in the short axis. In *D. woodii* a single cell in the seed testa is highly elongate and accounts for most of the seed length (Plate 2.4C). The anticlinal walls of adjacent testa cells are fused, and protrude from the seed surface (Plate 2.4D) as a result of the concave and adpressed periclinal walls. The fusion of the anticlinal walls is variously incomplete, and a groove indicates its origin from two different cells. A triangular concavity highlights the corner where three cells meet (Plate 2.4E). The degree of fusion between testa cells impacts on impermeability, where the depth and narrowness of the intercellular groove (compounded by anticlinal wall protrusion) indicates relative cell fusion. *Disa pulchra* (Plate 2.1C) and *D. cornuta* (Plate 2.1F) differed in this regard, as do many of the other members of the genus. These differences do not correlate to taxonomic grouping, phytogeography or habitat.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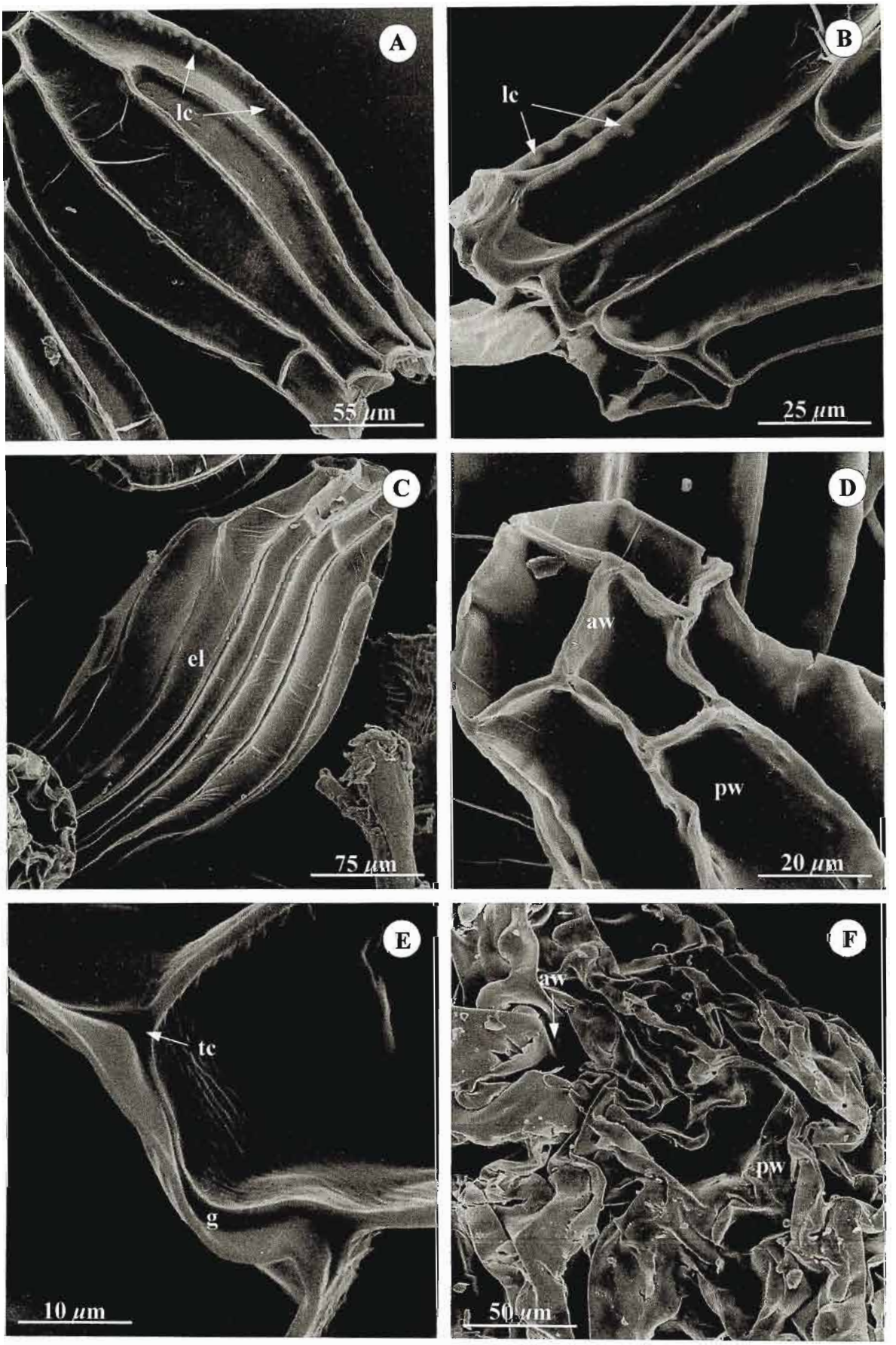
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In *D. uniflora* – type seeds an average of six and nine cells make up testa length and width respectively. However, the determination of cell margins is comparatively difficult due to the raised and undulating nature of the cell walls. Cell shape may be highly irregular (Plate 2.4F).

Beneath the cells of the testa in the *Satyrium* – type *Disa* species is a rudimentary inner integument that sheaths the embryo in a carapace. Because of its hydrophobic nature (VEYRET, 1969) and continuous coverage, the cellular detail of the embryo is obscured (Plate 2.5A). The thickness of the testa, made visible by the separation of testa cells, is highlighted in Plate 2.5B.

A sub-globular to globose embryo (l/w ratio = 1.13) was visible through the translucent testa of the *Satyrium* – type seed and was usually situated centrally (KURZWEIL, 1993). Usually the embryo occupies almost the entire width of the seed, with the mean diameter being only slightly smaller than that of the seed itself (Table 2.4 and Table 2.5). The embryo of the *D. uniflora* – type seed is not visible externally, but is situated centrally within the seed (KURZWEIL, 1993). All seeds have a prominent opening on the suspensor (Plate 2.5C), but this is blind-ended and does not penetrate to the embryo (VEYRET, 1969, 1974).

Embryos from *Satyrium* – type seeds measured 0.17 ± 0.02 x 0.15 ± 0.03 mm (mean ± SD; Table 2.5). These embryos were significantly shorter ($F_{1,148}$ = 303.75, $P < 0.01$) but not significantly narrower ($F_{1,148}$ = 2.157, P = 0.144) than their *D. uniflora* – type counterparts (0.35 ± 0.05 x 0.2 ± 0.04 mm; Figure 2.4). These data are in keeping with dimensions reported for *D. uniflora* embryos by THORNHILL & KOOPOWITZ (1992). No significant morphometric differences were recorded within *D. uniflora* ($F_{2,84}$ = 0.115, P = 0.891) and *Satyrium* – type ($F_{20,168}$ = 0.412, P = 0.988) embryos. These differences were non-significant within and between species of the same type (Figure 2.4).

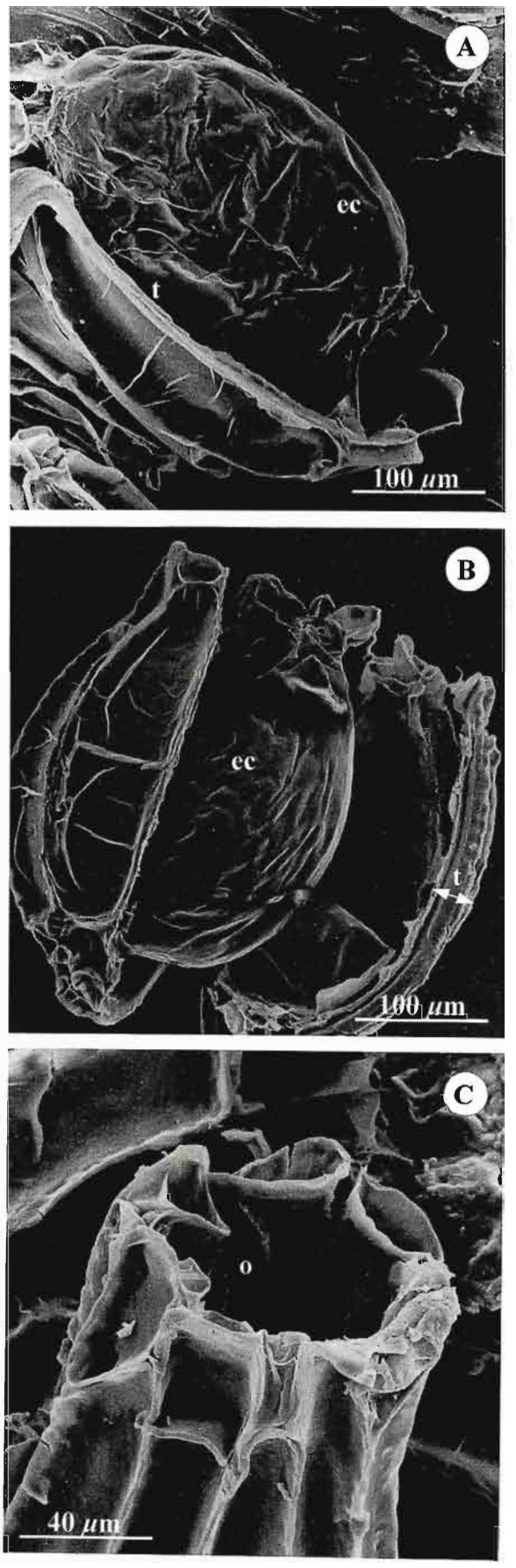
Embryo shape of the *D. uniflora* – type is subglobular, with a length : width ratio of approximately 1.8 (Table 2.5). Embryos are comparatively elongate, with mean length being significantly greater than mean embryo width ($F_{1,84}$ = 365.31, P < 0.01; Figure 2.4). *Satyrium* – type embryos are globose in shape and are non-significantly longer than they are wide ($F_{1,168}$ = 1.725, P = 0.336; Figure 2.4).

Table 2.5. Embryo morphometric data collected for 24 South African *Disa* species.

	Embryo					
	Length (l; mm)	Width (w; mm)	l/w	Volume (mm^3 x 10^{-3})	Seed volume / embryo volume	% air space
	mean (SD)	mean (SD)				
***Satyrium* - type embryo (*n* = 105)**	0.17 (0.02)	0.15 (0.04)	1.13	2.00	1.34	25.22
***Disa uniflora* - type embryo (*n* = 45)**	0.35 (0.05)	0.20 (0.04)	1.75	7.34	27.13	96.31

Percentage air space = [(seed volume – embryo volume) / seed volume x 100].

In the *D. uniflora* – type species the dimensions of the embryo are roughly one-third times as large as those of the entire seed. Therefore despite the significant discrepancy in seed size between the two seed types, embryo sizes – specifically width, are similar Figure 2.4. The relatively elongate embryos of the *D. uniflora* – type are comparable in length to the *Satyrium* – type seeds. Consequently, embryo volumes are markedly different between seed types, with the former being 3.6 times larger than the latter (7.34 and 2.00 mm^3 x 10^{-3} respectively; Table 2.5). Such data lends support to the speculation of endosperm in the *D. uniflora* – type species and may account for the rapid germination response observed in these species.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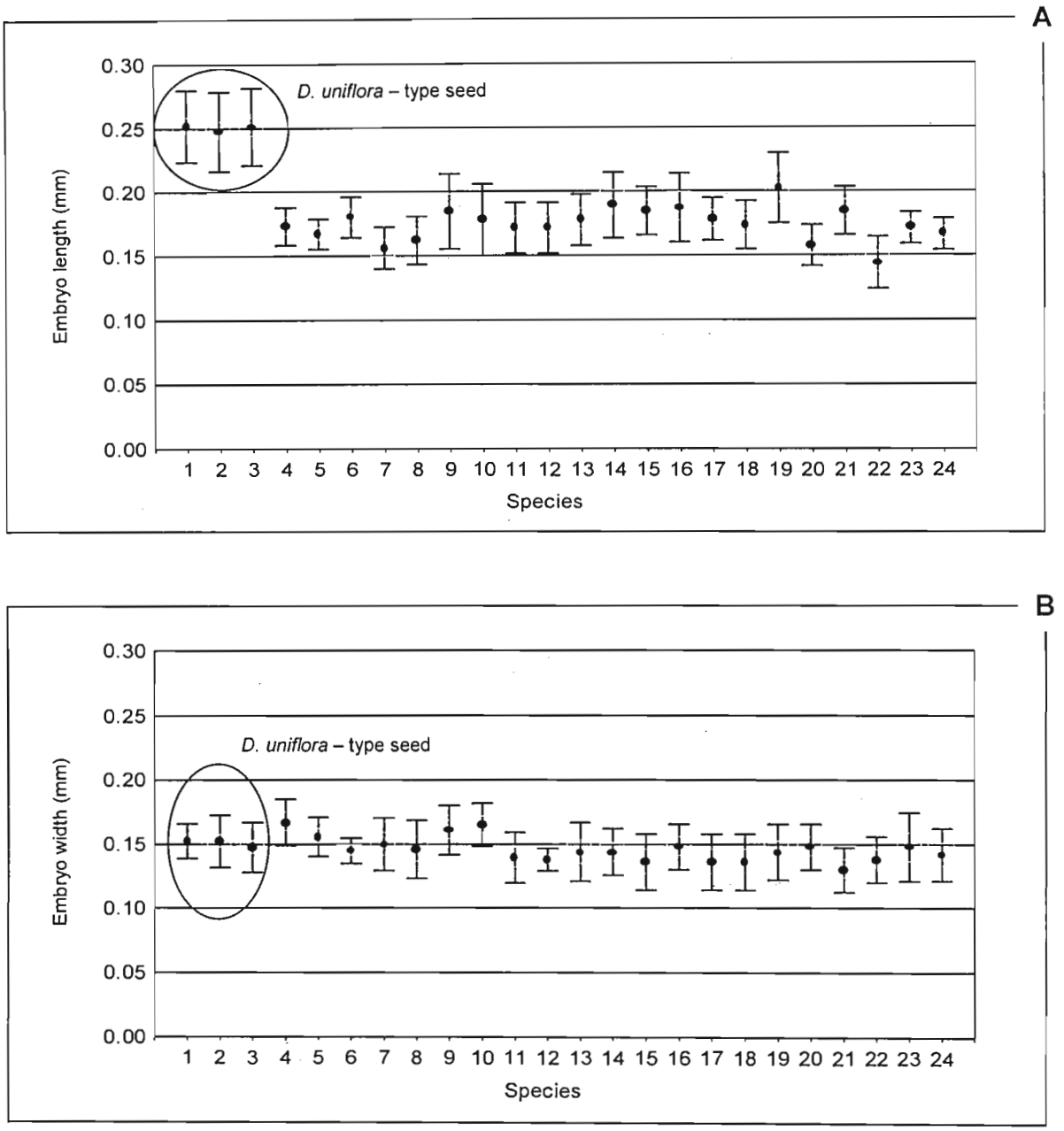


Figure 2.4. **Embryo dimensions in *Disa* (mean ± SD, *n* = 15 for *D. uniflora* - and 5 for *Satyrium* – type species). Specific embryo lengths** (A) **and specific embryo widths** (B). ***Disa uniflora* – type seeds are indicated. All measurements in mm. Species: 1 – *D. cardinalis*; 2 – *D. tripetaloides*; 3 – *D. uniflora*; 4 – *D. brevicornis*; 5 – *D. cephalotes*; 6 – *D. chrysostachya*; 7 – *D. cooperi*; 8 – *D. cornuta*; 9 – *D. crassicornis*; 10 – *D. draconis*; 11 – *D. fragrans*; 12 – *D. longicornu*; 13 – *D. nervosa*; 14 – *D. patula*; 15 – *D. polygonoides*; 16 – *D. pulchra*; 17 – *D. racemosa*; 18 – *D. sagittalis*; 19 – *D. saxicola*; 20 – *D. stachyoides*; 21 – *D. thodei*; 22 – *D. versicolor*; 23 – *D. woodii*; 24 – *D. uncinata*.**

However, large embryos and increased germinability are not mutually exclusive, since several species with small embryos are also germinated *in vitro* (ARDITTI, 1982; VOGELPOEL, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997; THOMPSON *ET AL.*, 2001, 2002).

Hydrochory affords *D. uniflora* and species with the same seed type, the luxury of large seeds and embryos, which germinate immediately post-dispersal. A seed volume : embryo volume ratio of approximately 27 and a resultant percentage air space of 96 % make such seeds relatively buoyant and theoretically suited to wind dispersal. However, the large seed and embryo volumes and associated increased seed mass preclude this. A small percentage of seeds are undoubtedly dispersed by wind, which explains colonization of new habitats and may be the only mode of seed dispersal in species of this type growing away from watercourse margins (KURZWEIL, 1993, 2000). This should be regarded as a disadvantage since the majority of the wind dispersed seeds will be deposited in habitats where they have no chance of survival. Many characteristics of the seed that suit anemochory, especially a high percentage air space, are equally advantageous in hydrochory. ARDITTI *ET AL.* (1979, 1980) and HEALEY *ET AL.* (1980) recorded the percentage air space, in six northern hemisphere anemochorous terrestrial genera, as between 63 % and 96 %.

The perforated testa displayed in two *D. uniflora* – type species nullifies the advantages offered by a high percentage air space. It is conceivable that such seeds become waterlogged fairly rapidly, facilitating dispersal. In the case of *D. uniflora*, plants are found only in the upper reaches of mountain streams (LINDER, 1981; VOGELPOEL, 1987; LACROIX & LACROIX, 1997; LINDER & KURZWEIL, 1999).

Satyrium – type *Disa* species are exclusively wind dispersed and display a seed volume : embryo volume ratio of approximately 1.3 and a corresponding air space of 25 % (Table 2.5), far lower than values reported in the literature (ARDITTI *ET AL.*,1979, 1980; HEALEY *ET AL.*, 1980; RASMUSSEN, 1995; ARDITTI & GHANI, 2000).

Considerable variation can exist in embryo and seed volumes (ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; RASMUSSEN, 1995) but percentage air space is relatively uniform because the embryo occupies a comparatively small portion of the total seed volume. Even relatively large increases in embryo dimensions do not appreciably decrease the percent air space of the seed. In *Disa*, it is primarily the large differences in seed sizes between the *Satyrium* and *Disa uniflora* – type seeds that produces the different percentage air spaces (25 % vs. 96 % respectively).

Taxonomic studies reliant on orchid seed morphometrics are abundant in the literature, particularly for the delimitation of subfamilial or tribal taxa (BARTHLOTT, 1976; ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; TOHDA, 1983, 1985, 1986; CHASE & PIPPEN, 1988, 1990; KURZWEIL *ET AL.*, 1991; PETTERSSON, 1991; KURZWEIL, 1993; 2000; ARDITTI & GHANI, 2000). However, this study has revealed highly variable seed morphology across *Disa*, with the recognition of two distinct seed types. Seed type, colouration and testa ornamentation appear to be convergent within this genus.

CHAPTER THREE

ASYMBIOTIC SEED CULTURE I: ASSESSING OLD IDEAS AND ESTABLISHING *DISA* GERMINABILITY *IN VITRO*

Summer-rainfall *Disa* species have not been germinated previously *in vitro*. Four first-time germinations are reported here, from media with i) increased water availability (decreased viscosity or reduced inorganic salt concentrations) and ii) charcoal supplementation. The control of germination in these small-seeded *Disa* species is proposed as a trade-off between water availability and the presence of phyto-inhibitors in the environment of the embryo – two features typical of seeds exhibiting water-impermeable dormancy. Germination rate (> 12 weeks), percentage (< 30 %) and synchrony were most favourable under conditions of increased water availability, but are not in agreement with the values reported for easily germinable, winter-rainfall species (> 80 % germination in eight weeks). Not all small-seeded, summer-rainfall species responded to media manipulation – delimiting three germinability categories within *Disa*; i) easily germinable species, ii) poorly germinable species through media manipulation, and iii) ungerminated species despite protocol modification. Ease of germinability was related to phytogeography, climate and, to a lesser degree, seed type. Immature seed culture of the summer-rainfall *Disa* species revealed optimal excision times of six weeks, which is congruent with the remainder of the genus. Germination of immature seed in the absence of media modification was comparable to mature seed germination under modified conditions, providing further evidence of the role of the seed testa in regulating germination. Despite method modification and immature seed culture, the bulk of summer-rainfall *Disa* species remain ungerminated *in vitro*, fuelling conservation fears.

3.1 INTRODUCTION

To conserve rare and threatened orchids many authors have suggested *in vitro* seed germination as a suitable propagation method (ARDITTI, 1967, 1979, 1982; ARDITTI, MICHAUD & OLIVA, 1981; CLEMENTS, 1982; FAST, 1982; HADLEY, 1982; BALLARD, 1990; THORNHILL & KOOPOWITZ, 1992; ZETTLER & MCINNIS,

1993). However, most terrestrial orchid seed – particularly of temperate origin, is difficult to germinate both *in* and *ex vitro* due to specific requirements for nutrients and environmental conditions (LIDDELL, 1944; ARDITTI, 1982; VAN WAES & DEBERGH, 1986a; RASMUSSEN, 1995; VUJANOVIC, ST-ARNAUD, BARABÉ & THIBEAULT, 2000). These requirements may vary considerably within a genus (MCALPINE, 1947; STOUTAMIRE, 1964, 1974; ARDITTI, 1967; ARDITTI *ET AL.*, 1981; CLEMENTS, 1982; HADLEY, 1982; OLIVA & ARDITTI, 1984). Many species therefore remain ungerminated using asymbiotic methods (VEYRET, 1969; ARDITTI, ERNST, YAM & GLABE, 1990; RASMUSSEN, JOHANSEN & ANDERSEN, 1991; RASMUSSEN, 1995; ZETTLER & HOFER, 1998; MICHEL, 2002). Other species, although responsive *in vitro*, have resisted *ex vitro* transfer (CLEMENTS *ET AL.*, 1986).

Much attention has focussed on determining the nutritional and cultural requirements for optimal germination and early seedling development *in vitro* (HARRISON, 1970; HARVAIS, 1974; CLEMENTS & ELLYARD, 1979; CLEMENTS, 1988; YAM & WEATHERHEAD, 1988; MASUHARA & KATSUYA, 1989; YAM, ERNST, ARDITTI, NAIR & WEATHERHEAD, 1990; GEORGE, 1993; RASMUSSEN, 1995). RASMUSSEN (1995) reported 40 media as being capable of sustaining terrestrial orchid germination *in vitro*. Beneficial modifications include reduced concentrations of inorganic salts and increased organic compounds (HARVAIS, 1972, 1973, 1974; THOMPSON, 1974; ARDITTI *ET AL.*, 1979, 1980; ARDITTI, 1982; FAST, 1982; NAKAMURA, 1982; ARDITTI & ERNST, 1984; OLIVA & ARDITTI, 1984; MITCHELL, 1989; ANDERSON, 1991; CHU & MUDGE, 1994; RASMUSSEN, 1995). Early elimination of mineral salts is advantageous (ARDITTI, 1982; RASMUSSEN, 1995) as a high water potential is desirable for germination, but will not sustain seedling development. Liquid substrates also ensure a high water potential, yet they are not generally used in orchid germination as they offer little mechanical stability (LINDQUIST, 1960; ARDITTI, 1982; RASMUSSEN, 1995). GALUNDER (1984) recommended the use of soft agar (6 g L^{-1} agar) as a practical compromise, although KOHL (1962) and CHU & MUDGE (1994) reported beneficial, or at worst, neutral effects after using liquid culture.

Organic compounds in the form of carbohydrates, vitamins, amino acids and growth regulators may be incorporated into media directly, or through undefined additives such as coconut milk, banana homogenate or potato extract (ARDITTI, 1982; ARDITTI & ERNST, 1984; CHU & MUDGE, 1994) – resulting in increasingly complex media in which no single factor can be identified as the germination-stimulating agent. DOWNIE (1940, 1943), STOUTAMIRE (1964, 1974), HADLEY (1982), VAN WAES & DEBERGH (1986b) and RASMUSSEN (1995) established the minimum germination requirements for a number of Holarctic species and revealed that some species germinated readily on a basal medium, whilst others had variously intricate nutrient requirements. RASMUSSEN (1995) concluded that the germination percentage for a particular species generally increased with increasing media complexity. The few data that exist regarding the efficacy of individual substrate constituents are summarized in Table 3.1.

Table 3.1. Substrate constituents reported as having positive (+), or at worst, neutral (/) effects on percentage germination in terrestrial orchids.

Broad substrate additive	Constituent and effect on germination
Carbohydrate[1]	glucose (+), sucrose (+)
Nitrogen	organic compounds (amino acids, +), NH_4^+ (/)
Other ions	Ca^{2+} (/), Cl^- (+), K^+ (+),
Plant growth regulators	auxins (specifically IAA, IBA and NAA, /)
	cytokinins (specifically BA and kinetin, +)
Vitamins	nicotinic acid (+), pyridoxine (+), thiamine (+)
Undefined organic additives[2]	fruit and vegetable homogenates (+)
" inorganic additives	activated charcoal (+)

After HENDRICKSON, 1951; RAGHAVAN & TORREY, 1964; ARDITTI, 1967, 1982; BORRIS, 1969; HARVAIS, 1972, 1973; PIERIK & STEEGMANS, 1972; SMITH, 1973; MEAD & BULARD, 1975, 1979; STRAUSS & REISINGER, 1976; LUCKE, 1977; ARDITTI & ERNST, 1984; VAN WAES & DEBERGH, 1986b; VAN WAES, 1987; YAM & WEATHERHEAD, 1988; BUTCHER & MARLOW, 1989; YAM *ET AL.*, 1990; GEORGE, 1993; MIYOSHI & MII, 1995; RASMUSSEN, 1995; MICHEL, 2002.

[1] carbon sources suitable for germinating orchid seed were reported by ARDITTI (1967), ERNST (1967), ERNST, ARDITTI & HEALEY (1971), ARDITTI (1979, 1982), BUTCHER & MARLOW (1989) and reviewed by ARDITTI & ERNST (1984) and RASMUSSEN (1995).

[2] chemically complex and contain a variety of soluble carbohydrates, amino acids, vitamins and plant growth regulators.

Optimal *in vitro* parameters viz. temperature and light, are subject to specific variation and reflect taxonomic affiliation and differences in habitat. Optimum incubation temperatures were reported in the range 22 – 25 °C (BORRIS, 1969; STOUTAMIRE, 1974, 1990; ARDITTI, 1982; OLIVA & ARDITTI, 1984; RASMUSSEN, 1995; MICHEL, 2002). Alternatively, RASMUSSEN, ANDERSEN & JOHANSEN (1990) reported that temperate orchids required temperatures somewhat lower (< 20 °C) than those reported elsewhere in the literature. However, data are available that have revealed the positive effects of cold and warm stratification seed treatments prior to long-term incubation (WITHNER, 1959; NAKAMURA, 1976; HADLEY, 1982; FAST, 1982; PRITCHARD, 1985; BALLARD, 1987; VAN DER KINDEREN, 1987; VAN WAES, 1987; COKE, 1990; STOUTAMIRE, 1990; YANETTI; 1990; RASMUSSEN, 1992; DE PAUW & REMPHREY, 1993; MIYOSHI & MII, 1998).

Many terrestrial species benefit from incubation in continuous darkness for a minimum of 14 days following sowing (HARVAIS & HADLEY, 1967; FAST 1982; VAN WAES & DEBERGH, 1986b; BALLARD, 1987; VAN WAES, 1987; RASMUSSEN *ET AL.*, 1990; RASMUSSEN & RASMUSSEN, 1991; RASMUSSEN, 1992, 1995). However, variants include continuous low-light and continuous darkness treatments and a range of low-light : dark photoperiods (KNUDSON, 1943; STOUTAMIRE, 1963, 1964, 1974; ARDITTI, 1967, 1979; HARVAIS, 1972, 1973; CLEMENTS, 1982; ANDERSON, 1991; RASMUSSEN & RASMUSSEN, 1991; ZETTLER & MCINNIS, 1994).

In addition, differences in seed maturity influence *in vitro* germination success. Immature seed ('green-podding') is widely used in orchid seed culture for the germination of terrestrial species (STOUTAMIRE, 1964, 1974; BORRIS, 1969; SAULEDA, 1976; LINDÉN, 1980, 1992; VOGELPOEL, 1980, 1987; ARDITTI, 1982; CLEMENTS, 1982; FAST, 1982; OLIVA & ARDITTI, 1984; PRITCHARD, 1989; ANDERSON,1990, 1991; RASMUSSEN *ET AL.*, 1991; RASMUSSEN & RASMUSSEN, 1991; ZETTLER & MCINNIS, 1992; RASMUSSEN, 1995; LACROIX & LACROIX, 1997; WODRICH, 1997; CROUS, 1999, PERS. COMM; VOGELPOEL, 2001, PERS. COMM; WODRICH, 2001, PERS. COMM; MICHEL, 2002). However, the appropriate developmental stage for

excision of the developing seeds must be assessed for each species, with MICHEL (2002) reporting optimal harvest times in the range 21 – 360 days post-pollination for tropical terrestrial species. By contrast, RASMUSSEN (1995) reported a maximum of 63 days for Holarctic terrestrials.

'Green-podding' removes the need for seed decontamination, which is desirable since orchid seed displays specific sensitivity to decontaminants (BERGMAN, 1995, 1996; RASMUSSEN, 1995). However, exposure of the mature seed to decontaminants is not necessarily disadvantageous as chemical scarification increases the percentage germination in several Holarctic terrestrials (STOUTAMIRE, 1963; HARVAIS & HADLEY, 1967; PURVES & HADLEY, 1976; HARVAIS, 1980; LINDÉN, 1980; ARDITTI, 1982; VAN WAES & DEBERGH, 1986a, b; RASMUSSEN, 1992, 1995; MIYOSHI & MII, 1995). BERGMAN (1995), RASMUSSEN (1995) and MIYOSHI & MII (1995, 1998) demonstrated the hypochlorites, particularly calcium hypochlorite – $Ca(OCl)_2$, as the reagents of choice for decontaminating orchid seed. Hydrogen peroxide (H_2O_2) and ethanol (EtOH) were offered as alternative decontaminating agents by MASUHARA & KATSUYA (1989) and YANAGAWA, NAGAI, OGINO & MAEGUCHI (1995).

The germination of *Disa* species *in vitro* has been documented (LINDQUIST, 1960; STOUTAMIRE, 1974; VOGELPOEL, 1980, 1987, 1993; ARDITTI, 1982; LACROIX & LACROIX, 1997; WODRICH, 1997; MICHEL, 2002). However, these limited data suggest that despite comparatively high germination percentages (80 – 90 %), germination was achieved for only a small proportion (29 of 163 spp., approximately 18 %) of species (Table 3.2). This is in keeping with LACROIX & LACROIX (1997), who reported that critically few terrestrial orchids from Africa have been reared successfully when germinated asymbiotically.

Only the 11 species (7 % of the genus) registered with the RHS (Table 3.2) are germinated consistently *in vitro* and are in widespread cultivation. These *Disa* species form the parent base for all cultivated *Disa* hybrids – many of which have

been in cultivation for over 200 years (COLLETT, 1971; ARDITTI, 1982; ORCHARD, 1999, PERS. COMM.).

Table 3.2. *Disa* species germinated *in vitro*. Sectional classification (LINDER & KURZWEIL, 1999), seed type (*sensu* KURZWEIL, 1993; s – small, l - large) and rainfall-mediated endemism are included; w – winter rainfall endemic, ws – widespread species. Threatened *Disa* species are highlighted in grey (HILTON-TAYLOR, 1996a, b, 1997; GOLDING, 2002; VICTOR, 2002) whilst an asterisk denotes those currently registered with the Royal Horticultural Society (RHS; MICHEL, 2002).

Species	Section	Seed-type	Endemism
*D. atricapilla**	*Disa*	s	w
*D. aurata**	*Disa*	l presumed	w
D. barbata	*Herschelianthe*	s	w
*D. bivalvata**	*Disa*	s	w
*D. cardinalis**	*Disa*	l	w
*D. caulescens**	*Disa*	l	w
D. cornuta	*Repandra*	s	ws
D. cernua	*Monadenia*	s	w
D. draconis	*Coryphaea*	s	w
D. elegans Reichb.f	*Disa*	s	w
D. fasciata Lindl.	*Disa*	s	w
D. ferruginea	*Stenocarpa*	s	w
D. graminifolia Ker-Gawl.*	*Herschelianthe*	s	w
*D. longicornu**	*Phlebidia*	s	w
D. lugens	*Herschelianthe*	s	w
D. maculata	*Phlebidia*	s	w
D. marlothii	*Coryphaea*	s	w
D. obtusa	*Disella*	s	w
D. ophrydae (Lindl.) Bolus	*Monadenia*	s	w

Table 3.2. (continued)

Species	Section	Seed-type	Endemism
D. purpurascens Bolus	*Herschelianthe*	s	w
*D. racemosa**	*Disa*	s	w
D. sagittalis	*Coryphaea*	s	ws
D. spathulata	*Herschelianthe*	s	w
D. tenuicornis	*Disella*	s	w
D. tenuifolia Sw.	*Disa*	s	w
*D. tripetaloides**	*Disa*	l	ws
D. uncinata	*Disella*	s	w
*D. uniflora**	*Disa*	l	w
*D. venosa**	*Disa*	s	w

Germination data after LINDQUIST, 1960; COLLETT, 1971; STOUTAMIRE, 1974; VOGELPOEL, 1980, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997; CROUS, 1999, PERS. COMM; ORCHARD, 1999, PERS.COMM; VOGELPOEL, 2001, PERS. COMM; WODRICH, 2001, PERS. COMM; MICHEL, 2002.

With the exception of *D. graminifolia* (section *Herschelianthe*) and *D. longicornu* (section *Phlebidia*), these species belong to *Disa* section *Disa* (LINDER & KURZWEIL, 1999; Appendix One). Many are evergreen (*D. racemosa*, *D. venosa* and the *D. uniflora* sub-clade; Appendix One), being associated with perennial water (WODRICH, 1997; LINDER & KURZWEIL, 1999; ORCHARD, 1999, PERS. COMM.). Members of the *D. uniflora* sub-clade (Appendix One) produce anomalous, large seeds (KURZWEIL, 1993; Chapter Two). On a broader scale, phytogeographic evaluation revealed that all *Disa* species germinated previously *in vitro* are winter-rainfall endemics or widespread species.

In general the media, culture conditions and the means of decontamination recommended for *Disa* germination (COLLETT, 1971; STOUTAMIRE, 1974; VOGELPOEL, 1980, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997; CROUS, 1999, PERS. COMM; ORCHARD, 1999, PERS. COMM; VOGELPOEL, 2001, PERS. COMM; WODRICH, 2001, PERS. COMM; MICHEL, 2002) are consistent with those reported more widely in the literature for other terrestrial taxa. If anything, *Disa*

germination is cited as being comparatively easy and simple, with their germination requirements being likened to those of tropical species (COLLETT, 1971; STOUTAMIRE, 1974; MICHEL, 2002). Several authors (COLLETT, 1971; VOGELPOEL, 1980, 1987) have reported germination in *D. uniflora* despite the absence of exogenous carbohydrates.

Information regarding the *in vitro* germination of *Disa* seed is fragmented. No reports deal with the germination of any southern African summer endemic *Disa* species (43 spp.). Although this may be attributed in part to neglect, lack of visual appeal or provenance, cultures for these species that have been attempted have been unsuccessful. Germination therefore has not been, or cannot be, achieved for over 80 % of *Disa* species, a critical statistic considering that over 50 % (68 of 131 spp.) of South African species were considered threatened by HILTON-TAYLOR (1996a, b, 1997) and VICTOR (2002).

3.2 AIMS

- To establish a suitable decontamination protocol for representative small-seeded *Disa* species.
- To document *in vitro* germinability for mature, small-seeded *Disa* species.
- To determine the optimal harvest times for immature *Disa* seed culture.
- To examine the *in vitro* germination requirements for previously ungerminated *Disa* species.

3.3 MATERIALS AND METHODS

Undehisced capsules from 13 summer-rainfall endemic *Disa* species (Table 3.3) were collected in autumn (February – April; 1998 – 2000) from wild

plants. A minimum of 15 capsules were removed from the spikes of at least 10 plants. Capsules were collected in small paper envelopes and air-dried at ambient laboratory temp (25 ± 2 °C). Dehiscence typically took place within 72 h. For each species, mature seed was combined and passed through a nylon mesh to remove debris. Seed was stored, in darkness, in sealed glass vials at ± 4 °C with 0 % relative humidity (CLEMENTS, 1982; ZETTLER & MCINNIS, 1992). Storage time was kept to a minimum (LINDQUIST, 1960; ARDITTI, 1982) and varied from several days to four weeks.

Table 3.3. *Disa* species for which *in vitro* seed germination was attempted during the study period 1998-2000. All species are summer rainfall endemics and produce small-type seeds (*sensu* KURZWEIL, 1993). Sectional classification after LINDER & KURZWEIL (1999) is included in parentheses.

D. brevicornis (*Monadenia*)	*D. patula* var. *transvaalensis* (*Emarginatae*)
D. cephalotes subsp. *cephalotes* (*Stenocarpa*)	*D. pulchra* (*Stenocarpa*)
D. chrysostachya (*Micranthae*)	*D. saxicola* (*Stenocarpa*)
D. cooperi (*Hircicornes*)	*D. stachyoides* (*Emarginatae*)
D. crassicornis (*Hircicornes*)	*D. versicolor* (*Hircicornes*)
D. fragrans (*Micranthae*)	*D. woodii* (*Micranthae*)
D. nervosa (*Emarginatae*)	

In addition to mature seed culture, 'green-pod' trials were conducted for *D. chrysostachya*, *D. cooperi*, *D. pulchra* and *D. versicolor* (Table 3.3) across four broad seed maturity classes. Where possible, a minimum of five spikes towards the end of their flowering period were collected for each species and removed to the laboratory, where they were placed in water. Sequential acropetalous maturation meant that capsules spanning six weeks in age were located on individual spikes, with dehiscence occurring eight weeks after pollination. Capsule maturity was defined on capsule diameter and colour:

- First quarter – two weeks after pollination (flower senesced, capsule unswollen and green)
- Second quarter – four weeks after pollination (capsule moderately swollen and green)
- Third quarter – six weeks after pollination (capsule fully swollen and beginning to colour)
- Fourth quarter – dehiscent capsules, eight weeks after pollination (capsule swollen and coloured).

Capsules remained attached to the spike until cultures were initiated, which took place within two days of collection (ARDITTI *ET AL.*, 1981).

3.3.1 DECONTAMINATION PROTOCOLS

Mature seed from *D. cooperi* and *D. versicolor* was subjected to the following decontamination treatments:

Table 3.4. Decontamination protocols initiated for mature *Disa* seed. All decontaminants were applied as liquid pretreatments unless otherwise indicated.

Treatment	Duration	Reference
1.75 (w/v) % NaOCl	5, 10, 20, 30, 60 and 120 min	VAN WAES & DEBERGH (1986a, b), RASMUSSEN (1995)
0.01 % NaOCl	media additive	YANAGAWA *ET AL.* (1995)
15 % H_2O_2	10, 15 and 20 min	MASUHARA & KATSUYA (1989), YANAGAWA *ET AL.* (1995), CROUS (1999, PERS. COMM.)
0.01 – 1 % H_2O_2	media additive	YANAGAWA *ET AL.* (1995)
70 % EtOH	2 – 10 min	MASUHARA & KATSUYA (1989), YANAGAWA *ET AL.* (1995)
1 % Benlate®	5, 10 and 15 min	BECK (1999)

Table 3.4. (continued)

Treatment	Duration	Reference
1 % Virkon®	5, 10 and 15 min	BECK (1999)
Cl^{2-} (gas)[1]	10, 20 & 30 min	LAST (1999, PERS. COMM.)
Paraformaldehyde (gas)[2]	60, 120 & 180 min	LAST (1999, PERS. COMM.)
1 % Plant Preservative Mixture® (*PPM*)	10, 20 & 30 min	BECK (1999), FORD (1999)
0.1 – 1 % *PPM*®	media additive	BECK (1999), FORD (1999)

[1] prepared by adding 3 ml 1 *M* HCl to 100 ml 3.5 % NaOCl
[2] prepared by adding 1 ml sterile dH_2O to 5 ml paraformaldehyde powder

Seeds clumped together during aqueous pretreatments. Consequently small seed quantities (approximately 5 ml) were mixed with five times the volume of sterilant. One percent (v/v) Tween-20 was also added to all liquid decontamination treatments to enhance contact between seed and sterilant (RASMUSSEN, 1995). Decontamination was terminated by triple rinses in sterile dH_2O, except when treated with H_2O_2 or EtOH where seeds were cultured unrinsed (YANAGAWA *ET AL.*, 1995; CROUS, 1999, PERS. COMM.). Customized, autoclavable nylon sieves were used to move seed between solutions. Gaseous decontamination was conducted by exposing open culture vessels, after seed placement, to the sterilant within a bell-jar. Treatments were applied singly or in combination.

Decontamination of undehisced *Disa* capsules was conducted by submersion in 3.5 % NaOCl (5, 10, 15 and 30 min) or by alternating flaming (10 sec each) with three rinses (10 sec each) in 100 % EtOH. Capsules were plunged into cold, sterile dH_2O between flaming. Again, treatments were applied singly and in combination. Ascorbic acid (100 mg L^{-1}; 10 and 20 min), citric acid (150 mg L^{-1}; 10 and 20 min) and 1 % polvinylpyrrolidone (*PVP*; 10 and 20 min) aqueous rinses were used to inhibit phenolic browning of immature tissues (BECK,

1998, PERS. COMM.). The latter was reported by TOMITA & TOMITA (1997) to enhance germination of *Cypripedium macranthos* Sw. *in vitro*.

Disinfection trials were carried out on full strength MS media salts (MURASHIGE & SKOOG, 1962), supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol, 0.1 mg L^{-1} thiamine, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine and 2 mg L^{-1} glycine. The media *p*H was adjusted to 5.8 and solidified (8 g L^{-1} UNILAB agar) in 65 mm (internal diameter; ø) plastic petri dishes for mature seed and glass culture jars (ø 60 mm) for 'green-pod' cultures. Dishes were sealed with Parafilm. Incubation took place at 25 ± 2 °C under continuous illumination of 8.5 μmol m^{-2} s^{-1} supplied by cool white fluorescent tubes. Percentage disinfection was recorded seven days after sowing and represents the mean count from a minimum of fifty cultures. Where possible, cultures were viewed under a Wild Heerbrugg M400 photomicroscope. The optimum decontamination regimes identified here for mature seed and capsules respectively were used in all subsequent asymbiotic seed cultures.

3.3.2 SEED CULTURE *IN VITRO*: GERMINATION AND EARLY SEEDLING DEVELOPMENT

Many media have been recommended for asymbiotic seed germination (ARDITTI, 1982; RASMUSSEN, 1995). KNUDSON C (ARDITTI, 1982 modified after KNUDSON, 1946) and MS media, in various formulations, have been used extensively for temperate terrestrial germination (ARDITTI, 1982; CLEMENTS, 1982; FAST, 1982; RASMUSSEN, 1995; MICHEL, 2002) and were investigated in order to establish their value in the context of asymbiotic *Disa* seed culture (Table 3.3). A number of *Disa* species have been germinated on these media (VOGELPOEL, 1980, 1993; WODRICH, 1997; CROUS, 1999, PERS. COMM; ORCHARD, 1999, PERS. COMM; VOGELPOEL, 2001, PERS. COMM; WODRICH, 2001, PERS. COMM; MICHEL, 2002).

ARDITTI (1982) solidified KNUDSON C medium with 12 – 15 g agar, whilst the modifications of STOUTAMIRE (1964) and ANDERSON (1990) employed 8 and 6 g L^{-1} agar respectively. In this study both KNUDSON C and MS media were solidified with 8 g L^{-1} agar to regulate diffusion, absorption and water potential effects (GEORGE, 1993). Media were solidified in petri dishes (15 ml media), glass culture tubes (ø 25 mm; 10 ml media) and culture jars (25 ml media). Liquid cultures were continuously agitated by means of an orbital shaker operating at 50 cycles min^{-1} (OLIVA & ARDITTI, 1984; CHU & MUDGE, 1994). Decontaminated mature seed was dispersed over the media surface in jar cultures in approximately 1 ml dH_2O using a sterile spatula, or onto Whatman No. 1 filter paper discs that were placed onto the media surface in dish cultures. Sowing density was 150 – 250 seeds per vessel.

i) KNUDSON C medium (ARDITTI, 1982 after KNUDSON, 1946)

$Ca(NO_3)_2.4H_2O$	1 g L^{-1}
$(NH_4)_2SO_4$	0.5 g L^{-1}
$MgSO_47H_2O$	0.25 g L^{-1}
KH_2PO_4	0.228 g L^{-1}
$FeSO_4.7H_2O$	25 mg L^{-1}
K_2HPO_4	7.8 mg L^{-1}
$MnSO_4.4H_2O$	7.5 mg L^{-1}
$ZnSO_4$	0.331 mg L^{-1}
H_3BO_3	0.056 mg L^{-1}
$CuSO_4$	0.04 mg L^{-1}
MoO_3	0.016 mg L^{-1}
Sucrose	20 g L^{-1}
	*p*H adjusted to 5.8

ii) MS medium (MURASHIGE & SKOOG, 1962), excluding vitamin additives

KNO_3	1.9 g L^{-1}
NH_4NO_3	1.65 g L^{-1}
$CaCl_2.2H_2O$	0.44 g L^{-1}
$MgSO_4.7H_2O$	0.37 g L^{-1}
KH_2PO_4	0.17 g L^{-1}
NaFe EDTA	40 mg L^{-1}
$MnSO_4.4H2O$	22.3 mg L^{-1}
$ZnSO_4.7H_2O$	8.6 mg L^{-1}
H_3BO_3	6.2 mg L^{-1}
KI	0.83 mg L^{-1}
$Na_2MoO_4.2H_2O$	0.25 mg L^{-1}
$CoCl_2.6H_2O$	0.025 mg L^{-1}
$CuSO_4.5H_2O$	0.025 mg L^{-1}
Sucrose	30 g L^{-1}
Myo-inositol	0.1 g L^{-1}
	*p*H adjusted to 5.8

Decontaminated capsules were cut into a minimum of six transverse sections (2 – 4 mm thick, depending on the length of the capsule), with the two end sections being discarded to reduce contamination. Capsule sections were placed, cut surface down, onto the germination media. Alternatively, the seed was scraped onto the media surface (OLIVA & ARDITTI, 1984). Sowing density was two capsule sections or 150 – 250 loose seeds per tube culture and four sections or 400 – 500 seeds per jar culture. The number of seeds per culture vessel was regulated to avoid widespread seed loss due to contamination (MICHEL, 2002).

KNUDSON C and MS media, solidified with agar and supplemented with vitamins, were used as controls. Mature seeds were tested with various media additives, inorganic salt concentrations and media viscosity manipulations (Table 3.5), as well as a range of illumination and temperature regimes (Table 3.6).

Treatments were applied independently. 'Green-pod' cultures involved charcoal, inorganic salt concentration and media viscosity manipulation, which were applied as for the mature seed.

Table 3.5. Plant growth regulators and undefined compounds as independently manipulated media additives for the *in vitro* mature seed culture of 13 South African *Disa* species. Basal media were KNUDSON C and MS in original formulation with vitamin supplements[1]. Manipulations of inorganic salts (water potential) and media viscosity are also included.

Treatment	Reference
Plant growth regulators[2]	
2,4-D (auxin) at 0, 0.2, 0.5, 1 and 2 mg L^{-1}	ARDITTI & ERNST (1984), VAN WAES (1987), YAM *ET AL.* (1990), RASMUSSEN (1995), WODRICH (1997)
BA (cytokinin) at 0, 0.2, 0.5, 1 and 2 mg L^{-1}	
GA_3 (Gibberelic acid) at 0, 0.2, 0.5, 1 and 2 mg L^{-1}	
2,4-D : BA factorial grid at 0, 0.5 and 1 mg L^{-1}	
Undefined media additives[3]	YAM *ET AL.* (1990), CHU & MUDGE (1994), CROUS (1999, PERS. COMM.), MICHEL (2002)
Liquid endosperm (coconut milk) at 100 ml L^{-1}	
Banana homogenate at 100 ml L^{-1}	
Activated charcoal at 2 g L^{-1}	
Inorganic salts	
½ and ¼ strength MS salts	ARDITTI & ERNST (1984), CROUS (1999, PERS. COMM.)
½ and ¼ strength KNUDSON C salts	
Media viscosity	
Semi-solid media (4 g L^{-1} agar)	GALUNDER (1984), CHU & MUDGE (1994)
Liquid suspension cultures (0 g L^{-1} agar)	

[1] KNUDSON C (ARDITTI, 1982 after KNUDSON, 1946) and MS (MURASHIGE & SKOOG, 1962) media supplemented with thiamine (0.1 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), pyridoxine (0.5 mg L^{-1}) and glycine (2 mg L^{-1}). Culture conditions were a constant 25 ± 2 °C under continuous illumination of 8.5 μmol m^{-2} s^{-1} supplied by cool white fluorescent tubes.

[2] 2,4-D and BA were autoclaved whilst GA_3 was filter sterilized.

[3] Banana homogenate (BH) was prepared from equal masses of green banana pulp and H_2O. Coconut milk (CM) was supplied by M. GILLMER, Durban, South Africa. Both BH and CM were frozen (-10 ± 3 °C) until required. Activated charcoal (AC) was acid-rinsed, *BDH # 330324E*.

Table 3.6. Temperature and illumination manipulations for the *in vitro* seed germination of 13 South African *Disa* species. Basal media were KNUDSON C and MS in their original formulation, with vitamin supplements[1].

Treatment	Reference
Stratification	COKE (1990), STOUTAMIRE (1990), RASMUSSEN (1992), CHU & MUDGE (1994)
12 wks at 5 °C ▸ 25 ± 2 °C	
8 wks at 25 ± 2 °C ▸ 12 wks at 5 °C ▸ 25 ± 2 °C	
Illumination	
continuous darkness	CLEMENTS (1982), VAN WAES & DEBERGH (1986b), CROUS (1999, PERS. COMM.)
2 wks dark ▸ continuous low-light	
16:8 low-light : dark photoperiod	

[1] KNUDSON C (ARDITTI, 1982 after KNUDSON, 1946) and MS (MURASHIGE & SKOOG, 1962) media supplemented with thiamine (0.1 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), pyridoxine (0.5 mg L^{-1}) and glycine (2 mg L^{-1}). Control conditions were a constant 25 ± 2 °C under continuous illumination of 8.5 μmol m^{-2} s^{-1} supplied by cool white fluorescent tubes.

Seed of each species investigated did not experience all possible media and culture condition manipulations due to limited seed availability. The germination responses were graded according to modifications of the broad categories employed by WARCUP (1973), OLIVA & ARDITTI (1984), SMRECIU & CURRAH (1989) and LEROUX, BARABÉ & VIETH (1997):

i. No germination
ii. Testa split and embryo swollen
iii. Embryo enlarged, rhizoids present
iv. Protocorm considerably larger than testa
v. Differentiation of the apical meristem
vi. Greening of the primary leaf tissue.

Germination and early seedling development were recorded fortnightly up to a maximum of 18 months (OLIVA & ARDITTI, 1984; ZETTLER & HOFER, 1998), where primary seedling development was concluded by the concomitant initiation

of primary leaves and root initials. Percentages represent mean counts for a minimum of 100 randomly chosen seeds from at least five cultures.

Germinability was compared between *Disa* species by plotting their *in vitro* germination response onto the morphology cladograms of LINDER & KURZWEIL (1999; Appendix One). Cladograms were not available for *D. nervosa*, *D. patula* and *D. stachyoides* (section *Emarginatae*), *D. cephalotes*, *D. pulchra* and *D. saxicola* (section *Stenocarpa*) and *D. brevicornis* (section *Monadenia*). The molecular phylogeny of BELLSTEDT *ET AL.* (2001; Appendix Two) was not used because it reveals the relationships of comparatively fewer of the small-seeded *Disa* species examined here and is reliant on the sectional classification of LINDER & KURZWEIL (1999).

3.4 RESULTS AND DISCUSSION

3.4.1 DECONTAMINATION PROTOCOLS

No single decontamination treatment provided satisfactory levels (> 80 %) of pathogen-free mature seed cultures, with contamination being primarily fungal. Completely ineffective pretreatments included 70 % EtOH, 1 % Benlate®, 1 % Virkon®, Cl^{2-}, 1 % *PPM*® and paraformaldehyde. These data were irrespective of the duration of the treatment. Media additives 0.01 % NaOCl, 0.01 – 1 % H_2O_2 and 0.1 – 1 % *PPM*® were equally ineffective when incorporated into solid germination media. *PPM*® (1 %) as an additive was only effective when incorporated into semi-solid and liquid media, resulting in 58 % disinfection. However, the cost of *PPM*® made this sterilant unsuitable for widespread experimentation.

Decontamination with 15 % H_2O_2 for the *in vitro* culture of several winter endemic *Disa* species (CROUS, 1999, PERS. COMM.) produced a maximum

disinfection efficacy of 40 % after 20 min exposure. Peroxide as a decontaminating agent has an advantage over NaOCl since the former need not be rinsed before plating (YANAGAWA *ET AL.*, 1995). A maximum of 46 % disinfection was recorded in cultures where seeds were pretreated for 20 min with 1.75 % NaOCl. These data are in keeping with ORCHARD (1999, PERS. COMM.), who reported 'adequate' decontamination following a pretreatment of 10 – 20 min with this sterilant. Increased exposure up to 60 min did not decrease the percentage of contaminated cultures. Corrosive damage to the seed testa was noted where the duration of the pretreatment was increased to 120 min.

The low occurrence of disinfection reported in this study was attributed to i) the reticulate-foveate arrangement of the testa cells and ii) the difficulty experienced in wetting the seed – a problem that typifies terrestrial orchids (BURGEFF, 1959; ARDITTI, MICHAUD & OLIVA, 1982a; VAN WAES & DEBERGH, 1986a). Air-pockets persisted in the grooves between adjacent testa cells, despite the inclusion of 1 % Tween-20 in liquid decontaminants. A 70 % EtOH seed surface spray prior to, or a 2 min ultrasound pulse during the NaOCl rinse had the effect of enhancing contact between seed testae and the sterilant – improving disinfection efficacy by 10 %. Combination treatments involving fungicides produced a similar effect. The following decontamination regime was adopted for all mature seed cultures:

70 % EtOH spray ► 1.75 % NaOCl + 1 % Tween-20 (20 min)
► 1 % Benlate® (10 min) ► 3 x rinses in sterile dH_2O.

This protocol was, at best, 65 % effective. These data for the summer endemic *Disa* species contrast with MICHEL (2002), who reported using dilute sterilants and reduced exposures for the large-seeded *Disa* species as a consequence of their porous testae.

Capsule decontamination using 3.5 % NaOCl or 100 % EtOH proved highly successful (> 90 %) in achieving pathogen-free cultures, especially when used in combination. Anti-oxidation treatments (ascorbic and citric acid and *PVP*) were shown to be unnecessary. Consequently, the following decontamination regime was adopted for all 'green-pod' cultures:

3.5 % NaOCl (10 min) ▸ 3 x [100 % EtOH rinse (10 sec) ▸ flamed (10 sec) ▸ cold, sterile dH_2O rinse] ▸ final rinse in sterile dH_2O (5 min).

3.4.2 SEED CULTURE *IN VITRO*: FIRST TIME GERMINATION RECORDS

The asymbiotic *in vitro* germination response for the 13 summer-rainfall *Disa* species investigated proved very poor. Germination was only achieved for four species (Table 3.7) and never under control conditions. Media manipulation was therefore obligate in eliciting germination in *D. cooperi*, *D. nervosa*, *D. pulchra* and *D. woodii*, which are reported from this study as first time germination records.

Media with increased water potential (liquid and semi-solid media, and media with reduced inorganic salt concentrations; GEORGE, 1993) were required for the germination of mature seed of these species (Figure 3.1). Germination percentages never exceeded 30 % (*D. pulchra*, semi-solid ½ strength MS media supplemented with charcoal; Table 3.7), nor occurred in less than 12 weeks (*D. cooperi* and *D. woodii*, liquid ½ strength MS and liquid ¼ strength KNUDSON C media respectively; Table 3.7). Germination on a solid medium, or on a medium with a full compliment of inorganic salts, was only achieved when supplemented with charcoal (Figure 3.1). However, these germination percentages were lower and the times taken to germination longer than those recorded for media with increased water availability (Table 3.7).

Table 3.7. Media manipulations that induced germination in four small-seeded summer rainfall *Disa* species. Mean percentage and time taken to first germination are presented in parentheses. Percentages represent mean counts of 100 randomly chosen seeds from a minimum of five cultures.

Species	Media manipulation
D. cooperi	i. liquid ½ strength KNUDSON C and MS media (24 %; 12 weeks)
	ii. semi-solid ½ strength MS media supplemented with charcoal (2 g L^{-1}) (15 %; 18 weeks)
	iii. solid ½ and ¼ strength KNUDSON C and MS media supplemented with charcoal (2 g L^{-1}) (13 %; 19 weeks)
D. nervosa	i. solid full and ½ strength KNUDSON C media supplemented with charcoal (2 g L^{-1}) (9 %; 22 weeks)
D. pulchra	i. liquid ½ and ¼ strength MS media (29 %; 13 weeks)
	ii. semi-solid full and ½ strength MS media supplemented with charcoal (2 g L^{-1}) (30 %; 16 weeks)
	iii. solid full and ½ strength KNUDSON C and MS media supplemented with charcoal (2 g L^{-1}) (14 %; 19 weeks)
D. woodii	i. liquid ½ and ¼ strength KNUDSON C and MS media (27 %; 12 weeks)
	ii. semi-solid ½ and ¼ strength MS media (7 %; 18 weeks)
	iii. solid ½ and ¼ strength KNUDSON C and MS media supplemented with charcoal (2 g L^{-1}) (7 %; 20 weeks)

Full-strength KNUDSON C and MS media, solidified with agar and supplemented with 0.1 mg L^{-1} thiamine, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine and 2 mg L^{-1} glycine, represent controls for all experiments. Cultures were incubated at 25 ± 2 °C under a continuous low-light intensity of 8.5 μmol m^{-2} s^{-1} supplied by cool white fluorescent tubes.

WODRICH (1997), CROUS (1999, PERS. COMM.) and ORCHARD (1999, PERS. COMM.) reported the benefits of reduced inorganic salts and complex undefined additives (charcoal) on large-seeded and winter-rainfall *Disa* germination. Full-strength MS medium never supported *Disa* germination, which is consistent with

data for other orchid taxa (ARDITTI, 1982; RASMUSSEN, 1995). MS medium has a total ionic concentration (m*M*) = 95.75 (GEORGE, 1993), approximately three times higher than KNUDSON C medium (33.39 m*M*; GEORGE, 1993). Consequently, full-strength MS medium has a substantially lower water potential than KNUDSON C medium and is comparable to the latter only when in reduced formulation (half or quarter-strength).

In contrast to previous studies (HARVAIS, 1972, 1973, 1982; CLEMENTS, 1982; ARDITTI & ERNST, 1984; VAN WAES & DEBERGH, 1986b; VAN WAES, 1987; YAM *ET AL.*, 1990; CHU & MUDGE, 1994; RASMUSSEN, 1995; WODRICH, 1997; CROUS, 1999, PERS. COMM; MICHEL, 2002), plant growth regulators (2,4-D, BA and GA_3), coconut milk and banana homogenate did not promote germination. ARDITTI (1982) reported that the addition of banana might hinder germination. In *D. cooperi*, where a media trial combined charcoal and complex organic additives, germination was attributed solely to the presence of the former supplement. Although not quantified statistically, germination percentage and germination rate were similar on KNUDSON C and MS media.

CHU & MUDGE (1994) defined germination synchrony as the occurrence of the majority of protocorms in a treatment at a given developmental stage at approximately the same time. Asynchronous germination stems from variable germination requirements within individuals of a seed population (BEWLEY & BLACK, 1994) and carries an ecological advantage. *In vitro*, asynchronous germination is undesirable as it hampers seedling maintenance and transflasking. In addition, DALLA ROSA & LANERI (1977) reported that seedlings matured from late germinating seeds were comparatively under-developed. In *D. cooperi*, *D. pulchra* and *D. woodii*, synchronous germination was recorded in liquid media – with the period between emergence of the first protocorm and emergence of the last protocorm being four to eight weeks. New germinations were not observed after this period. Comparable times were reported by CHU & MUDGE (1994) for liquid suspension seed cultures of several North American *Cypripedium* spp. On

solid media, newly emerged *D. nervosa* protocorms were still observed 24 weeks after the initial germination response.

ICHIHASHI & YAMASHITA (1977) reported that the effects of cold stratification and liquid germination media were almost identical in *Bletilla striata* Rchb. f., resulting in decreased time taken to germination, increased germination percentage and greater synchrony. Similar data were reported for *Cypripedium calceolus* Willd. by CHU & MUDGE (1994). In contrast, cold stratification did not promote germination in *Disa*, with 0 % germination being recorded in all temperature-manipulated trials. Warm stratified seed cultures of *Disa* were equally unresponsive.

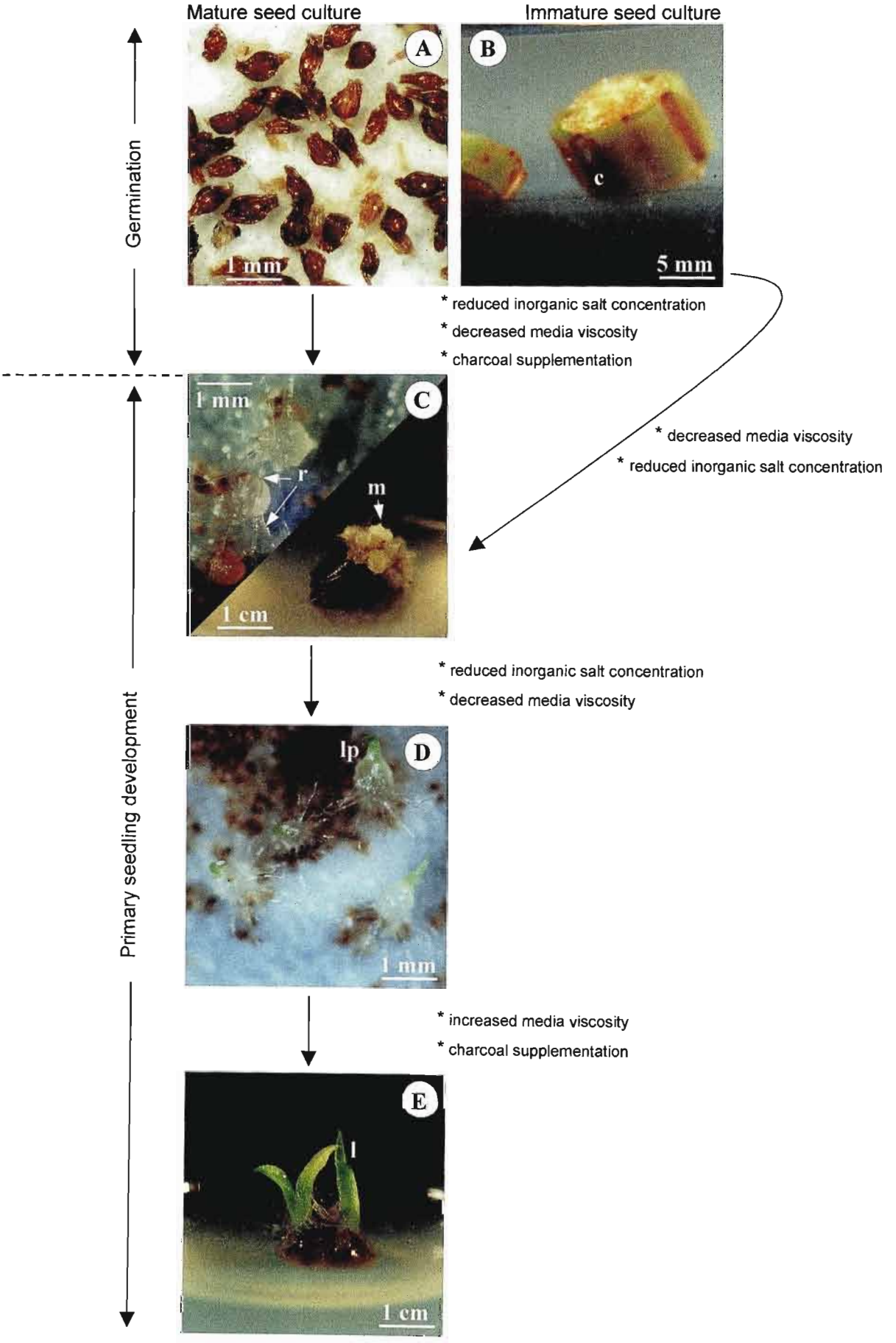
Photoperiod manipulation did not obviously influence germination rate or percentage relative to controls in *D. cooperi*, *D. nervosa* and *D. pulchra*. In addition, illumination manipulation did not induce germination in otherwise ungerminated species.

'Green-pod' cultures of *D. chrysostachya*, *D. cooperi*, *D. pulchra* and *D. versicolor* germinated under control conditions with percentages similar to those reported for mature seed under experimental conditions (Figure 3.1). Seeds originating from first quarter capsules never germinated (0 %), with the implication that in seeds at two weeks after pollination, the embryos were insufficiently developed to germinate. In addition, seed taken from second, third and fourth quarter capsules of *D. chrysostachya* and *D. versicolor* was ungerminated. By contrast, second and third quarter seed of the remaining species (*D. cooperi* and *D. pulchra*) germinated under control conditions, whereas manipulation of fourth quarter seeds was required (Figure 3.1). Germination percentages for immature seed ranged between 8 – 28 % for these species and were always highest in the six-week-old seeds. MICHEL (2002) reported similar optimal capsule excision times of between 35 – 40 days for the large-seeded *Disa* species. The time taken to germination of immature seed was cited as three weeks for the large-seeded

species (CROUS, 1999, PERS. COMM; MICHEL, 2002). The protrusion of the first protocorm never occurred in less than eight weeks in immature seed cultures of *D. cooperi* and *D. pulchra*. MICHEL (2002) reported germination success in excess of 80 % from immature seed of *D. uniflora* and *D. tripetaloides*, in combination with reduced strength medium. A reduction in media salts and a decrease in viscosity raised percentage germination of immature seed to over 50 %, the highest percentage recorded for the summer-rainfall *Disa* species.

Disa protocorms were initially spherical and white (Figure 3.1C). Alternatively, CROUS (1999, PERS. COMM.) reported green protocorms in several large-seeded *Disa* species. From protrusion, each protocorm displayed rhizoids and a single apical meristem. Protocorms of *D. cooperi*, *D. nervosa*, *D. pulchra* and *D. woodii* matured similarly and displayed leaf primordia and the apical development of chlorophyllous leaflet tissue four to six weeks after germination (Figure 3.1D). However, not all protocorms attained this level of differentiation, with increased necrosis (> 70 %) in all species on solid and full-strength media, as well as on half-strength MS medium. True leaves and roots were observed 8 – 10 weeks after germination (Figure 3.1E), although root initiation was limited on semi-solid and liquid media. A maximum of 68 % root initiation was recorded for *D. cooperi* protocorms cultured on solid media supplemented with charcoal. In the presence of charcoal, *Disa* roots displayed negative phototropism, as reported for other orchid taxa by ERNST (1967), WERKMEISTER (1971), UDEA & TORIKATA (1972) and DALLA ROSA & LANERI (1977).

Primary seedling development was similar on KNUDSON C and half and quarter-strength MS media. Shoot development in the absence of roots, as observed for these *Disa* species, was interpreted by HADLEY (1982) and RASMUSSEN (1995) as evidence of germination in habitats with freely available water and the need for seed dormancy and temporally controlled dehiscence in fluctuating environments.


Mature seed culture
Immature seed culture
A
B
c
1 mm
5 mm
Germination
* reduced inorganic salt concentration
* decreased media viscosity
* charcoal supplementation
* decreased media viscosity
* reduced inorganic salt concentration
1 mm
C
r
m
1 cm
* reduced inorganic salt concentration
* decreased media viscosity
lp
D
1 mm
Primary seedling development
* increased media viscosity
* charcoal supplementation
E
l
1 cm

South African *Disa* species *in vitro* therefore formed an aggregated continuum from easily germinable to intractable. A germinability index (GI; Figure 3.2), based on time taken to and total percentage germination *in vitro*, was assigned to each of the 13 species investigated in this study and to 29 species germinated previously. Species that germinated with > 80 % success in a maximum of 8 weeks were allocated GI = 1. These winter-rainfall and widespread species included the large-seeded species of the *D. uniflora* sub-clade. The GI = 1 species did not require media or protocol manipulation to germinate from mature seed, although reduced concentrations of inorganic salts and charcoal supplementation were recommended by WODRICH (1997), CROUS (1999, PERS. COMM.) and ORCHARD (1999, PERS. COMM.).

The four summer-rainfall species germinated through this research were allocated a GI = 2 (Figure 3.2). Mature seed was characterized by < 30 % germination in a minimum of 12 weeks (Table 3.7) and only as a result of media and protocol manipulations. Germination was independent of sectional classification (Table 3.3). Intractable / ungerminated summer-rainfall species were ascribed a GI = 3 (Figure 3.2). These species remained ungerminated *in vitro*, despite manipulation.

The effect of free water on germination suggests that the seed testa contributes towards controlling germination in the summer-rainfall *Disa*. Previous studies (ARDITTI, 1967; VAN WAES & DEBERGH, 1986a, b; HARBORNE, 1989; LINDÉN, 1992; CHU & MUDGE, 1994; RASMUSSEN, 1995) have attributed enhanced germination in liquid suspension cultures to leaching of the seed testa and the dilution of phyto-inhibitors – features typically associated with testa-imposed seed dormancy (VAN DER KINDEREN, 1987; RASMUSSEN, 1995; BASKIN & BASKIN, 1998). The occurrence of germination in the presence of charcoal, which irreversibly binds – amongst others, toxic polyphenolics (VAN WAES & DEBERGH, 1986a; WATERMAN & MOLE, 1994; MIYOSHI & MII, 1995), supports this notion.

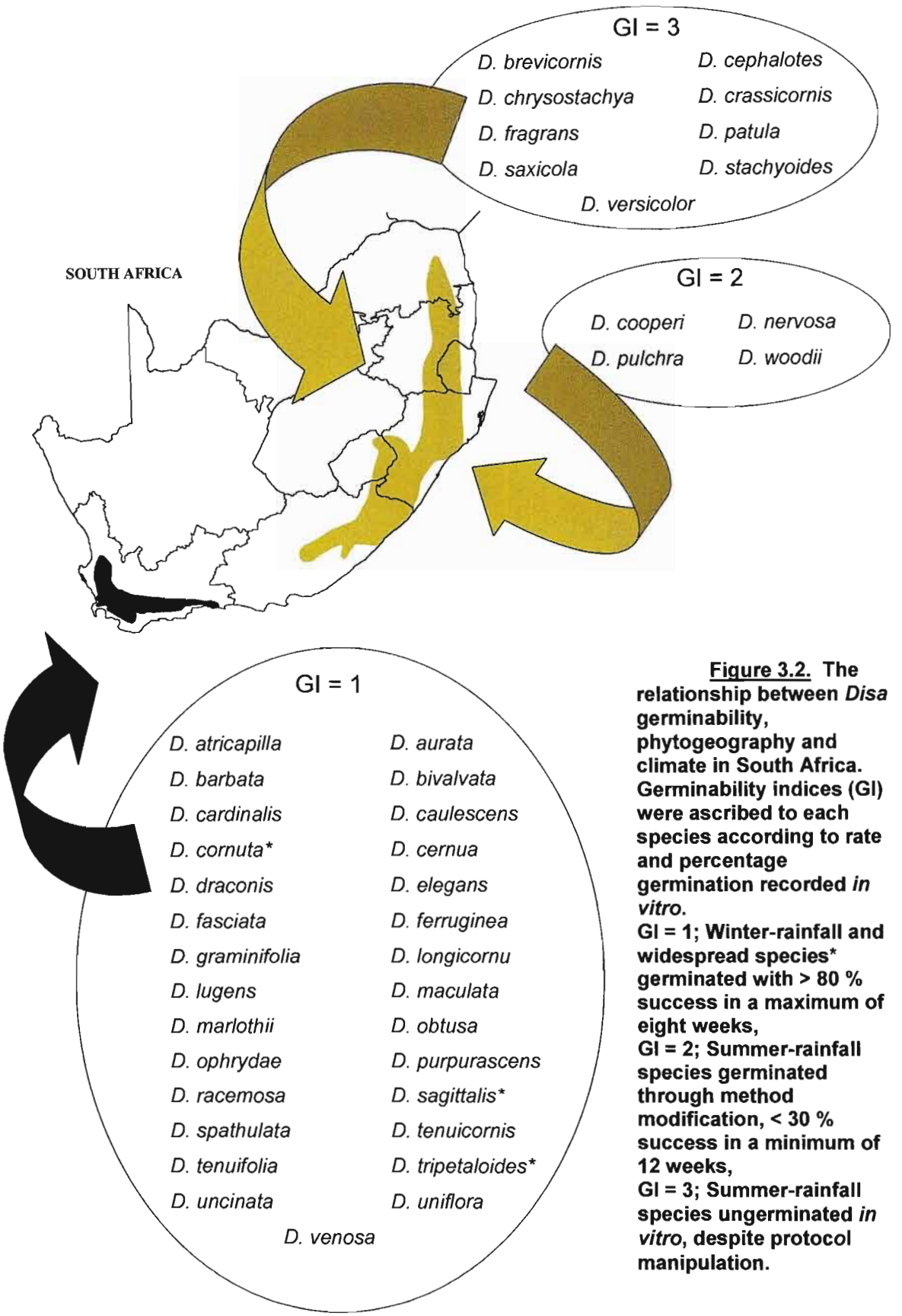


Figure 3.2. The relationship between *Disa* germinability, phytogeography and climate in South Africa. Germinability indices (GI) were ascribed to each species according to rate and percentage germination recorded *in vitro*.
GI = 1; Winter-rainfall and widespread species* germinated with > 80 % success in a maximum of eight weeks,
GI = 2; Summer-rainfall species germinated through method modification, < 30 % success in a minimum of 12 weeks,
GI = 3; Summer-rainfall species ungerminated *in vitro*, despite protocol manipulation.

Germination of immature seed – which have underdeveloped and theoretically permeable testae (SAULEDA, 1976; PRITCHARD, 1989; RASMUSSEN *ET AL.*, 1991; SHOUSHTARI, HEYDARI, JOHNSON & ARDITTI, 1994; RASMUSSEN, 1995), in similar percentages to those achieved through media manipulation (Figure 3.1), provides further evidence for this.

Germination in at least four summer-rainfall *Disa* species, that was prohibited under control conditions by the seed testa, can be modelled as a trade-off between water availability and the presence of phyto-inhibitors (Figure 3.3). An increase in the former and a corresponding decrease in the latter are presented here as the reason for the first time germination of *D. cooperi*, *D. pulchra*, *D. nervosa* and *D. woodii* under modified *in vitro* conditions. However, decreased media viscosity – which also promoted protocorm survival, was not satisfactory for primary seedling development (Figure 3.1).

Immature seed were uncontaminated and germinated on solid media (Figure 3.1), offering potential as source material for *in vitro* propagation. However, MIYOSHI (1997) cautioned against immature seed culture as it permits the transmission of plant viruses.

These findings provide several clues regarding the germination requirements of *Disa*. However, not all *Disa* species investigated responded to manipulation of water availability and phyto-toxins *in vitro*. In addition, germination percentages in this study were considerably lower than those reported for other member of the genus (VOGELPOEL, 1980, 1993; ARDITTI, 1982; LACROIX & LACROIX, 1997; WODRICH, 1997; MICHEL, 2002). As a genus *Disa* is clearly divided – as attested to by the *in vitro* germinability indices, with regard to the germination requirements of individuals that experience winter or summer rainfall and annual or perennial water.

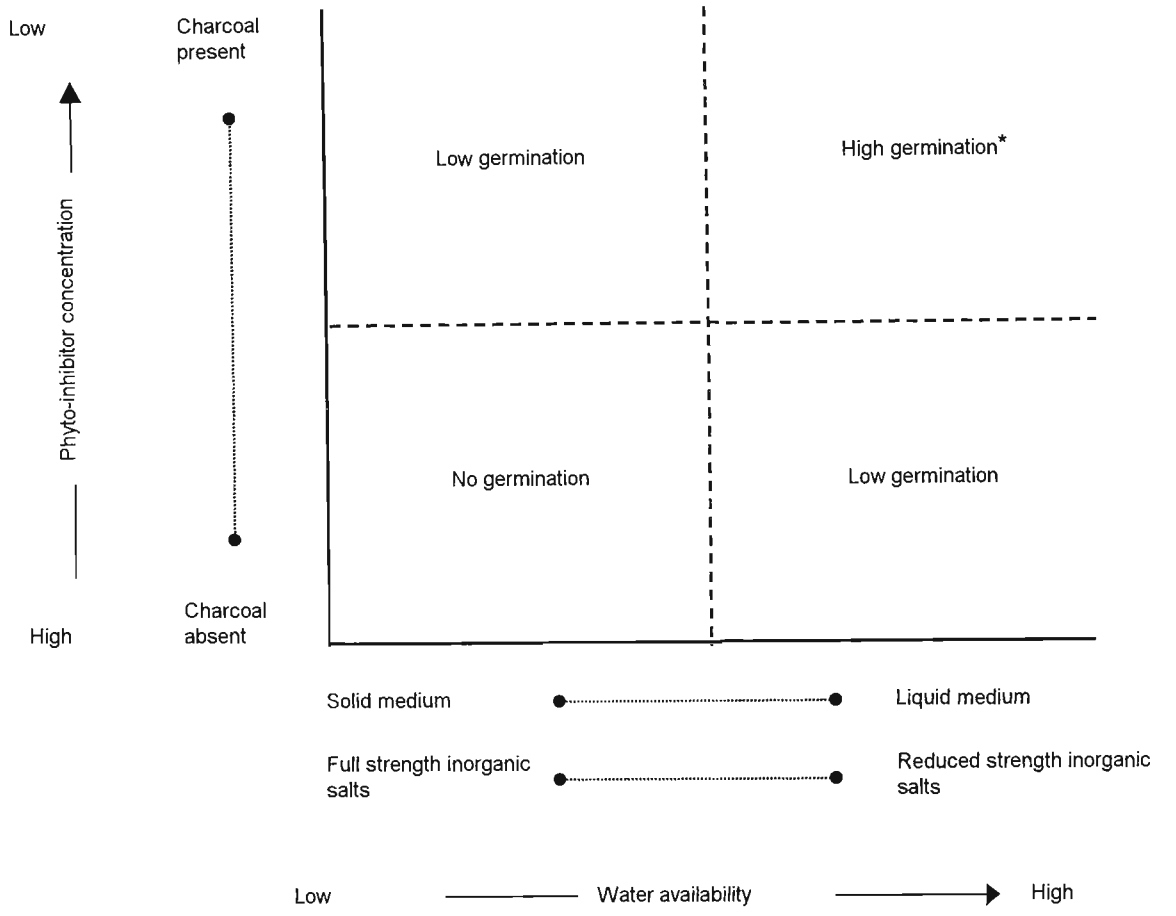


Figure 3.3. Model predicting *Disa* germinability as a function of water availability and phyto-inhibitor concentration *in vitro*. Increased water availability was achieved through i) decreased media viscosity or ii) decreased inorganic salt concentration (half or quarter-strength; nature of basal medium). Charcoal supplementation (2 g L^{-1}) decreased the concentration of phyto-inhibitors in the culture vessel. A medium with multiple modifications is expected to further increase percentage germination* and result in additional first time germination records for *Disa*.

The lack of increased germination in summer-rainfall *Disa* seed incubated with plant growth regulators or undefined media additives need not be interpreted as a lack of response to these supplements. Rather, in the case of testa-imposed dormancy, the additives are not reaching their site of action – the isolated embryo.

In addition to speculation over testa-imposed dormancy in the Orchidaceae, previous authors have cited;

i. limited endogenous reserves (ARDITTI, 1982; ARDITTI & ERNST, 1984; MANNING & VAN STADEN, 1987; SMRECIU & CURRAH, 1989; RASMUSSEN, 1995);
ii. reliance on mycotrophy (STOUTAMIRE, 1974; CLEMENTS *ET AL.*, 1986; ZETTLER & MCINNIS, 1992; RASMUSSEN, 1995; ZETTLER & HOFER, 1998; BATTY *ET AL.*, 2001a, b); and
iii. a lack of viable seed (FAST, 1982; HARVAIS, 1982)

as determinants of poor or non-existent germination for terrestrial orchids *in vitro*.

CHAPTER FOUR

NUTRIENT RESERVES AND PATTERNS OF SEED GERMINABILITY IN THE SOUTH AFRICAN *DISA*

Theory dictates that *Disa* seed should be energy poor, since orchids lack endosperm and are supposedly reliant on symbiosis to germinate naturally. Transmission electron microscopy of mature *Disa* embryos revealed significant nutrient reserves in all species. Large-seeded, easily germinable species contained mostly starch – the presence of which is unusual in orchids. Starch hydrolysis could facilitate the rapid, autonomous germination observed in these species. Small-seeded, often intractable *Disa* species were shown to store lipids and proteins. The difference between poorly germinable and intractable species was not correlated to variable lipid and protein reserves. Poorly germinable species accumulated starch post-germination, a phenomenon not observed in the intractable species. Gas chromatography revealed that the embryo protoplasts of all species contained significant free sugars, irrespective of germinability. First records of ribose, xylose, sorbose, galactose, inositol and trehalose result. Decreased sucrose / increased fructose representation correlated significantly with decreased seed germinability. The interplay between these key sugars, rather than the total free sugar complement of the embryo, is critical. Immature seeds from typically intractable *Disa* species displayed the free sugar status of mature, germinable species – providing evidence for the germination of the former under 'green-pod' conditions.

4.1 INTRODUCTION

The minute size and lack of endosperm (ARDITTI, 1982) that typifies orchid seeds dictates that they should contain insufficient reserve nutrients to allow for germination and the production of autotrophic seedlings (KNUDSON, 1929; RASMUSSEN, 1995; LEROUX *ET AL.*, 1997). In this regard ARDITTI & GHANI (2000) argue that orchids produce characteristically large numbers of seeds only because of the minimal energy expended on seed reserves.

Past research (HARRISON, 1977; ARDITTI & ERNST, 1981; MANNING & VAN

STADEN, 1987; RASMUSSEN, 1990; RICHARDSON, PETERSON & CURRAH, 1992; BEWLEY & BLACK, 1994; RASMUSSEN, 1995) has demonstrated that orchids can accumulate appreciable amounts of nutrient reserves within the embryo. Although present, these reserves are insoluble and not easily metabolized by the orchid embryo (ARDITTI & ERNST 1981, 1984; MANNING & VAN STADEN, 1987; RICHARDSON *ET AL.*, 1992; RASMUSSEN, 1995), hence their reliance on fungal symbiosis to facilitate germination.

The literature is depauperate in studies assessing the nutritional status of orchid seeds, specifically in the absence of mycotrophy or conventional germination studies. However, seed size necessitates limited and concentrated reserve storage (RASMUSSEN, 1995; ARDITTI & GHANI, 2000), where the preferential storage of lipids and proteins carries advantage. By volume, lipids and proteins are more energy rich than storage carbohydrates (SIMON, 1984; BRADBEER, 1988; BEWLEY & BLACK, 1994). Research confirms that orchid seed reserves are predominantly lipids (up to 32 % based on w/w determination; HARRISON & ARDITTI, 1978), although significant quantities of proteins may also be present (ARDITTI & ERNST, 1984; MANNING & VAN STADEN, 1987; ARDITTI, 1992; ARDITTI & GHANI, 2000). MANNING & VAN STADEN (1987) reported protein bodies numbering as many as 35 per cell in a medial seed section in the terrestrial orchid genus *Huttonaea* Harv.

The literature indicates that mature embryonic cells are un-vacuolate, with lipid and protein bodies occupying the dense cytoplasm to the almost total exclusion of organelles (CARLSON, 1940; STOUTAMIRE, 1964, 1974; HARVAIS, 1974; HARRISON, 1977; MANNING & VAN STADEN, 1987; RASMUSSEN, 1990, 1995; RICHARDSON *ET AL.*, 1992; LEROUX *ET AL.*, 1997). The distribution of insoluble reserves in the mature orchid embryo is fairly uniform (MANNING & VAN STADEN, 1987; LEROUX *ET AL.*, 1997), although epidermal cells may contain less than centrally situated cells (HARRISON, 1977; RASMUSSEN, 1990, 1995). *Cattleya aurantiaca* (Bateman) P.N. Don embryos have a polarized reserve distribution, with protein bodies concentrated at the chalazal pole (HARRISON, 1977).

Mature orchid embryos are not usually starchy (CARLSON, 1940; HARVAIS, 1974; NAKAMURA, 1976; ARDITTI & ERNST, 1984; MANNING & VAN STADEN, 1987; NISHIMURA, 1991; RICHARDSON *ET AL.*, 1992; RASMUSSEN, 1995; LEROUX *ET AL.*, 1997), although starch grains were reported by BURGEFF (1959) and ERNST, ARDITTI & HEALEY (1970). HARRISON (1977) and LEROUX *ET AL.* (1997) confirmed that these are present only in proplastids, which are absent from the mature embryo. Starch is therefore regarded as characteristic of the developing orchid embryo and is abundant in *Disa polygonoides* (MANNING & VAN STADEN, 1987) and representatives from other genera leading up to seed maturity (STOUTAMIRE, 1964; HARVAIS, 1974; HARRISON, 1977; RICHARDSON *ET AL.*, 1992; RASMUSSEN, 1995; LEROUX *ET AL.*, 1997; ARDITTI & GHANI, 2000).

As embryo maturation proceeds, lipid and protein deposition occurs at the expense of the amyloplasts, which become reduced in size, number and in the number of starch grains that they contain (MANNING & VAN STADEN, 1987). This differs from other monocotyledons, where seed tissues act as permanent, irreversible sinks for starch throughout maturation (BEWLEY & BLACK, 1994; COIMBRA & SALEMA, 1994; SIVAK & PREISS, 1995; ZAMSKI, 1995; ZIEGLER, 1995).

The absence of starch from mature seeds typifies most orchid species. *Calypso bulbosa* (L.) Oakes, *Bletilla striata* Rchb. f. and *Cymbidium ensifolium* Sw., all northern hemisphere terrestrials, are exceptions. The first species is notable in that it lacks protein storage (YEUNG & LAW, 1992), whilst *B. striata* is one of only 10 orchids that have been shown to possess rudimentary cotyledons (NISHIMURA, 1981). All three species are easily germinated under asymbiotic laboratory conditions (MEI-SHENG, FU-XIONG, NAN-FEN & AN-CI, 1985; SHUN-XING & JIN-TANG, 1990; RASMUSSEN, 1995).

Despite the presence of inert storage compounds in seeds, increasing evidence from work on non-orchidaceous species suggests that it is not the reserve compounds, but rather soluble carbohydrates housed within the

protoplasts, that serve as the source of respiratory energy during germination (BEWLEY & BLACK, 1978, 1994; SIMON, 1984; ZIEGLER, 1995). At the very least, simple sugars and oligosaccharides serve as energy and synthesis substrates until the mobilization of more inert and polymeric reserves has progressed sufficiently to supply the increasing demands of germination (ZIEGLER, 1995). However, soluble sugars are rarely the main storage carbohydrate in non-orchidaceous species and are found as only minor reserves within embryonic and / or endosperm tissues (BRADBEER, 1988; BEWLEY & BLACK, 1994; ZIEGLER, 1995).

ARDITTI (1982) and RASMUSSEN (1995) report on the presence of appreciable amounts of glucose, fructose, sucrose and mannose within the mature embryo protoplasts of orchids. Apart from these, several other kinds of soluble sugars (viz. arabinose, rhamnose and maltose) have been detected in South African terrestrial orchid species, including *D. polygonoides* (MANNING & VAN STADEN, 1987).

The absence of starch precludes the possibility of soluble, preformed sugars within the mature orchid seed arising as a result of post-dispersal hydrolysis (HALMER, 1985). The catabolism of lipid reserves demands a high level of activity in embryonic cells, with lipolysis being regarded as the crucial step in orchid seed germination (MARRIOT & NORTHCOTE, 1975; ARDITTI & ERNST, 1981; ARDITTI, 1982; BHANDARI & CHITRELLEKHA, 1984; MANNING & VAN STADEN, 1987; RASMUSSEN, 1995).

HARRISON (1977) and MANNING & VAN STADEN (1987) demonstrated that glyoxysomes are not present in mature terrestrial orchid seed. Consequently, they lack the appropriate enzyme systems responsible for the conversion of storage lipids into metabolically active sugars (DOIG, COLBORNE, MORRIS & LAIDMAN, 1975; MANNING & VAN STADEN, 1987; RASMUSSEN, 1995; LEROUX *ET AL.*, 1997). *Cattleya aurantiaca* seeds lack the necessary enzyme systems to metabolize polysaccharides and lipids (MUIR, 1989; SMRECIU & CURRAH, 1989) and require an

exogenous carbon supply for an initial period over which time they construct these systems (MUIR, 1987). Free sugars are therefore required to support respiration until the initiation of lipolysis (HARRISON, 1977; MANNING & VAN STADEN, 1987) and subsequent starch accumulation. The hydrolysis of protein bodies is a water-dependent process that occurs passively following seed and embryonic cell hydration (ADAMS, NORBY & RINNIE, 1985).

The detection of free sugars in the mature orchid seed is therefore reliant solely on small amounts of these compounds being permanently present within the embryonic protoplasts. Unfortunately the free sugar status of developing seeds was not addressed by MANNING & VAN STADEN (1987).

The *in vitro* germination responses of orchids (STOUTAMIRE, 1964, 1974, 1990; ERNST *ET AL.*, 1970; WARCUP, 1981; ARDITTI, 1982; ARDITTI *ET AL.*, 1982a, b; CLEMENTS *ET AL.*, 1986; VAN WAES & DEBERGH, 1986b; RASMUSSEN, 1990, 1995; ZETTLER & MCINNIS, 1992, 1993, 1994; WODRICH, 1997) under asymbiotic conditions support the notion that soluble carbohydrates are crucial in germination. Three responses are noted; species which germinate in the absence of an external carbon source, those which germinate only when exogenous carbon is provisioned and lastly, those species which do not germinate *in vitro*, despite carbohydrate augmentation.

Several species of *Dactylorhiza* and *Orchis* Linn. germinate in water and remain alive for several weeks without receiving external nutrients (ARDITTI, 1967; RASMUSSEN, 1995). Here the exogenous application of a soluble carbohydrate *in vitro* does not improve germination (ARDITTI, 1982). Following germination and during the subsequent 'waiting time' (VERMEULEN, 1947), the seedling remains autonomous (RASMUSSEN, 1995). Such seeds, in the presumed absence of both starch and lipid catabolism, must contain sufficient quantities of soluble reserves to facilitate germination in the absence of external carbon.

Alternatively, the obligatory reliance of some species on exogenous carbohydrate supplementation *in vitro* implies a depauperate internal energy source incapable of fuelling respiration (ERNST *ET AL.*,1971; STOUTAMIRE, 1974; ARDITTI & ERNST, 1981, 1984; ARDITTI, 1982; CLEMENTS, 1982; CLEMENTS *ET AL.*, 1986; RASMUSSEN, 1995). *Cypripedium reginae* Walt. (HARVAIS, 1982), *Goodyera repens* R.Br. (DOWNIE, 1941) and *Galeola serpentrionalis* (NAKAMURA, 1982) all require the addition of soluble sugars if they are to germinate *in vitro*. Soluble sugars, if present in the protoplasts of the embryonic cells of these species, are insufficient to facilitate lipid catabolism (MARRIOT & NORTHCOTE, 1975; MANNING & VAN STADEN, 1987). Consequently seeds may imbibe and undergo protein hydrolysis, but remain ungerminated due to a paucity of soluble sugars.

Many less exacting examples exist where species, although capable of germination in the absence of a specific external carbohydrate, do show an increased germination percentage in their presence (ARDITTI, 1982; RASMUSSEN, 1995). It is therefore conceivable that individual seeds differ in their endogenous nutritional status. It makes sense that species, which do not germinate even when provisioned with specific soluble sugars, remain so due to factors independent of the nutrient status of the embryo. Unfortunately there is no explicit data to confirm the correlation between high endogenous levels of accessible sugars and the capacity to germinate in the absence of external carbon. In fact, no specific attempts have been made to correlate endogenous seed reserves with *in vitro* germination performance.

The accumulation of starch following germination as a result of lipolysis is characteristic of orchids, being particularly pronounced in asymbiotic seed cultures (ARDITTI, 1967; ARDITTI & ERNST, 1981). Starch has been detected in the post-imbibition *D. polygonoides* seed where gluconeogenesis, shown by the presence of glyoxysomes and lipolysis, resulted in substantial starch deposition two weeks after culture initiation (MANNING & VAN STADEN, 1987). Similar reports exist for germinating seeds from other orchid taxa (PURVES & HADLEY, 1976;

HARRISON, 1977; RASMUSSEN, 1995; LEROUX *ET AL.*, 1997). However, the persistence of amyloplasts and starch in the developing seedling is unknown, but presumably extends beyond the period during which the young plant is strictly heterotrophic (RASMUSSEN, 1995).

4.2 AIMS

- To document the nutrient reserves of mature *Disa* seeds.
- To document the free sugars in mature and developing *Disa* seeds.
- To correlate seed type (Chapter Two), *in vitro* germination performance (Chapter Three) and dominant nutrient profile for 10 South African *Disa* species.
- To investigate variability in seed germinability as a function of endogenous lipid, protein and carbohydrate reserves.

4.3 MATERIALS AND METHODS

Mature seed ultra-structure of 10 *Disa* species (Table 4.1) was investigated with regard to their nutrient reserves. Unless otherwise acknowledged, seed was collected from wild populations of *Disa*. Where possible fresh seed was analysed. However, the variable timing of capsule dehiscence within and between species meant that for the most part, seeds experienced some time in cold storage at ± 4 ^{0}C with 0 % relative humidity.

Representatives of both the large (*D. uniflora* – type *sensu* KURZWEIL, 1993) and small (*Satyrium* – type *sensu* KURZWEIL, 1993) seeds are included, which form an aggregated continuum from easily germinable to intractable on modified media *in vitro* (Chapter Three; Figure 3.2; page 77).

This investigation was complemented by gas chromatography of free sugars. Soluble sugars were identified using co-chromatography with authentic reference standards. Additionally, the free sugar profiles were determined for *D. cooperi* (GI = 2; Table 4.1) and *D. versicolor* (GI = 3; Table 4.1) seed across four broad seed maturity classes. Seed used in the analyses originated from each of the four capsule developmental quarters outlined in Chapter Three (page 61).

Table 4.1. *Disa* species in which nutrient reserve status was assessed. Seed types are included in parentheses. A germinability index (GI), based on time taken to and percentage germination *in vitro*, is included for each species.

D. chrysostachya (small)	3	*D. pulchra* (small)	2
D. cooperi (small)	2	*D. stachyoides* (small)	3
D. cornuta (small)	2	*D. tripetaloides* (large)[1]	1
D. crassicornis (small)	3	*D. uniflora* (large)[1]	1
D. nervosa (small)	2	*D. versicolor* (small)	3

In vitro germinability (GI) indices: 1 – easily germinated with > 80 % success in less than eight weeks; 2 – germinated with < 30 % success in a minimum of 12 weeks; 3 – intractable / ungerminated *in vitro* on modified media. [1]Seed of the winter-rainfall species was supplied dry following capsule dehiscence by DR. L. VOGELPOEL, Cape Town, South Africa.

4.3.1 SEED ULTRA-STRUCTURE

Seeds were fixed in 3 % gluteraldehyde in a sodium cacodylate buffer at room temperature until the fixative was absorbed and the seeds had sunk. Post-fixation with 2 % osmium tetroxide (4 h) was carried out, followed by 1 h block staining with uranyl acetate (MOLLENHAUER, 1974; BANCROFT & STEVENS, 1986), before samples were dehydrated in a graded EtOH series and embedded in Epon / Araldite resin. Sections were cut to 50 – 70 nm with a diamond knife on an LKB Ultratome (III) and stained with lead citrate. Sections were viewed with a Philips CM 120 BioTWIN transmission electron microscope at an accelerating voltage of 80 – 100 kV.

Lipids were recognizable by their almost transparent appearance as they are less dense than other cellular components, with densitometer readings of 78 – 81 % transmission (MANNING & VAN STADEN, 1987). Protein bodies are usually well defined, dense in appearance (densitometer reading of 30 % transmission, MANNING & VAN STADEN, 1987) and are typically larger than the lipid bodies. Starch grains were crystalline in appearance, with approximately 20 – 30 % transmission (MANNING & VAN STADEN, 1987).

Where possible only median longitudinal sections were used for quantification and illustration. Where possible, mean values represent counts from at least three cells from median sections of a minimum of three seeds. Difficulties experienced during the sectioning of both testa and starch-containing material resulted in torn sections and a limited availability of suitable sections for all species. Preliminary observation revealed that the distribution and relative amounts of lipid and protein storage did not differ visually between species with the same seed type. Consequently, comparisons between species of the same seed-type were not analyzed statistically. Rather, mean size and quantity values stem from data that were pooled for each seed type. The difference between cell and nucleus volumes (μm^3) can be used to give an indication of the volume of total reserve storage (LEROUX *ET AL.*, 1997), but only in the absence of cellular organelles. However, the validity of such a calculation is also reliant on medial cell sections with maximum volume. The volume of reserve material was therefore not calculated. The difficulties outlined with respect to torn sections result in much of these data being un-illustrated.

Seeds being prepared for paraffin wax embedding were placed into small paper envelopes and collectively housed within plastic cassettes. These cassettes were bathed in 10 % neutral buffered formal saline until required (DRURY & WALLINGTON, 1980; DAMJANOV, 1996). Tissue was processed overnight using a Leica Histokinette 2000 automatic tissue processor, during which time the seeds were dehydrated in an EtOH series, rinsed in chloroform and infiltrated with

liquid paraffin wax (BANCROFT & STEVENS, 1986). Cassettes were retained for a minimum of 0.5 h in a final bathing solution of liquid wax (CULLING, ALLISON & BARR, 1985). Paper envelopes were transferred to individually labeled cassettes and embedded in paraffin wax. Sections, cut at 3 μm, were prepared from the paraffin blocks using a LKB Bromma 2218 Historange microtome. Sections were stained with haemotoxylin and the counter-stain eosin *Y* (CULLING *ET AL.*, 1985; DAMJANOV, 1996) that, amongst other staining reactions, result in blue nuclei and pink triglycerides (lipids). For a control, lipids were extracted with methanol : chloroform (1:1) before staining (COIMBRA & SALEMA, 1994). Staining proceeded as follows:

- removed paraffin wax with xylene (1 x 5 min rinse)
- 2 x rinses in 100 % EtOH (1 min each)
- washed under running water (30 sec)
- stained with haemotoxylin (6 min)
- washed under running water (30 sec)
- differentiated in acid / alcohol until only nuclei remained blue
- washed under running water (30 sec)
- 1 x rinse in SCOTTS' tap water (10 sec)
- washed under running water (30 sec)
- stained with 1 % aqueous eosin *Y* (3 min)
- washed under running water (1 min)
- 3 x rinses in 100 % EtOH (30 sec each)
- 2 x rinses in xylene (1 min each)

Sections were mounted onto glass slides and viewed under an Olympus BH-2 light microscope. Again, only median longitudinal sections were used for quantification and illustration.

4.3.2 FREE SUGAR ANALYSIS

One mg (f/w) of seed was extracted overnight in 4 ml 80 % EtOH before being filtered through Whatman No.1 filter paper and rinsed copiously with 80 % EtOH. Samples were then dried down under nitrogen. Sugars were converted to their oximes by the addition of 400 μl of 25 mg ml^{-1} hydroxylamine hydrochloride solution dissolved in pyridine, and the reaction allowed to proceed for 40 min at 35 °C. After cooling the samples were re-dried under nitrogen and the sugars silylised for 20 min at room temperature by the addition of 100 μl Sylon BT (SWEELEY, BENTLEY, MAKITA & WELLS; 1963). The trimethylsilyl derivatives were separated on a Varian 3700 gas chromatograph fitted with a 2 m glass column packed with 3 % OV-17 on 80 / 100 Chromosorb W HP and detected by flame ionization. The chromatograph was programmed as follows:

- Chromatographic conditions; injection temperature 200 °C, detector temp. 300 °C, and initial oven temp. 155 °C
- Program conditions; 3 min hold, increased at 4 °C min^{-1} to 284 °C, and a final hold of 3 min
- Integrator parameters; attenuation 4, ECD 10^{-10}

Standards of the sugars arabinose, rhamnose, sorbose, fructose, galactose, glucose, inositol (a sugar alcohol), mannose, sucrose, trehalose, cellobiose and maltose at concentrations of 10 μg ml^{-1} were treated accordingly. It must be noted that several standards failed to produce repeatable results and for this reason the specific levels of sugars (μg mg^{-1} fresh weight) contained within seeds were not quantified. Where non-repeatable chromatographs were obtained, retention times for standard sugars were obtained from PODD (2000).

The total free sugar contents (total CHO) of species were compared according to the following ratios, which were calculated using the individual peak areas from the chromatograph detector response. All ratios were plotted against ease of seed germinability. Species were grouped according to their sugar profile and compared to groupings based on seed type, phylogeny and germinability data. Linear and polynomial regressions were fitted to the data using Statistica (STATSOFT INC., 1998), where r^2 represents the proportion of variation accounted for by the equation. An r^2 value that approaches 1 indicates a significant goodness-of-fit (ZAR, 1996).

- fructose : glucose : sucrose (F:G:S)
- combined FGS fraction : all remaining sugars (FGS:other CHO's)
- sucrose : other C_{12} sugars (S:C_{12} CHO's)
- C_5 : C_6 : C_{12} sugars (C_5:C_6:C_{12})

The evolution of *Disa* seed reserves were investigated by plotting seed characters onto morphological cladograms (LINDER & KURZWEIL, 1999; Appendix One). The characters plotted were the presence of starch, the representation of individual free sugars and the relative dominance of glucose, fructose and sucrose respectively. Unfortunately cladograms were not available for *D. nervosa*, *D. stachyoides* (section *Emarginatae*, LINDER & KURZWEIL, 1999) and *D. pulchra* (section *Stenocarpa*, LINDER & KURZWEIL, 1999). In the absence of these cladograms, the parsimonious evolution of nutrient reserve characters remains relatively uninformative and has not been illustrated. However, taxonomic affiliations and germinability implications are reported where appropriate. The molecular phylogeny of BELLSTEDT *ET AL.* (2001; Appendix Two) was not used because it reveals the relationships of comparatively fewer small-seeded *Disa* species and is reliant on the sectional classification of LINDER & KURZWEIL (1999).

4.4 RESULTS AND DISCUSSION

4.4.1 SEED ULTRA-STRUCTURE

The mature embryos of all *Disa* species investigated contained significant storage products (Plate 4.1 and Plate 4.2). MANNING & VAN STADEN (1987) showed that mature *D. polygonoides* seed contains abundant lipid and protein reserves, but no starch. Similar results were obtained for *D. chrysostachya*, *D. cooperi*, *D. cornuta*, *D. crassicornis*, *D. nervosa*, *D. pulchra*, *D. stachyoides* and *D. versicolor* (Plate 4.1).

The distribution, relative size and relative abundance of storage bodies did not differ between these 8 species, all of which produce small seeds (Plate 4.1A – D). Organelles were not visible in the cells of the small-seeded *Disa* embryo, as is typical for orchid embryos. Vacuoles were absent. In these species, the bulk of the protoplast apart from the nucleus, was occupied by contiguous lipid and protein bodies. Amyloplasts and starch grains were absent from the seeds of all species at maturity. Limited seed availability, coupled with the requirement of mature seed for germination trials, dictated that seed ultra-structure was not investigated in immature *Disa* seeds. Consequently we remain reliant on previous studies in this regard, particularly MANNING & VAN STADEN (1987).

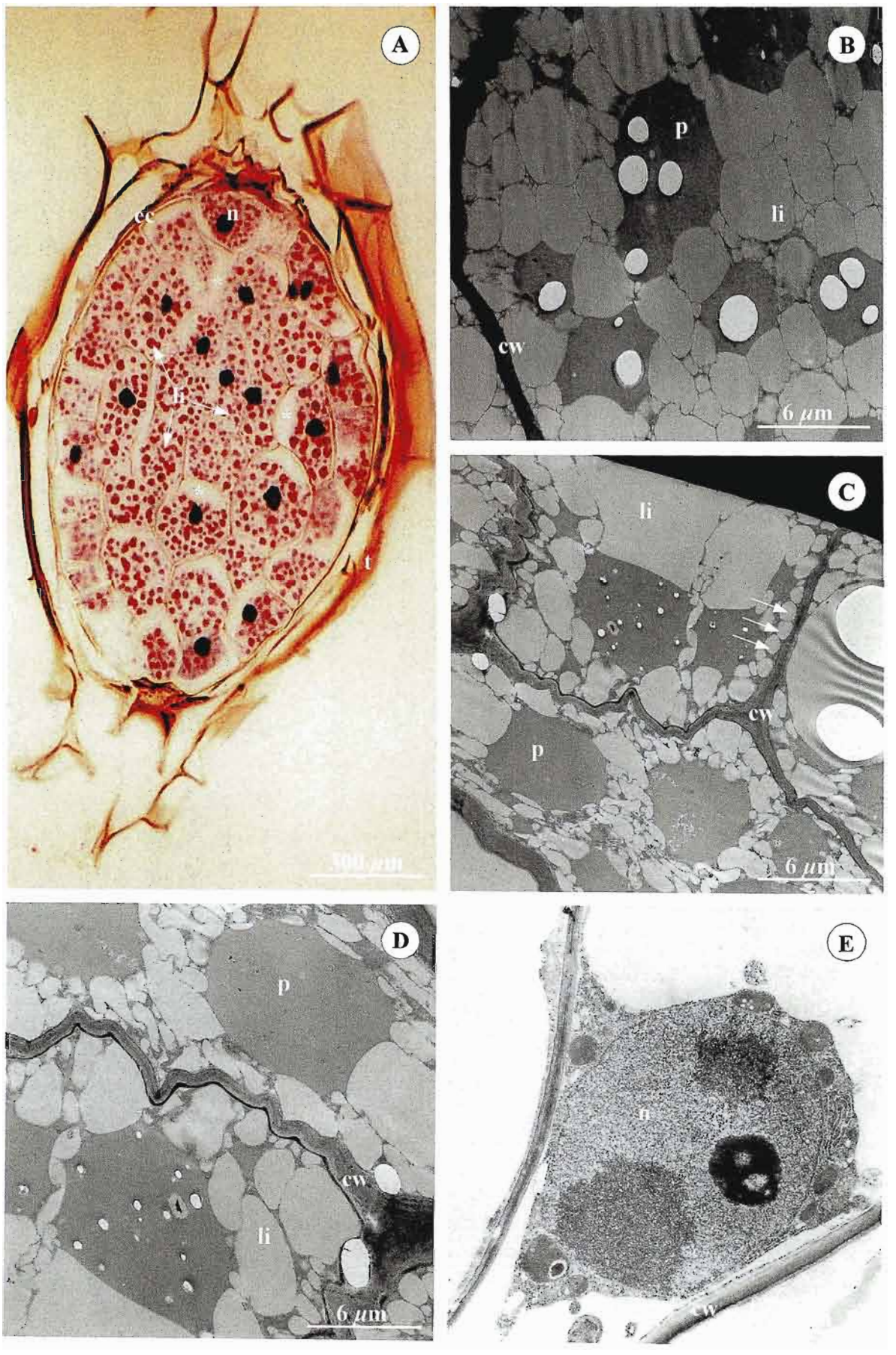
Lipid bodies ranged between 0.2 – 5.0 μm (1.4 ± 1.8 μm; mean ± SD, n = 300) at their widest point. Large and small bodies were found in all small-seeded species. Typically, smaller bodies ($\leq$ 1 μm) formed a layer adjacent to the plasmalemma, as illustrated for *D. crassicornis* (Plate 4.1C). The presence of this layer was consistent between cells and between species. A similar pattern was reported for *D. polygonoides* (MANNING & VAN STADEN, 1987). The number of lipid bodies per cell in medial section was recorded as 127 ± 7 (mean ± SD, n = 45).

Light microscopy (Plate 4.1A) revealed that lipid bodies are distributed uniformly throughout *Disa* embryos, with no relationship being identified between epidermal and centrally situated or polar and non-polar cells in any of the species investigated. Such a distribution was expected based on previous studies (LEROUX *ET AL.*, 1997). *Disa cornuta*, *D. crassicornis* and *D. pulchra* (Plate 4.1A) contained several cells with relatively large areas that do not contain lipid reserves. These areas are commonly adjacent to the plasmalemma towards the interior or micropyle of the embryo.

The regions that are free from reserves are reminiscent of cellular vacuoles, the presence of which was not confirmed with electron microscopy. The occurrence of these cells is unrelated to germination response since they occur sporadically within the embryos of three species which display germinability indices of two, three and two respectively.

Protein bodies occurred in all small-seeded species investigated (Plate 4.1B – D), averaging 9 ± 2 (mean ± SD, n = 45) per medial cell section. A maximum of 14 protein bodies were recorded in *D. stachyoides*. At their widest point, protein bodies measured 4.2 ± 2.3 μm (mean ± SD, n = 160). The volume of lipid and protein bodies was not calculated directly as a result of their largely variable dimensions and irregular shape. Additionally, the presence of reserve-free regions within occasional cells in *D. cornuta*, *D. crassicornis* and *D. pulchra* embryos precluded the calculation of a per cell or per embryo nutrient reserve volume.

None of the lipid and protein rich, small-seeded *Disa* species were easily germinable *in vitro*, with germinability indices of two or three being recorded under the seed culture conditions outlined in Table 4.1. The nutrient reserve status of these species did not allow for separation based on the nature, abundance or relative size of storage bodies.


A
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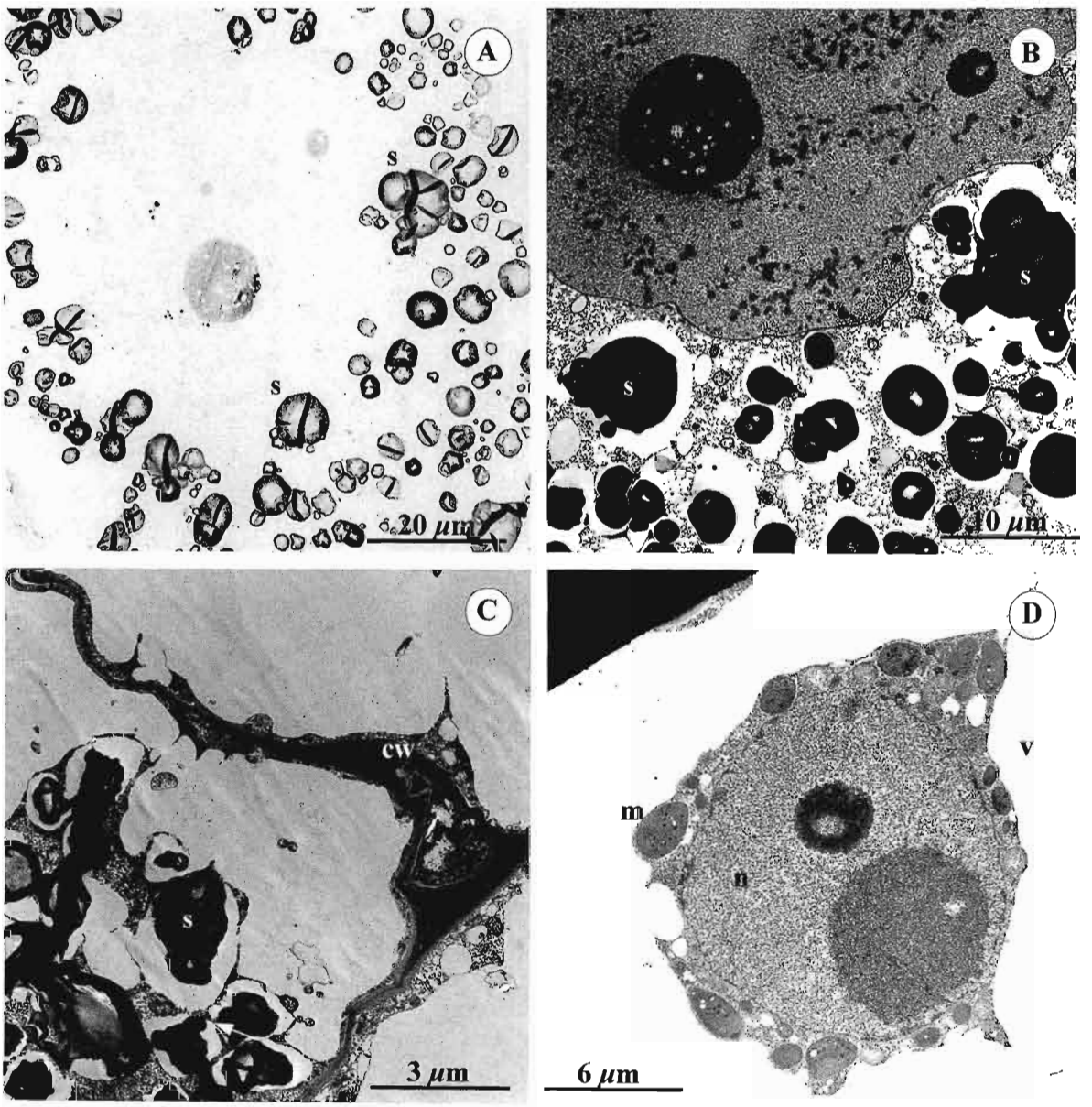
Consequently, the difference between poorly germinable (*D. cooperi*, *D. cornuta*, *D. nervosa* and *D. pulchra*) and intractable species (*D. chrysostachya*, *D. crassicornis*, *D. stachyoides* and *D. versicolor*) cannot be explained in terms of variable lipid or protein reserves. Specific trials to quantify the influence of *ex situ* aging on *Disa* seed viability and germination were beyond the scope of this study. However, the higher germinability indices (GI = 3) ascribed to the intractable *Disa* species cannot be attributed to lost seed viability as a consequence of cold storage, since non-stored seed of these species was also intractable *in vitro*. By the same token, the poorly germinable *Disa* species consistently displayed germinability indices of two, irrespective of pre- or post-storage culture.

Small seeds are synapomorphic within *Disa*. The restriction of the dominance of storage lipids to such seeds implies that lipid storage is the primitive condition within *Disa*. The presence of lipids as the primary storage compound is reported from several other genera within the Orchidoideae (MANNING & VAN STADEN, 1987).

Contrary to previous reports (HARRISON, 1977; ARDITTI & ERNST, 1984; MANNING & VAN STADEN, 1987; RICHARDSON *ET AL.*, 1992; RASMUSSEN, 1995) starch was recorded in *D. tripetaloides* and *D. uniflora* seeds (Plate 4.2). The presence of starch in the mature seeds of these species is significant, bearing in mind that starch reserves are characteristic of immature and post-imbibition orchid embryos and that starch was not detected in the other *Disa* species examined. *Disa tripetaloides* and *D. uniflora* both germinate readily *in vitro*, displaying germinability indices of one. Additionally, these two species are known to produce relatively large, seeds classified as belonging to the *D. uniflora* – type (Table 4.2). Such seeds are apomorphic in the genus and are restricted to a single clade in section *Disa*.


A
s
s
20 μm
B
s
s
10 μm
C
cw
s
3 μm
D
v
m
n
6 μm

This restriction implies that seed size and nutrient status evolved in affiliation with one another, making the occurrence of starch in the *Disa* embryo a secondary condition. KURZWEIL (1993) speculated that the presence of anomalous 'endosperm' in these derived species accounted for their rapid germination response. However, this study refutes such a claim, with the cells of the embryo remaining undifferentiated at seed maturity.

Increased seed size, which was interpreted as an adaptation to hydrochorous seed dispersal (KURZWEIL, 1993), therefore also permits the storage of insoluble reserves (starch). In the absence of endosperm, as expected for a member of the Orchidaceae, starch storage occurred without pattern in the cells of the *Disa* embryo.

Starch grains measured 3.9 ± 1.4 μm (mean ± SD, n = 30). Lipid bodies were also detected in the large-seeded *Disa* species, but in insignificant quantities (Plate 4.2B). These bodies were significantly smaller than those found in the small-seeded species, only reaching a maximum diameter of 1.2 μm. Protein bodies were not detected in either *D. tripetaloides* or *D. uniflora* (Table 4.2).

Table 4.2. The presence or absence and relative abundance of lipid, protein and starch storage bodies in 10 South African *Disa* species. Species have been grouped according to seed type and germinability indices (GI) are included.

	GI	Lipid	Protein	Starch
Small-seeded *Disa* species	2 / 3	✓	✓	X
Large-seeded *Disa* species	1	*	X	✓

* indicates negligible storage.

It seems logical that the starch-containing *Disa* species will be reliant on starch hydrolysis to facilitate rapid, autonomous germination, as has been documented for non-orchidaceous species (BEWLEY & BLACK, 1994; COIMBRA & SALEMA, 1994; SIVAK & PREISS, 1995; ZIEGLER, 1995).

VOGELPOEL (1980) reported that *D. uniflora* is useful as a representative species because unlike most other orchids, viable seeds can be germinated rapidly without resorting to aseptic tissue culture practices. Several authors (COLLETT, 1971; VOGELPOEL, 1980, 1987) reported germination in *D. uniflora* despite the absence of exogenous carbohydrates. Germination under such conditions must be facilitated by endogenous storage reserves, the presence of which have now been confirmed in this study. This capacity appears restricted to those species that contain starch at maturity. The easily germinated *Calypso bulbosa* and *Bletilla striata*, which also contain starch at maturity (NISHIMURA, 1981; YEUNG & LAW, 1992), add support to this argument. However large-seeded *Disa* species, such as *D. uniflora*, are atypical with regard to nutrient storage. The small-seeded *Disa* species, which constitute the main diversity within the genus, do not contain starch at maturity and are not germinable when sown in the absence of exogenous carbohydrates.

Starch grains were detected in low frequencies in *D. cooperi*, *D. cornuta*, *D. nervosa* and *D. pulchra* embryos between two to four weeks after *in vitro* culture initiation. This is characteristic of orchid seeds which contain sufficient active reserves to facilitate the initiation of lipolysis (ARDITTI & ERNST, 1981, 1984; MANNING & VAN STADEN, 1987). These four species display germinability indices of two. The low and variable percentage germination achieved *in vitro* on modified media, that categorizes these species, suggests that seeds of a single species may vary with respect to their soluble reserves. This has been proposed previously by RASMUSSEN (1995). Seeds displaying a germinability index of three did not accumulate starch during the same period. Light microscopy revealed that the lipid reserves of these species remained immobilized for up to six months after culture initiation. Such a result implies that although more-than-ample lipid reserves are present within the embryos of the intractable *Disa*, they are not accessible through the inability to initiate gluconeogenesis.

During germination, the cells of the embryos of both the large and small-

seeded *Disa* species become highly vacuolate and organelles become visible (Plate 4.1E and Plate 4.2D). Vacuolation typically occurs as a result of storage body depletion during germination (HARRISON, 1977; MANNING & VAN STADEN, 1987; RASMUSSEN, 1995), confirming the role of lipids as precursors to metabolically active substrates.

Starch, although present in mature, large-seeded *Disa* embryos both pre- and post-imbibition and in the mature, germinable small-seeded *Disa* post-imbibition, was not observed in protocorms over the age of 8 – 10 weeks. Starch therefore represents a transitory carbohydrate that can be mobilized by the germinating *Disa* embryo. This corresponds to the maturation of leaf primordia and the terminal development of primary leaf tissue with the visible appearance of chlorophyll. However, an external carbohydrate source needs to be maintained indefinitely since photosynthesis does not function sufficiently to support the seedling *in vitro* (Chapter Three). This has been reported previously for several Holarctic orchids (HARRISON & ARDITTI, 1978; RASMUSSEN, 1995: LEROUX *ET AL.*, 1997; ARDITTI & GHANI, 2000).

The relationship between starch accumulation in the post-imbibition seed and the ability of that seed to germinate is clear, with species that do not accumulate starch remaining intractable *in vitro*. The presence of amyloplasts in the post-imbibition seeds of poorly germinable species is expected in terms of previous studies (ARDITTI, 1967; HARRISON, 1977; ARDITTI & ERNST, 1981; MANNING & VAN STADEN, 1987; RASMUSSEN, 1995) and represents a crucial step in the germination process. Starch in the mature seed of *D. tripetaloides* and *D. uniflora* is therefore indicative of seeds that are nutritionally primed for the germination process.

4.4.2 FREE SUGAR ANALYSIS

The mature *Disa* seed examined contained significant levels of free

(soluble) sugars within the embryo protoplasts (Figures 4.1 – 4.3). The presence of these soluble carbohydrates in *Disa* embryos is noteworthy since the mature seeds were examined dry. Consequently, they are not the result of post-imbibition lipid or starch hydrolysis. Rather, previous studies (BEWLEY & BLACK, 1994; ZIEGLER, 1995) have indicated that soluble sugars are required to support these processes, which facilitate germination. This is further supported by the presence of soluble sugars in those *Disa* species that did not germinate *in vitro* on modified media (Figure 4.3). Free sugars in the small-seeded *Disa* species cannot result from starch hydrolysis post-dispersal, since starch is absent from mature embryos of these species.

Glucose, fructose, arabinose, rhamnose, sucrose, mannose and maltose are all reported from mature seeds of terrestrial orchid species, including *D. polygonoides* (ARDITTI, 1982; MANNING & VAN STADEN, 1987; RASMUSSEN, 1995). Fifteen sugars were encountered in the 10 *Disa* species investigated, with fructose, glucose and sucrose occurring in all species (Table 4.3). The widespread occurrence of sucrose is in keeping with previous research on terrestrial orchids (ARDITTI, 1982; MANNING & VAN STADEN, 1987; RASMUSSEN, 1995). First records of ribose, xylose, sorbose, galactose, inositol and trehalose from orchid embryos are reported from this study. The presence of galactose (in *D. crassicornis*; Figure 4.3B), which is toxic to plant tissues at relatively low concentrations (ERNST *ET AL.*, 1970; ARDITTI, 1979, 1982), was unexpected and this result should be viewed with caution. Mannose, which is common in many terrestrial orchid species (ARDITTI, 1982; RASMUSSEN, 1995), was absent from the *Disa* species investigated.

These sugars occurred without taxonomic (sectional) or germinability affiliation across the 10 *Disa* species investigated. The plotting of individual sugar types onto the morphological cladograms of LINDER & KURZWEIL (1999; Appendix One) revealed that none of the sugars occurred exclusively in any particular section, nor were they restricted to species with shared germinability indices.

Even sugars with low occurrence (Table 4.3) did not reveal taxonomic or germinability relationships.

Three sugars (CHO's$_{1-3}$) were encountered which were not identifiable from co-chromatography. These made very little overall contribution (< 5 %) in the majority of species and were ignored for the purposes of ratio calculation. However, in *D. cornuta* (Figure 4.2B) and *D. versicolor* (Figure 4.3D) CHO_1 made significant contributions of 15.9 % and 5.7 % respectively. In these cases, ratio calculation was unavoidably influenced by their omission. As with the identified free sugars, CHO's$_{1-3}$ were distributed without apparent taxonomic or germinability links. No relationship was found between the total soluble sugar content (Figures 4.1 – 4.3) and the level of germinability in *Disa* ($y = 2924.3x + 485601$, $r^2 = 0.0034$), with intractable *D. versicolor* seed (Figure 4.3D) containing approximately twice as much soluble sugar (74 000 units) as the easily germinable *D. uniflora* (Figure 4.1A; 40 000 units).

Fructose, glucose and sucrose were the most commonly encountered and the most abundant sugars in the 10 *Disa* species examined. Typically they constituted over 50 % of the total soluble sugar content when combined and are regarded as the dominant free sugars in the *Disa* protoplasts (Figures 4.1 – 4.3). Despite this, the contribution of a combined fructose, glucose and sucrose fraction towards total sugar content decreased non-significantly with decreasing germinability ($y = -2.3362x + 82.768$, $r^2 = 0.3122$). Looking strictly at this combined fraction (Figure 4.4), a decreased percentage contribution by sucrose was correlated significantly with decreasing germinability ($y = -5.0802 x + 63.79$, $r^2 = 0.7791$). Sucrose also made a significant contribution to the total C_{12} sugar content of the seed ($y = -0.7836 x + 84.895$, $r^2 = 0.7132$). Such data are not surprising since current research on the seeds of endospermous species has shown the ratio of sucrose to other C_{12} or larger sugars as being influential in germination (BEWLEY & BLACK, 1994).

Table 4.3. Sugars encountered in the 10 *Disa* species investigated. The occurrence of particular sugars (up to a maximum of 10, highlighted in grey) and the number of sugars per species are included.

		Disa species										
Sugar	Retention time (min)	*chrysostachya*	*cornuta*	*cooperi*	*crassicornis*	*nervosa*	*pulchra*	*stachyoides*	*tripetaloides*	*uniflora*	*versicolor*	Occurrence
ribose	6								✓			1
arabinose	8.5		✓	✓	✓		✓	✓	✓	✓	✓	8
xylose	10.5			✓		✓		✓			✓	4
rhamnose	11.5	✓	✓		✓	✓	✓		✓		✓	7
sorbose	13				✓	✓	✓	✓			✓	5
fructose	14.5	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	10
galactose	15				✓							1
glucose	16	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	10
inositol	18						✓		✓			2
CHO_1	20		✓	✓	✓		✓			✓	✓	6
CHO_2	24			✓			✓				✓	3
CHO_3	28.5	✓									✓	2
sucrose	30	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	10
trehalose	32.5					✓	✓			✓	✓	4
maltose	33.5	✓	✓	✓	✓	✓	✓		✓	✓	✓	9
Species total		6	7	8	9	8	11	6	8	7	12	

Similarly, a significant increase in fructose relative to the combined fructose, glucose and sucrose fraction correlated with decreasing germinability ($y = 2.5358 x + 6.0633$, $r^2 = 0.6818$; Figure 4.4). A variable glucose contribution was not correlated significantly with differential germinability in *Disa*. However, the contribution made by C_6 sugars (glucose and fructose) towards total sugar content increased significantly with decreasing seed germinability ($y = 4.8735 x + 20.727$, $r^2 = 0.6067$), primarily due to the influence of fructose. C_5 and C_{12} sugar contributions were insignificantly correlated to germinability (Figure 4.5).

Table 4.4. The relationship between the dominance of glucose, fructose and sucrose and germinability in 10 South African *Disa* species. Species have been grouped according to seed type. Dominant (✓) and ancillary (x) sugars are recorded.

	fructose	glucose	sucrose
Large-seeded, germinable *Disa* species	x	✓	✓
Small-seeded, germinable *Disa* species	✓	✓	✓
Small-seeded, intractable *Disa* species	✓	✓	x

Consequently, easily germinable species (GI = 1) such as *D. tripetaloides* and *D. uniflora* displayed free sugar profiles dominated by sucrose and, to some extent, glucose, whilst being comparatively depauperate in fructose (Figure 4.1A, B). Germinable small-seeded species (*D. cooperi*, *D. cornuta*, *D. nervosa* and *D. pulchra*; GI = 2) also have free sugar profiles dominated by sucrose and glucose, together with increased fructose (Figure 4.2A – D). Intractable *Disa* species (*D. chrysostachya*, *D. crassicornis*, *D. stachyoides* and *D. versicolor*; GI = 3) were rich in both glucose and fructose, but comparatively poor in sucrose (Figure 4.3A – D). A continuum exists in which germinable small-seeded species represent a nutritionally intermediate state between the easily germinable, large-seeded species and the small-seeded intractable species (Table 4.4).

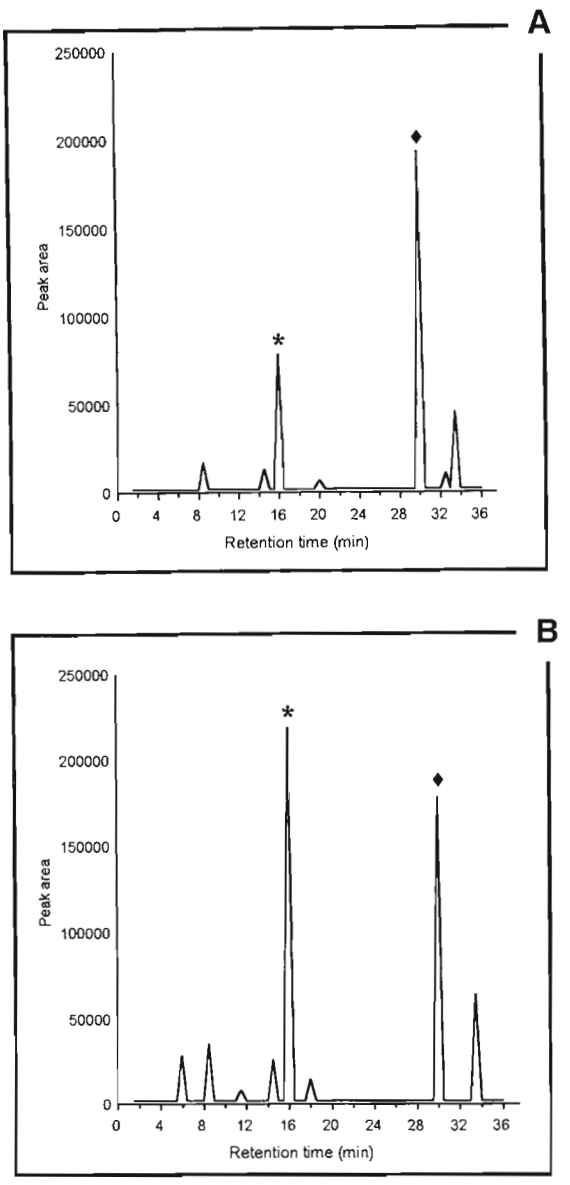


Figure 4.1. Representative sugar chromatographs for the germinable (GI = 1), large-seeded *Disa* species. *Disa uniflora* (A) **and *D. tripetaloides*** (B). **Attention is drawn to the dominant peaks at 16 and 30 minutes, the retention times of glucose (*) and sucrose (♦).**

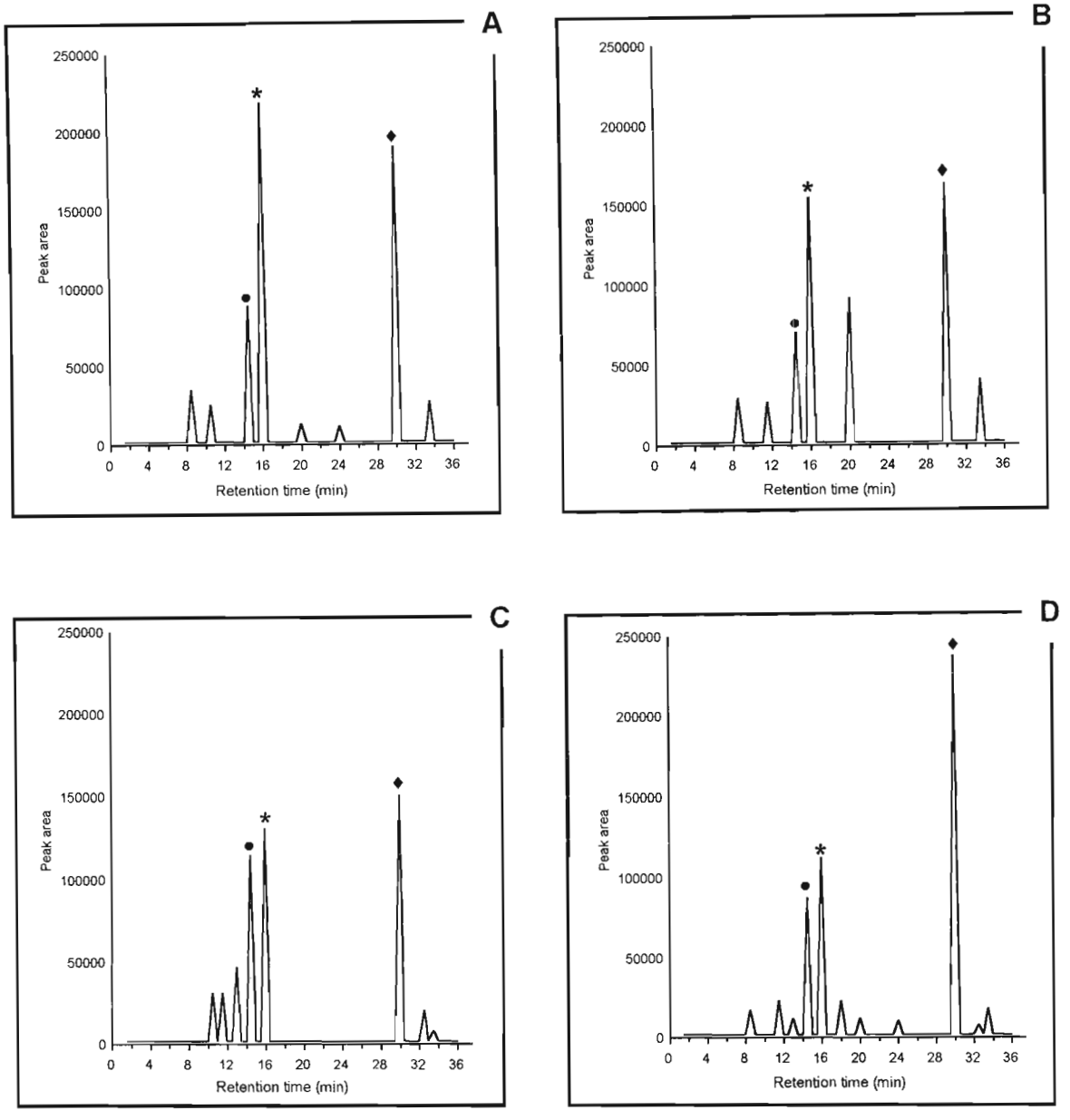


Figure 4.2. Representative sugar chromatographs for the germinable (GI = 2), small-seeded *Disa* species. *Disa cooperi* (A), *D. cornuta* (B), *D. nervosa* (C) and *D. pulchra* (D). Attention is drawn to the dominance of fructose (•), glucose (*) and sucrose (♦), with retention times of 15, 16 and 30 minutes respectively.

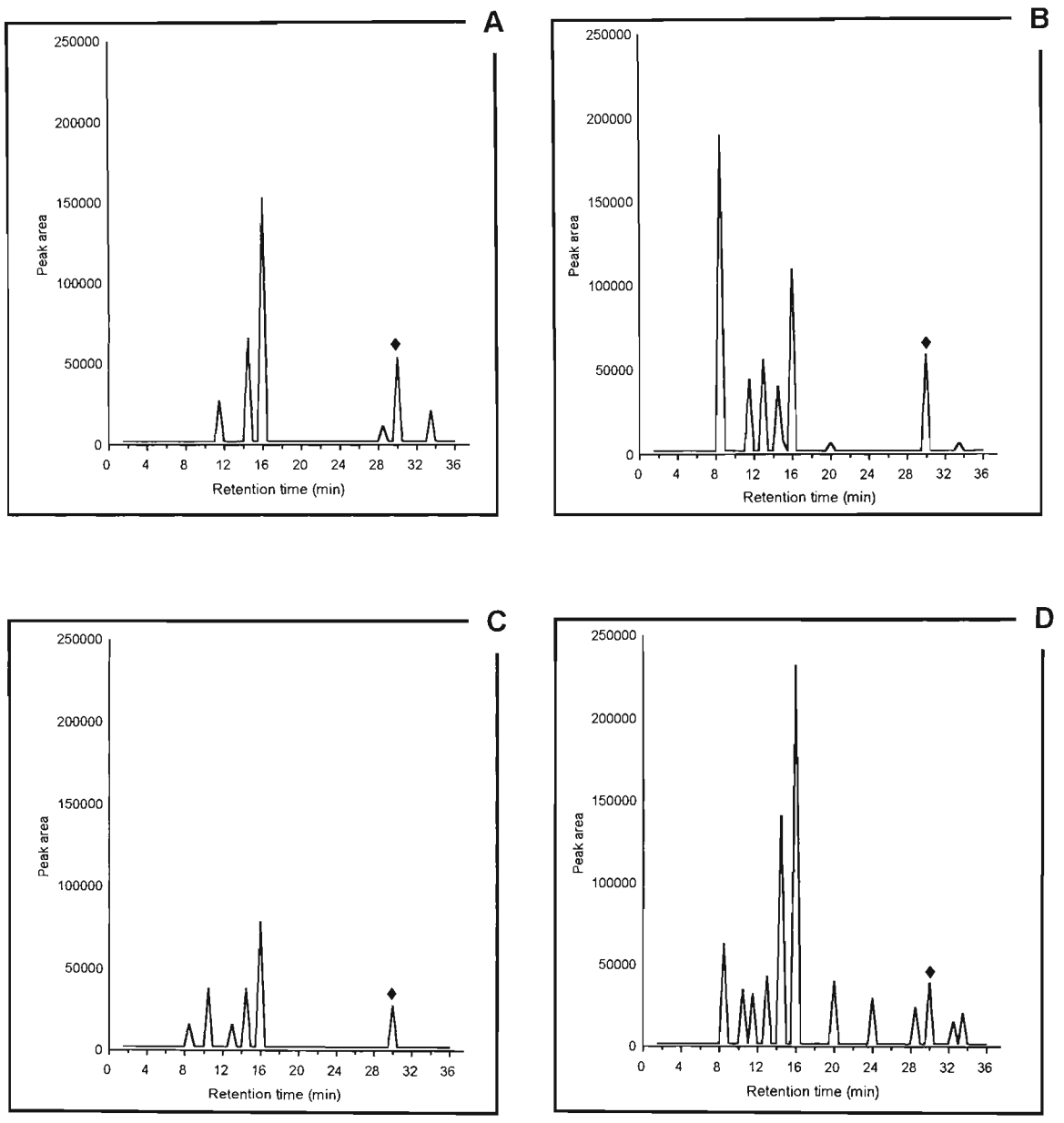
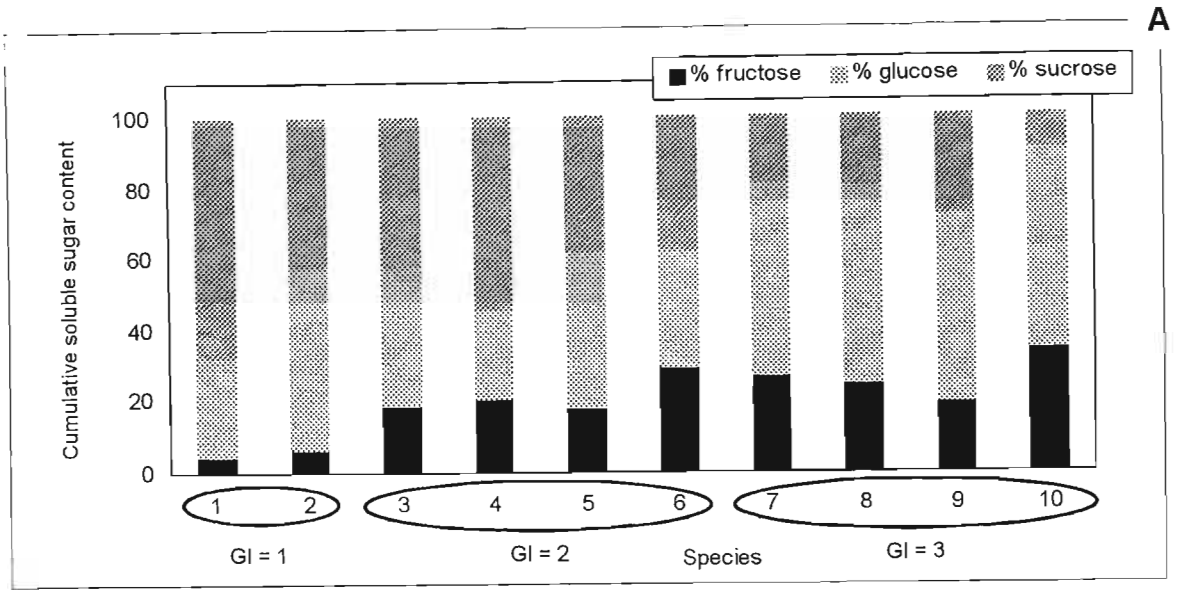


Figure 4.3. Representative sugar chromatographs for the intractable (GI = 3), small-seeded *Disa* species. *Disa chrysostachya* (A), *D. crassicornis* (B), *D. stachyoides* (C) and *D. versicolor* (D). Attention is drawn to the absence of a dominant peak at 30 minutes, the retention time of sucrose (♦).



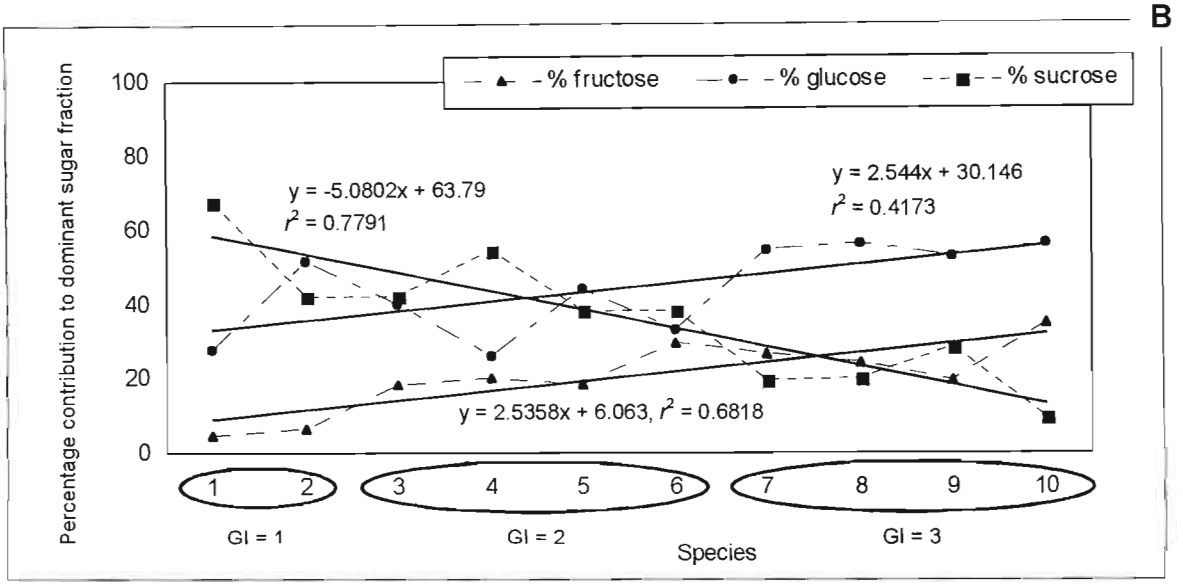
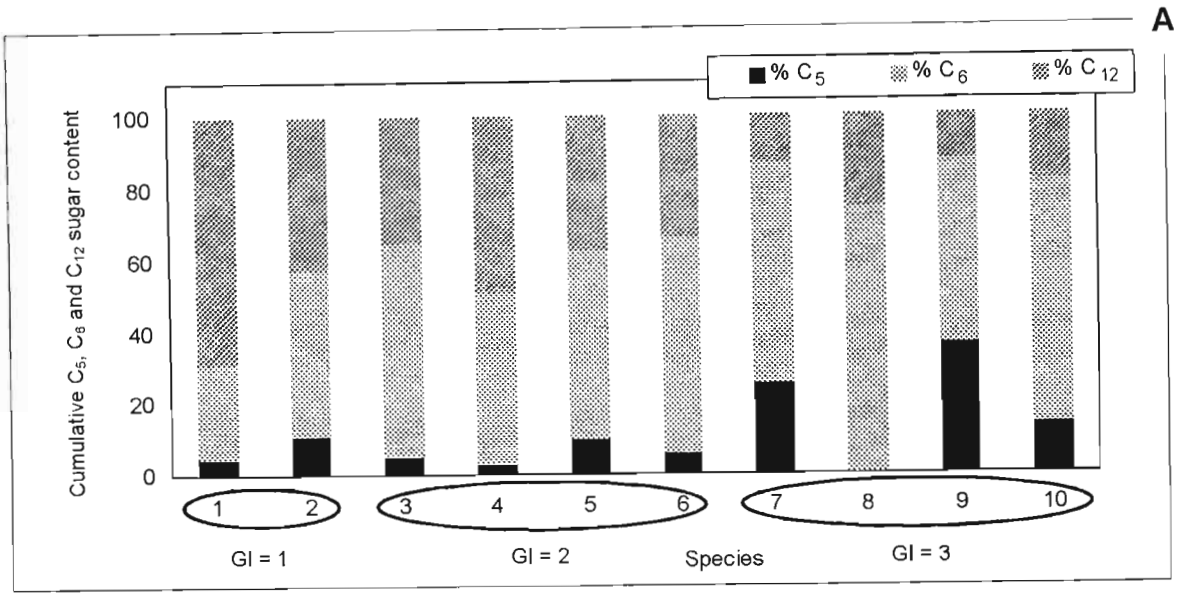


Figure 4.4. Relative percentage contributions made by the dominant free sugars fructose, glucose and sucrose towards a combined sugar total. Graphic representation (A) **and statistical analyses** (B) **of the combined fraction reveals a negative correlation between ease of germinability and the percentage fructose contribution (y = 2.5358x + 6.063, r^2 = 0.6818). A positive correlation exists between ease of germinability and the percentage sucrose contribution (y = -5.0802x + 63.79, r^2 = 0.7791). Germinability indices are included and species are ranked in order of germinability: 1 - *D. uniflora*; 2 - *D. tripetaloides*; 3 - *D. cornuta*; 4 - *D. pulchra*; 5 - *D. cooperi*; 6 - *D. nervosa*; 7 - *D. stachyoides*; 8 - *D. chrysostachya*; 9 - *D. crassicornis*; 10 - *D. versicolor*.**



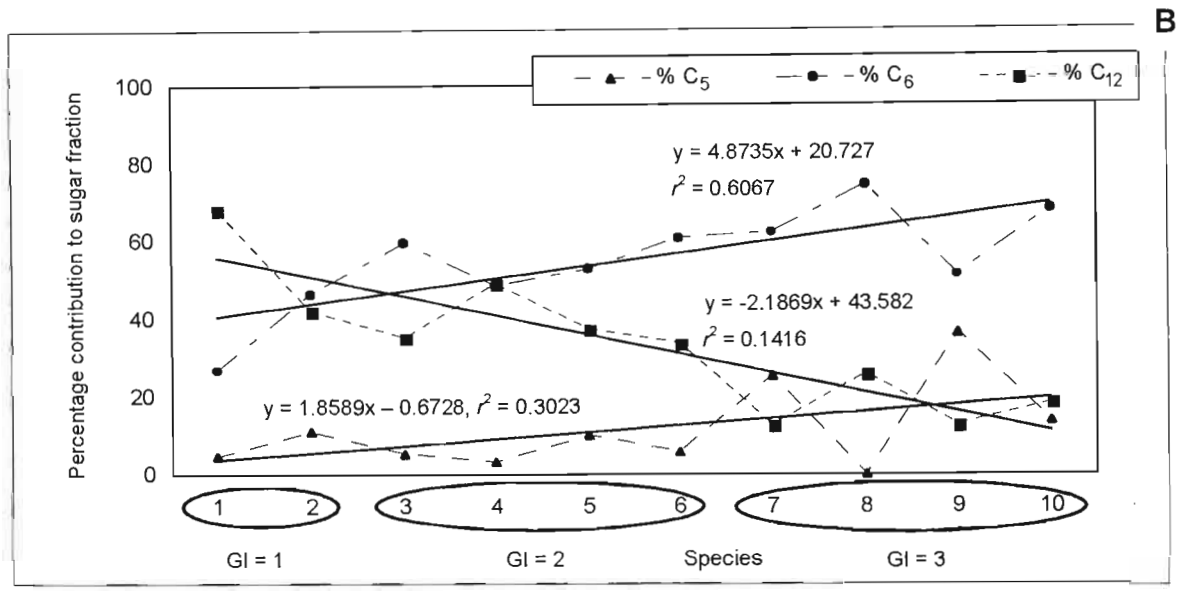


Figure 4.5. Relative percentage contributions made by C_5, C_6 and C_{12} sugars towards a combined sugar total. Graphically (A), several trends between sugar representation and species germination are noted. However, only the contribution made by C_6 sugars correlates significantly with germinability (B). This negative correlation is represented by $y = 4.8735x + 20.727$, $r^2 = 0.6067$. Germinability indices are included and species are ranked in order of germinability: 1 - *D. uniflora*; 2 - *D. tripetaloides*; 3 - *D. cornuta*; 4 - *D. pulchra*; 5 - *D. cooperi*; 6 - *D. nervosa*; 7 - *D. stachyoides*; 8 - *D. chrysostachya*; 9 - *D. crassicornis*; 10 - *D. versicolor*.

The free sugars identified in this study therefore serve as the source of carbohydrates needed for the initiation of lipolysis and germination. In large-seeded *Disa* species, these soluble sugars are present in sufficient quantities to maintain metabolism prior to the catabolism of storage products. In these species the storage compound was starch, which is easily cleaved to liberate glucose, sustaining its presence in the protoplast. Consequently, starch storage allows for autonomous germination that is maintained beyond the period where metabolism is reliant on the complement of free sugars present in the embryo.

The distribution of both starch storage and the dominance of glucose within the free sugar pool of mature *Disa* seeds reveals that size and nutrient status evolved jointly in *D. tripetaloides* and *D. uniflora*. This suite of characters is presumably apomorphic for the sub-clade within section *Disa*. Assessing the nutrient status of *D. cardinalis* and *D. caulescens* will confirm this. Large seeds belonging to the *D. uniflora* – type have been explained as an adaptation to hydrochory (KURZWEIL, 1993). The presence of starch and elevated levels of free sucrose in the mature embryos serves to ensure rapid germination and seedling establishment in these species, which are restricted to streamside localities under the winter-rainfall conditions of the western Cape.

However, several small-seeded species with comparatively higher soluble sugar contents than the large-seeded *Disa* species remain ungerminated *in vitro* (Figure 4.3), confirming that it is not the entire complement of sugars which determines seed germinability, but rather the presence or absence and relative quantities of specific key sugars. The correlations between sucrose, fructose and germinability have already been demonstrated. Consequently, a species such as *D. versicolor*, which has a comparatively high overall free sugar content, remains intractable due to a decreased sucrose, increased fructose content (Figure 4.3D) and an inaccessible store of lipids. The increased occurrence of sucrose in the small-seeded, germinable *Disa* species (Figure 4.2 and Figure 4.4) highlights the role of this specific sugar in facilitating germination in both starch-containing and

starch-deprived species (Table 4.4).

Free sugar accumulation in the maturing small-seeded *Disa* embryo (*D. cooperi* and *D. versicolor*; Figure 4.6 and Figure 4.7) was complete by the beginning of the fourth developmental quarter (> six weeks after pollination), being defined by the significant second order polynomial $y = -34874x^2 + 314973x - 9075$ ($r^2 = 0.991$). *Disa cooperi* and *D. versicolor* accumulated 97 % and 98 % respectively of their mature soluble sugar content during this period (Figure 4.8).

This period corresponds to the time frame over which accumulated starch disappeared from the developing *D. polygonoides* embryo (MANNING & VAN STADEN, 1987). Consequently, the indication is such that pre-maturation starch hydrolysis results in soluble glucose that is accumulated in the embryonic protoplasts.

Free sugar accumulation in the germinable *D. cooperi* was relatively rapid, with over 50 % of the total complement being present after the first developmental quarter (> two weeks). Halfway through development, seeds of the same species contain approximately 87 % of their final carbohydrate fraction. For the intractable *D. versicolor* seed, comparative values of 27 % and 55 % were recorded across these developmental stages (Figure 4.8). For *D. cooperi* this represented a two-fold increase in the quantity of sugar in the embryonic protoplasts over the first three developmental quarters (Figure 4.8). *Disa versicolor* had an initial sugar content approximately two times lower than the *D. cooperi* seed. However, by completion of the third quarter of development (six weeks after pollination), *D. versicolor* contained a greater soluble sugar complement. This difference was maintained through to seed maturity and represents a four-fold increase in sugar accumulation during maturation (Figure 4.8).

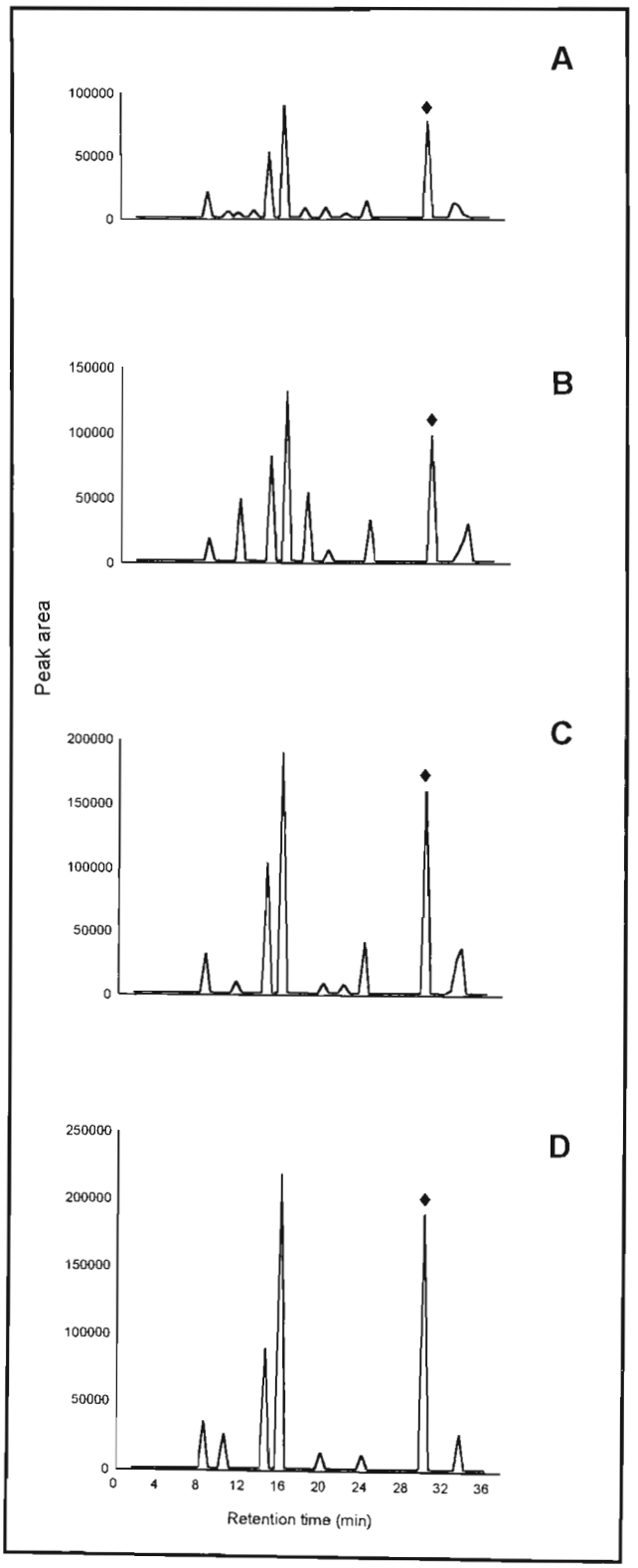


Figure 4.6.
Representative sugar chromatographs for developing *D. cooperi* seed. *Disa cooperi* is a small-seeded species with a germinability index of two. Seed used in the analysis originated from the following capsule developmental quarters:

First quarter – two weeks after pollination (A); **Second quarter – four weeks after pollination** (B); **Third quarter – six weeks after pollination** (C); **and Fourth quarter - dehiscent capsules, eight weeks after pollination** (D).

Attention is drawn to the sucrose peaks (♦) identified in all developmental quarters.

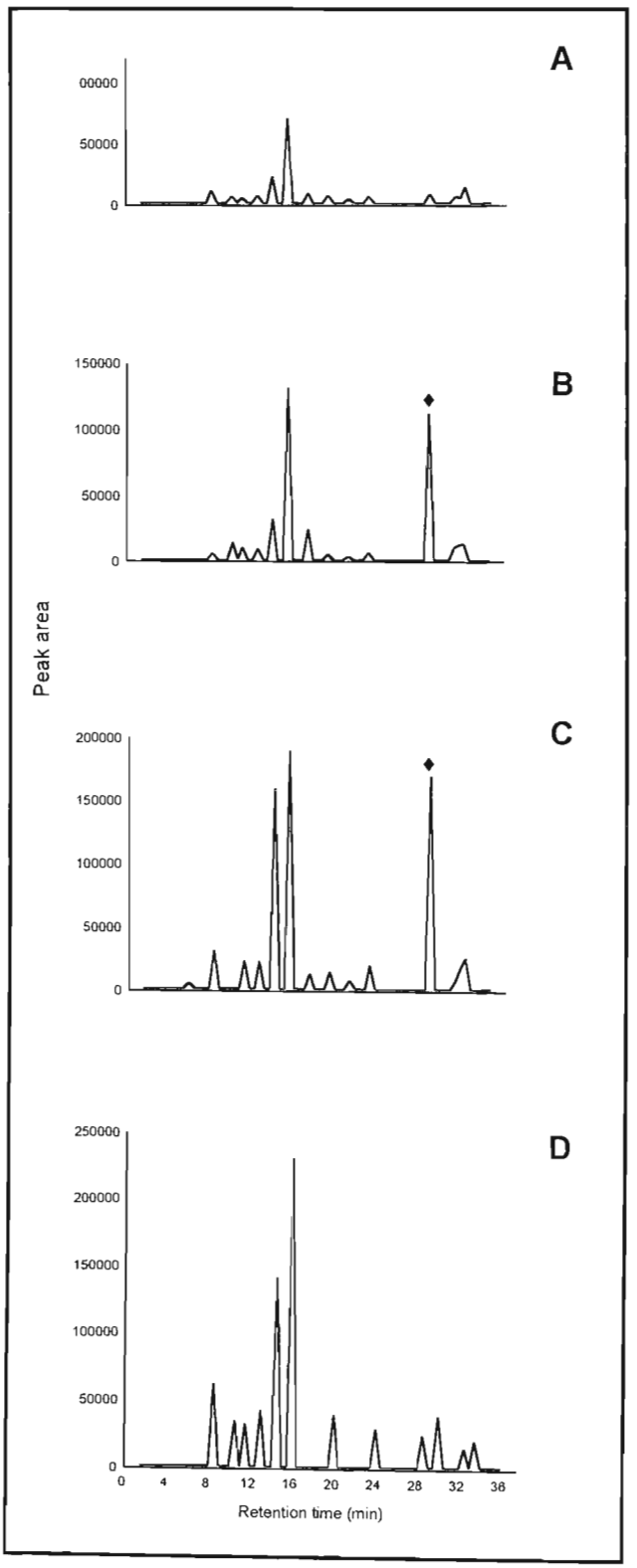


Figure 4.7.
Representative sugar chromatographs for developing *D. versicolor* seed. *Disa versicolor* is a small-seeded species with a germinability index of three. Seed used in the analysis originated from the following capsule developmental quarters:

First quarter – two weeks after pollination (A); **Second quarter – four weeks after pollination** (B); **Third quarter – six weeks after pollination** (C); **and Fourth quarter - dehiscent capsules, eight weeks after pollination** (D).

Attention is drawn to the sucrose peaks (♦) identified in seeds in the second and third developmental quarters.

At least 16 different sugars were detected in the developing seeds of these two species. Rhamnose, sorbose, glucose and an unidentified sugar (CHO_2) increased in both species throughout seed maturation. Other sugars, specifically fructose and maltose, are accumulated during the first three-quarters of development, after which time their levels decreased (Figure 4.6 and Figure 4.7). These results indicate that there is significant flux between sugar forms, especially during the last quarter of development, where the sugar profile reflects changes that are not associated with active carbohydrate accumulation.

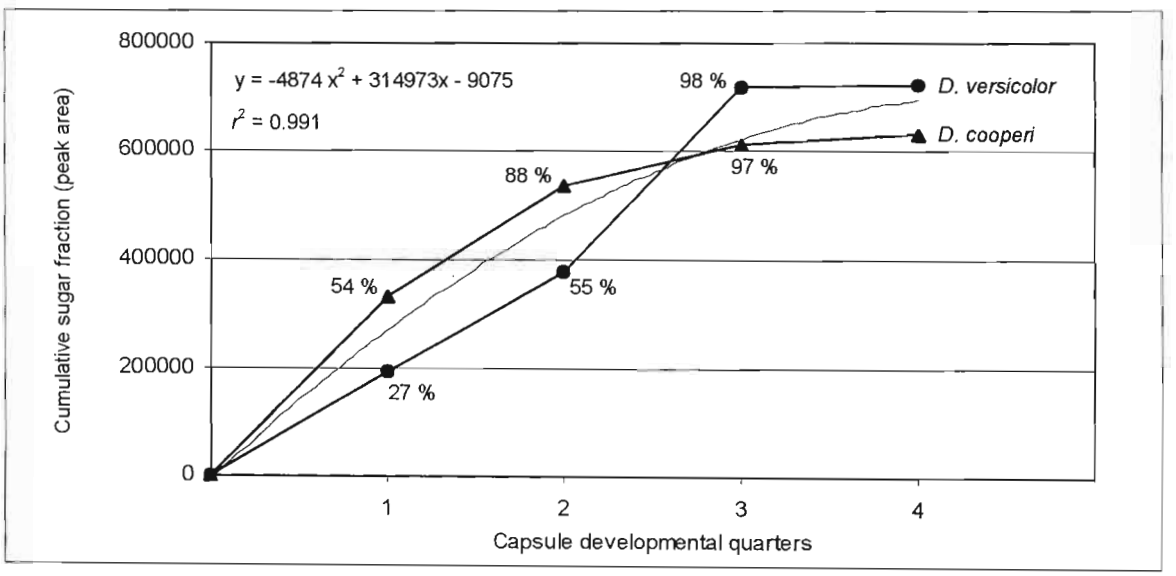


Figure 4.8. Cumulative soluble sugar content across four broad seed maturity classes for *D. cooperi* (GI = 2) and *D. versicolor* (GI = 3), as defined by a significant second order polynomial regression. The percentage of the total content achieved at each developmental stage is indicated. Seed used in the quantification of total free carbohydrates originated from each of four capsule developmental quarters: first quarter – 2 weeks after pollination; second quarter – 4 weeks after pollination; third quarter – 6 weeks after pollination and fourth quarter – dehiscent capsules, 8 weeks post pollination.

The cessation of free carbohydrate accumulation during the fourth quarter of development is dictated, at least in part, by the deposition and oxidation of phenols in the seed testa and the dehydration of the embryo. This study, in an

evaluation of seed testa phenolics and seed dormancy, has shown that phenol deposition takes place primarily during the latter half of seed maturation, as indicated by the progressive colouring of seeds in the third and fourth developmental quarters for both *D. cooperi* and *D. versicolor*. Carbohydrate flux until seed dehiscence, as observed in *Disa*, is also reported by FAST (1982), MITCHELL (1989) and NAGASHIMA (1989) for a number of northern hemisphere orchid species.

A window period therefore exists in the maturation of small-seeded *Disa* spp. during which time the embryo displays the almost total soluble sugar status of the mature embryo (Figure 4.8), but which has not yet been isolated from the external environment by an impenetrable seed testa. There are few obstacles to embryo rehydration if the seed coat has not yet acquired its moisture-repellent properties and the period of metabolic quiescence that usually follows embryo dehydration can be bypassed (RASMUSSEN, 1995).

The culture of immature seed, or 'green-podding', is a widely used technique in orchid seed culture and has proved extremely useful in achieving germination in difficult to germinate terrestrial orchid species (STOUTAMIRE, 1964, 1974; ARDITTI, 1982; CLEMENTS, 1982; FAST, 1982; ANDERSON, 1990, 1991; RASMUSSEN & RASMUSSEN, 1991; ZETTLER & MCINNIS, 1992; RASMUSSEN, 1995). *In vitro* germination results (VOGELPOEL, 1980, 1987; LACROIX & LACROIX, 1997; WODRICH, 1997; CROUS, 1999, PERS. COMM.) and the implementation of a novel dual-phase technique (Chapter Seven) reveal that 'green-podding' is also useful in the context of *Disa*, allowing for improved germination in species which were intractable (GI = 3) or only marginally germinable (GI = 2) under standard seed culture conditions (Table 4.1). The early accumulation of soluble carbohydrates by the *Disa* seed makes 'green-podding' a valuable technique to exploit the developmental window period, allowing the nutritionally primed embryo to germinate unrestricted.

Results have also shown that the free sugar profile of seeds of poorly germinable species is dominated by sucrose, fructose and glucose in the mature state. *Disa cooperi* seed displays this characteristic profile throughout maturation, with the key sugars dominating during all developmental quarters (Figure 4.6A – D). However, the seeds of *D. versicolor* (Figure 4.7), an intractable small-seeded species, were sucrose deficient at maturity. This is a state that was achieved only during the final quarter of seed development, since both second and third quarter maturation stages contained a relatively large sucrose complement (Figure 4.7B – C). Seed originating from third developmental quarter capsules of *D. versicolor* was rich in all three key sugars. Late maturation and sucrose deficiency in *D. versicolor* coincide with increased monosaccharide representation, specifically glucose (Figure 4.7D), with carbohydrate flux occurring through sucrose cleavage. Consequently, the immature (third quarter) intractable *Disa* seed (Figure 4.7C) displays the same sugar profile as the germinable *Disa* seed (Figure 4.6). This result emphasizes the potential of 'green-pod' culture as a viable method when trying to germinate small-seeded members of the genus *Disa in vitro.*

Reliance of the orchid seed on symbiosis is thought to be a byproduct of insufficient or inaccessible nutrient reserves (STOUTAMIRE, 1963, 1974; HARVAIS & HADLEY, 1967; WARCUP, 1971; SMRECIU & CURRAH, 1989; RASMUSSEN & WHIGHAM, 1998). This study has revealed a nutritional continuum within *Disa*, with species differing in the form of the storage reserves and in the profile of free carbohydrate accumulation in the embryonic cells. Consequently, the dependence of *Disa* species on fungi to facilitate germination is expected to be variable, as reported for many other orchid genera (WARCUP, 1971; PURVES & HADLEY, 1976; ARDITTI, 1982; RASMUSSEN & WHIGHAM, 1993, 1998; RASMUSSEN, 1995). Additionally, these results suggest that rather than simply being nutrient-deficient, species that are heavily reliant on symbiosis lack only those specific carbohydrate forms that are crucial for initiating and maintaining the germination process.

CHAPTER FIVE

MYCOTROPHY AND *DISA*: DEVELOPING SYMBIOTIC SEED CULTURES FOR SOUTH AFRICAN SPECIES

Symbiotic seed germination techniques are described for the first time for members of the South African *Disa*. Endophytes isolated from adult plants were restricted to limited groups of fungi, revealing congruency with Australian and Holarctic orchid mycorrhizae. Fungi obtained from the roots of 11 *Disa* hosts revealed mycorrhizal capacity *in vitro*, yet only a single isolate of *D. crassicornis* supported advanced seedling development in intraspecific association. These data confirm that endophytes isolated from roots do not necessarily support germination in seeds of the host species. Endophytes were shown to liberate soluble carbohydrate and non-carbohydrate compounds, providing evidence of the nutritional value of mycotrophy. Finally, it is suggested that fungal association may be functional in the release of the *Disa* embryo from testa-imposed dormancy.

5.1 INTRODUCTION

All orchids, at some point in their natural life cycle, are infected by and utilize mycorrhizal fungi as a source of energy (CLEMENTS *ET AL.*, 1986; RASMUSSEN *ET AL.*, 1990; RASMUSSEN, 1995, 2002; ZETTLER & HOFER, 1998; ZETTLER, BURKHEAD & MARSHALL, 1999; BATTY *ET AL.*, 2001a, b). The association between the Basidiomycotina (mycorrhizal fungi) and the Orchidaceae appears ancestral, with all three subfamilies exploiting fungal associations (WARCUP, 1981). The exact nature of the relationship between orchid and fungus, despite almost 150 years of research, remains undefined. Several researchers (BURGEFF, 1943; STOUTAMIRE, 1963; HADLEY, 1970; SMITH 1974) reported the lysis of hyphae within the plant tissue as a defense mechanism against parasitic infection. Others defended the idea of a mutualistic nutritional relationship (WILLIAMSON & HADLEY,

1970; WARCUP, 1973, 1981), with the bi-directional translocation of nutrients. However, the transfer of nutrients towards the fungus has never been unambiguously demonstrated (HADLEY & PURVES, 1974; PURVES & HADLEY, 1975; RASMUSSEN, 1995), although JACKSON & MASON (1984) present evidence that the orchid provides the mycorrhizal partner with an organic nitrogen source and undefined growth factors. Studies on chlorophyll-deficient orchid species revealed that the adult plants survived solely by parasitising fungi (ALEXANDER & HADLEY, 1985; SALMIA, 1988; BEYRLE & SMITH, 1993).

In the absence of autotrophy and the extended period of subterranean existence reported for the protocorms of many species, RASMUSSEN & WHIGHAM (1993, 1998) concluded that the embryo persists through parasitism, which relieves the need for an endogenous source of nutrients. However, it remains highly unlikely that the fungal hyphae will actively penetrate the living tissues of the embryo without benefit. It has been suggested that internal hyphae provide a means by which the endophyte may persist and regenerate when the external environment is conducive to fungal growth (RASMUSSEN, 1995), although DRESSLER (1981) speculated that in the Diseae mycorrhiza were established anew each growing season. In the absence of a concrete definition, the loose term 'symbiosis' remains widespread in the literature (WARCUP, 1981; CLEMENTS, 1982; CLEMENTS *ET AL.*, 1986; SMRECIU & CURRAH, 1989; ZETTLER & MCINNIS, 1992; JOHNSON, 1994; RASMUSSEN, 1995; DIJK, WILLEMS & VAN ANDEL, 1997; ZETTLER *ET AL.*, 1999).

The involvement of fungi in the initiation of orchid seed germination has been reported since the beginning of the 20th century (MACDOUGAL, 1899; SCHOSER, 1981; HARLEY & SMITH, 1983; HADLEY & PEGG, 1989). Contemporary interpretations (FAST, 1982; RASMUSSEN, 1990, 1995; ZETTLER *ET AL.*, 1999) propose that the penetration of the mycorrhiza into the embryo signifies the cue for post-imbibition germination. Experimentation has shown that orchid germination can be maintained entirely through fungal connections and that if

these connections are severed the protocorms die (MACDOUGAL, 1899; RASMUSSEN, 1995) – the indication being that development of the orchid embryo is not autonomous. Early translation of this data concluded that the fungal endophyte acquired essential compounds from the environment and transferred these to the embryo where they were taken up by the plant tissue through the lysis of internal hyphae.

Mycorrhizal associations were reported by STOUTAMIRE (1963, 1974), HARVAIS & HADLEY (1967), WARCUP (1971) and ARDITTI & ERNST (1984) as obligatory due to the insufficient or unavailable nutrient reserves of the orchid embryo. Under such a scenario the need for the fungus is undisputed and its role is essentially nutritive. However, the development of asymbiotic seed culture protocols and the employment of these techniques in germinating many terrestrial orchid species has demonstrated that the presence of the fungus is not essential for germination (HARVAIS & HADLEY, 1967; WARCUP, 1971; PURVES & HADLEY, 1976; ARDITTI *ET AL.*, 1981; ARDITTI, 1982; CLEMENTS, 1982; OLIVA & ARDITTI, 1984; CLEMENTS *ET AL.*, 1986; RASMUSSEN & WHIGHAM, 1993; RASMUSSEN, 1995). Two possible explanations exist; i) the germination requirements of the embryo, including the provision of nutrients, are met by the asymbiotic culture medium and ii) germination of the orchid seed is fuelled autonomously.

ARDITTI (1967) and HARRISON (1977) suggested that the varying degree to which orchid species rely on mycorrhiza under natural conditions is a consequence of the seeds' ability to metabolize nutrient reserves, rather than an inadequate energy supply. Orchid seeds vary considerably in their nutritional status at maturity, both in the form and quantity of the primary storage compounds and in the representation of soluble carbohydrates within the cell protoplasts (ARDITTI & ERNST, 1981, 1984; MANNING & VAN STADEN, 1987; RASMUSSEN, 1990, 1995; RICHARDSON *ET AL.*, 1992). The implication of variable nutrient reserves is that the dependence of orchids on fungi to facilitate germination may be equally variable. *In vitro* asymbiotic germination data confirms this (ERNST *ET AL.*, 1970,

1971; STOUTAMIRE, 1974; PURVES & HADLEY, 1976; ARDITTI, 1982; RASMUSSEN, 1995). In terms of fungal dependency during germination, RASMUSSEN (1995) proposed the following categories.

Orchid seeds which:

i. germinate in water or on water agar in the absence of fungal infection;
ii. germinate on water agar only in the presence of a compatible symbiont;
iii. germinate asymbiotically on substrates with specific ingredients; and
iv. germinate symbiotically only if certain substrate requirements are also met.

These criteria are not mutually exclusive and seeds from a single parent plant may vary in their individual requirements (RASMUSSEN, 1995). Consequently, the effect of increasingly complex germination conditions (i – iv above) is that a greater number of seeds are expected to germinate.

Contemporary interpretations (SMRECIU & CURRAH, 1989; ZETTLER & MCINNIS, 1993; RASMUSSEN, 1995, 2002; ZETTLER *ET AL.*, 1999) define orchid mycorrhiza as a source of energy for the embryo which either supplements, replaces or alternates with embryo nutrition. The mycorrhizal infection of orchid seeds is therefore important for two reasons:

i. the stimulation of gluconeogenesis and reserve mobilization by the provision of usable carbohydrates; and

ii. post-germinative nutritional support until the seedling becomes autotrophic.

There is also evidence of hormonal control in the gluconeogenic process (ARDITTI & ERNST, 1981; MANNING & VAN STADEN, 1987), but whether this control is complete or incomplete is unknown. The possible provision of growth regulating hormones by the fungus to the orchid embryo has been reported by HANKE & DOLLWET (1976), BARROSO, CHAVES NEVES & PAIS (1986) and WILKINSON, DIXON, SIVASITHAMPARAM & GHISALBERTI (1994). Additionally, several authors (HARVAIS & PEKKALA, 1975; ARDITTI, 1982; ARDITTI & ERNST, 1984; BRADY, 1984; RASMUSSEN, 1995) have proposed that vitamins (Table 5.1) and essential inorganic elements are also supplied to the post-imbibition orchid seed by the extensive network of fungal hyphae. Importantly, mycorrhizal fungi cannot oxidize ammonium compounds to usable nitrites or nitrates, nor can they fix elemental nitrogen into combined compound forms (BRADY, 1984; DIJK, 1990; RASMUSSEN, 1995).

Mycorrhizal fungi are also proficient in their ability to decompose organic residues. Cellulose, starch, disaccharide sugars and sugar alcohols and even proteins may be catabolized to constituent monomers by their activity (BRADY, 1984; BEYRLE & SMITH, 1993; RASMUSSEN, 1995). Glucose, trehalose and mannitol are all reported from mycorrhizal fungi (SMITH, 1973). In addition, orchid mycorrhiza digest phenolics such as lignin and suberin (JACKSON & MASON, 1984; VAN WAES & DEBERGH, 1986b; DIJK *ET AL.*, 1997). Consequently, the lysis of fungal hyphal coils (pelotons) within the cells of the developing orchid may liberate metabolic substrates, inorganic minerals, growth regulators (Table 5.1) and enzymes (Table 5.1), all of which are available for utilization by the embryo (SMRECIU & CURRAH, 1989). Complex organic molecules such as enzymes and growth regulators (HARVAIS & PEKKALA, 1975) are assimilated or synthesized by the fungus from the surrounding environment. In addition, WILKINSON *ET AL.* (1994) reported on the induction of endogenous hormone synthesis in the embryo by metabolites of the mycorrhizal fungus. JACKSON & MASON (1984) reported that a small amount of nutrient transfer from live hyphae exists, whilst the remainder undoubtedly occurs necrotrophically as intracellular pelotons are lysed. Fungi have been shown to secrete hydrolytic enzymes (BURGEFF, 1959; HADLEY &

PEROMBELON, 1963; SMITH, 1966, 1967; NIEUWDORP, 1972; BARROSO *ET AL.*, 1986; RASMUSSEN, 1995) and limited amounts of soluble carbohydrates (BURGES, 1936, 1939). However, the effect of these liberated compounds seems negligible, as ARDITTI *ET AL.* (1981) recorded that liquid media filtrate, originating from the culture of a mycorrhizal fungus, did not induce seed germination.

Table 5.1. Enzymes, growth regulators and vitamins detected in orchid mycorrhizal fungi.

Enzymes	Reference
Cellulase	BURGEFF, 1959; SMITH, 1966; NIEUWDORP, 1972; BARROSO *ET AL.*, 1986
Amylase	BURGEFF, 1959
β-Fructofuranosidase (invertase)	BURGEFF, 1959
α-D-Glucosidase (maltase)	BURGEFF, 1959
β-D-Glucosidase (emulsin)	BURGEFF, 1959
Pectinase	NIEUWDORP, 1972
Endopolygalacturonase	HADLEY & PEROMBELON, 1963
Endopolymethylgalacturonase	HADLEY & PEROMBELON, 1963
Polyphenoloxidase	BARROSO *ET AL.*, 1986
Proteolytic enzymes	BURGEFF, 1959
Growth regulators & vitamins	
IAA (Indoleacetic acid)	BARROSO *ET AL.*, 1986
Indole-ethanol	BARROSO *ET AL.*, 1986
Ethylene	HANKE & DOLLWET, 1976
Nicotinic acid	HARVAIS & PEKKALA, 1975
Thiamine	HARVAIS & PEKKALA, 1975

Adapted from RASMUSSEN (1995).

The exact time that infection of the seed must occur if symbiosis is to be established successfully is poorly documented and JACKSON & MASON (1984) report that this varies between species. Several authors (FAST, 1982; ALEXANDER

& HADLEY, 1983; MUIR, 1987; HADLEY & PEGG, 1989) report that mycorrhizal infection takes place through the suspensor of the embryo via the micropyle, or via rhizoids once the protocorm had emerged (RASMUSSEN, 1995). MOLVRAY (2002) confirmed the presence of the suspensor in the Orchideae and the Diseae whilst ARDITTI & GHANI (2000) reported that the suspensor end opening is wide enough to allow entry of fungal hyphae. In certain species infection may be facilitated by gaps between adjoining testa cells (MOLVRAY, 2002). Rhizoids as the site of infection suggests that the initial germination response is independent of a mycorrhizal association.

Controlled infection, which is dependent on the successive lysis of invasive pelotons, requires that host tissue be imbibed (BURGEFF, 1943; RASMUSSEN, 1995). Unimbibed embryos only become infected by means of pathogenic invasion (RASMUSSEN, 1995). However, JACKSON & MASON (1984) report that germination in the genus *Cypripedium* L. is initiated by hyphal penetration of the testa prior to infection of the suspensor cells. Consequently the mobilization of stored protein by the embryo, which is initiated as tissues imbibe (ADAMS *ET AL.*, 1985), is not necessarily dependent on mycorrhizal infection. Additionally, the bulk of protein storage occurs in the chalazal pole (HARRISON, 1977), away from the embryo suspensor. Lipolysis, which is reliant on metabolic function and the presence of soluble carbohydrates (MARRIOT & NORTHCOTE, 1975; ARDITTI & ERNST, 1981; ARDITTI, 1982; BHANDARI & CHITRALEKHA, 1984; MANNING & VAN STADEN, 1987; RASMUSSEN, 1995), may not take place in the absence of invasion by the fungus. In addition, RASMUSSEN *ET AL.* (1991) showed that immature ovules of *Listera obovata* (L.) R.Br. could be inoculated successfully with a mycorrhizal fungus, indicating that the capacity to resist parasitic infection and exploit lysed hyphae develops early during embryogenesis.

The infected embryo subsequently develops into a protocorm consisting of a basal region which houses the fungal pelotons and an apical, non-infected meristematic region (JACKSON & MASON, 1984; RASMUSSEN *ET AL.*, 1990). Three

stages of infection are recognized (BURGEFF, 1943; RASMUSSEN *ET AL.*, 1990; RASMUSSEN, 1995); primary infection with the formation of a hyphal coil, collapse and lysis of the peloton, and repeated infection. Healthy protocorms are characterized by uninfected apical meristems and protocorm steles (ARDITTI *ET AL.*, 1981; SMRECIU & CURRAH, 1989; RASMUSSEN, 1995; ZETTLER *ET AL.*, 1999).

Orchid endophytes isolated from naturally occurring protocorms are likely to have a role in germination (WARCUP, 1981; CLEMENTS *ET AL.*, 1986; DIXON, 1987; RASMUSSEN & WHIGHAM, 1993). Unfortunately, it is almost impossible to trace orchid dust seeds or recently emerged protocorms in the soil. RASMUSSEN & WHIGHAM (1993) provide a preliminary report on the development of a technique for sowing and retrieving seeds *in situ*, enabling the isolation of fungi that, with certainty, are involved with germination. Pelotons have also been isolated from roots, and occasionally from rhizomes or tubers of adult plants (HARVAIS & HADLEY, 1967; WARCUP & TALBOT, 1967; RASMUSSEN, 1995). Recent literature (ZETTLER *ET AL.*, 1999) has revealed that during the different developmental stages, an orchid host may be reliant on a sequence of different fungi. HADLEY (1970) and WARCUP (1975) found no direct correlation between the fungi living in the roots of older plants and those active in germination. Consequently, the fungi present in mature plants may not have the capacity to facilitate germination in the seeds of the same species. In addition, mature plants often house a variety of non-symbiotic fungi and pathogens (RASMUSSEN, 1995), compounding the problems associated with isolating a virulent endophyte. In rare cases, adult orchid plants may be mycorrhizae free, despite having grown through an earlier saprophytic phase (JACKSON & MASON, 1984).

The reliance of orchid seeds on fungal infection to facilitate germination and the capacity to isolate mycorrhizal pelotons from living tissue has been exploited *in vitro*, where seedlings are raised together with mycorrhizal fungi in symbiotic seed cultures. This has been particularly useful for many previously ungerminated Australian and Holarctic terrestrial orchid species (BURGEFF, 1959;

STOUTAMIRE, 1963, 1974; MCINTYRE, VEITCH & WRIGLEY, 1974; CLEMENTS, 1982; CLEMENTS *ET AL.*, 1986; DIXON, 1987; MUIR, 1987, 1989; SMRECIU & CURRAH, 1989; RASMUSSEN *ET AL*, 1990, 1991; ANDERSON, 1991; DIXON & SIVASITHAMPARAM, 1991; ZETTLER & MCINNIS, 1992, 1993, 1994; WILKINSON *ET AL.*, 1994; PERKINS & MCGEE, 1995; RASMUSSEN, 1995, 2002; DIJK *ET AL.*, 1997; ZETTLER *ET AL.*, 1999; BATTY *ET AL.*, 2001a, b). Symbiotic germination often results in higher percentage germination and a faster germination rate than in the absence of the endophyte (STOUTAMIRE, 1963, 1974; HADLEY & WILLIAMSON, 1971; WARCUP 1973; CLEMENTS *ET AL.*, 1986; WILKINSON *ET AL.*, 1994; RASMUSSEN, 1995). Despite significant advances, comparatively few species have been propagated using symbiotic techniques due to the unpredictable nature of the orchid-fungus association (ZETTLER & MCINNIS, 1992), a problem which may be compounded by the unquantified conditions of the sealed culture vessel (SMRECIU & CURRAH, 1989).

BURGEFF (1959) outlined seven orchid mycorrhizal relationships (i – vii below) that describe the nature of the association between seed and fungus *in vitro*. This continuum is based on the compatibility between seed and endophyte, where one participant may be weak and the other extremely virulent (SMRECIU & CURRAH, 1989; ZETTLER & MCINNIS, 1992, 1993; RASMUSSEN, 1995). With little or no modification these interactions have been employed by most researchers (DOWNIE, 1959; HADLEY, 1970; CLEMENTS, 1982; SMRECIU & CURRAH, 1989; RASMUSSEN, 1995; ZETTLER *ET AL.*, 1999).

i. No infection.
ii. Hyphae enter the suspensor and a few pelotons form in the basal part of the embryo. No orchid growth occurs.
iii. Seeds are infected and pelotons form. Seedling development is slow, but proceeds normally. Infection is restricted and many seeds remain ungerminated.

iv. Normal infection. The majority of seeds show signs of infection with pelotons concentrated in the basal part of the embryo. Seedling development occurs rapidly.

v. Infection is too heavy. Seedlings develop slowly and initiate the breakdown of pelotons. No new non-infected rhizoids appear.

vi. Over infection. Embryos are extensively infected and remain peloton free only at the chalazal end. No germination occurs and seeds eventually die.

vii. Instant over infection. Embryonic tissue is rapidly filled with hyphae and host tissues are broken down. No germination occurs.

Reports by HARVAIS & HADLEY (1967) and HADLEY (1970) referred to the association between orchid and fungus as opportunistic. Studies on select Australian terrestrial orchids by WARCUP (1971, 1973) suggested a closer relationship between fungus and orchid, with the linking of an endophytic fungus to a particular group of orchids. RASMUSSEN (1995, 2002) suggested that, in certain instances, the specificity of individual mycorrhizal fungi to a particular orchid species might exist. A wide diversity of fungi have been found associated with orchids, although the exact number is unknown. The majority have been isolated from the roots of wild growing adult plants and may therefore not have the capacity to initiate germination (MASUHARA & KATSUYA, 1994; ZETTLER *ET AL.*, 1999).

Most fungi isolated from orchids belong to the form-genus *Rhizoctonia* DC, which contains a variety of anamorphic (sterile) strains viz. *Ascorhizoctonia* Yang & Korf, *Epulorhiza* Moore, *Ceratorhiza* Moore and *Moniliopsis* Ruhland (ALEXOPOULOS & MIMS, 1979; MOORE, 1987; ZETTLER & MCINNIS, 1992, 1993; ANDERSEN & STALPERS, 1994; RASMUSSEN, 1995; DIJK *ET AL.*, 1997). It is difficult to correlate anamorphic mycelia with the reproductive telomorphs on which

traditional mycological classification is based. However, the atypical induction of reproductive features *in vitro* allows for anamorphic taxa to be referred to telomorphic genera (ANDERSEN, 1990). These include *Ceratobasidium* D.P. Rogers, *Thanatephorus* Donk, *Tulasnella* J. Schröt and *Sebacina* Tul. & C. Tul. (WARCUP & TALBOT, 1966, 1971; WARCUP, 1971, 1973; Moore, 1987; MILLIGAN & WILLIAMS, 1987, 1988; ANDERSEN, 1990; CURRAH & ZELMER, 1992; ANDERSEN & STALPERS, 1994; HAWKSWORTH, KIRK, SUTTON & PEGLER, 1995). The literature is confusing on this issue due to the inconsistent treatment and lack of precise classification of isolates by different authors (SMRECIU & CURRAH, 1989; HAWKSWORTH *ET AL.*, 1995). This is compounded by the use of variable and age-dependent mycelia characters as a means of taxonomic division (HARVAIS & HADLEY, 1967; ALEXOPOULOS & MIMS, 1979; ANDERSEN, 1990).

In vitro, isolates of *Rhizoctonia* from mature orchids have been shown to be compatible with a range of orchid seeds (DOWNIE, 1943, 1959; WARCUP & TALBOT, 1967; CAMPBELL, 1970; ARDITTI *ET AL.*, 1981; WARCUP, 1981, 1991; CLEMENTS, 1982; HADLEY, 1982; CLEMENTS *ET AL.*, 1986; DIXON, 1987; DIXON & SIVASITHAMPARAM, 1991; CURRAH & ZELMER, 1992; ZETTLER & MCINNIS, 1992, 1993; HAWKSWORTH *ET AL.*, 1995; RASMUSSEN, 1995; ZETTLER ET AL., 1999). *Tulasnella calospora* (Boudier) Juel and *Ceratobasidium cornigerum* (Bourdot) Rogers have been isolated from a range of European, Australian and North American terrestrial orchids (WARCUP & TALBOLT, 1967; WARCUP, 1981; MUIR, 1987; ANDERSEN, 1990), indicating wide distribution. Strains belonging to these species have proved to be compatible with numerous orchids *in vitro*. The former species is the most common root and seedling endophyte encountered and has been proposed as a universal orchid symbiont (DOWNIE, 1959; CLEMENTS & ELLYARD, 1979; MUIR, 1987, 1989; RASMUSSEN, 1995).

Numerous studies have dealt with the ability of fungal isolates from mature orchids to promote germination in a wide variety of Australian and Holarctic species (HADLEY, 1970; WARCUP, 1973; ARDITTI *ET AL.*, 1981; CLEMENTS, 1982,

1988; OLIVA & ARDITTI, 1984; CLEMENTS *ET AL.*, 1986; SMRECIU & CURRAH, 1989; RASMUSSEN *ET AL.*, 1990; ZETTLER & MCINNIS, 1992, 1993; WILKINSON *ET AL.*, 1994; ZETTLER *ET AL.*, 1999; BATTY *ET AL.*, 2001a, b; RASMUSSEN, 2002). There are no comparable studies of symbiotic germination in terrestrial orchid taxa in South Africa and no data exists to define the nature of the association in this context. This research was initiated as a means of elucidating the nature of mycotrophy and developing symbiotic seed culture protocols in the previously neglected *Disa*.

5.2 AIMS

- To isolate and evaluate the efficacy of endophytes within the small-seeded *Disa* species.
- To establish and optimize the *in vitro* culture of *Disa* mycorrhizae.
- To initiate symbiotic seed cultures and to quantify host-endophyte specificity for *Disa*.
- To define the nutritional contribution of *Disa* endophytes to germination.

5.3 MATERIALS AND METHODS

Extensive seasonal lysis of fungal tissue is reported to make the isolation of living pelotons from plant tissue difficult at certain times of the year (RASMUSSEN, 1995). To optimize fungal isolation, wild *Disa* plants (Table 5.2) were collected prior to flowering (spring of 1999 – 2001). Host plants originated from the same locality every year. Plants of all species originated from the summer-rainfall areas of South Africa, including *D. cornuta* that experiences winter, summer and yearlong rainfall across its distribution. The remaining species are summer-rainfall endemics. Germinability indices, as outlined in Chapter Three (Figure 3.2; page 77), were included for comparative purposes.

Host plants were transported dry (WARCUP, 1981) and subterranean plant tissues were processed within four days of collection. Roots and tubers were washed extensively with sterile water to remove all soil debris. Following a final rinse the tissue was submersed in sterile, distilled water and moved to a laminar flow bench. Isolation and extraction of pelotons was achieved by thinly dissecting short sections of tissue longitudinally under a Wild Heerbrugg M3 dissecting light microscope at 40 x magnification. Pelotons were teased from the infected orchid tissue using a sterilized scalpel blade tip and dissecting needle (RASMUSSEN, 1995) and placed onto isolation media.

Table 5.2. *Disa* species for which the isolation of mycorrhizal fungi was attempted during 1999, 2000 and 2001. All species produce seeds of the small-type and none are especially germinable *in vitro*. Germinability indices (GI) on modified media* are included in parentheses.

D. chrysostachya (3)	*D. pulchra* (2)
D. cooperi (2)	*D. sagittalis* (2)
D. cornuta (2)	*D. stachyoides* (3)
D. crassicornis (3)	*D. versicolor* (3)
D. fragrans (3)	*D. woodii* (2)
D. nervosa (2)	

In vitro germinability indices; 2 – germinated with < 30 % success in a minimum of 12 weeks; 3 – intractable *in vitro* on modified media. * modified with decreased media viscosity, decreased inorganic salt concentration or charcoal supplementation.

5.3.1 ENDOPHYTE CULTURE *IN VITRO*

Two media have been recommended for the *in vitro* culture and maintenance of orchid endophytes viz. CLEMENT'S (CLEMENTS, 1982) and CM_1 (CLEMENTS *ET AL.*, 1986; RASMUSSEN *ET AL.*, 1990) isolation media. The suitability of these media, developed for Australian and European mycorrhizae respectively, was investigated with regard to South African *Disa* species (Table 5.2).

Media were solidified in 65 mm plastic petri dishes and inoculated with multiple pelotons isolated from single plants. Culture vessels were sealed with Parafilm. A minimum of five replicate cultures were initiated from at least three representatives of each host species. Pathogen free cultures were established by regular, selective sub-culturing (twice weekly) and by allowing the fungal hyphae to grow through the site of inoculation (RASMUSSEN, 1995). Antibiotics were not used (RASMUSSEN *ET AL.*, 1991).

Endophytes were cultured at ± 25 °C in darkness and establishment took approximately 72 h. Once established, endophyte cultures were stored at < 5 °C. Sub-culturing was carried out monthly (ZETTLER & MCINNIS, 1992) to maintain the samples and to ensure that adequate fungal stock was available for symbiotic culture. Sub-culturing took place onto similar growth medium.

In certain instances the capacity of fungi to establish orchid mycorrhiza is lost or is significantly reduced *in vitro* since the growth conditions favor those fungal genotypes that are better adapted as free living saprophytes (MARX & DANIEL, 1976; ALEXANDER & HADLEY 1983). However, the time taken for complete mycorrhizal activity to be lost is variable and reports range between six months to over four years (DOWNIE, 1959; ALEXANDER & HADLEY, 1983). Stock cultures, unless shown to be effective mycorrhizal partners for *Disa*, were therefore not maintained beyond the end of the flowering season (six months from initiation) and were re-initiated the subsequent spring when peloton-containing tissue became available.

i) CLEMENTS' isolation medium for Australian orchid fungi (CLEMENTS, 1982)

$NaNO_3$	0.3 g L^{-1}
KH_2PO_4	0.2 g L^{-1}
$MgSO_4 7H_2O$	0.1 g L^{-1}
KCl	0.1 g L^{-1}
Yeast extract (Difco)	0.1 g L^{-1}
Sucrose	5.0 g L^{-1}
Streptomycin sulphate	0.05 g L^{-1}
Agar	10 g L^{-1}
	*p*H adjusted to 5.0

ii) CM_1 medium for European orchid fungi (RASMUSSEN *ET AL.*, 1990 after CLEMENTS *ET AL.*, 1986)

$Ca(NO_3)_2.4H_2O$	0.2 g L^{-1}
KH_2PO_4	0.2 g L^{-1}
$MgSO_4 7H_2O$	0.1 g L^{-1}
KCl	0.1 g L^{-1}
Yeast extract (Difco)[a]	0.1 g L^{-1}
Sucrose	4.0 g L^{-1}
Novobiocin[b]	0.05 g L^{-1}
Agar	12 g L^{-1}
	*p*H adjusted to 5.8

[a] Yeast extract contains both nitrate and organic nitrogen

[b] Remains antibiotic after autoclaving

Fungi were assumed to be mycorrhizal if they produced analogous hyphal tip coils *in vitro*, as is common for rhizoctonias (CLEMENTS, 1982; MOORE, 1987; ANDERSEN, 1990; RASMUSSEN, 1995, RASMUSSEN & WHIGHAM, 1998). It was anticipated that only those fungi that displayed mycorrhizal activity and supported

germination and seedling development in symbiotic trials would be classified. Alternatively, fungi were simply recognized and grouped according to mycelia branching arrangement and color and the rate and pattern of colony establishment in pure culture. In the absence of fructifications, isolates were grouped tentatively into either the Ceratobasidiaceae or the Tulasnellaceae, as is common for Holarctic and Australian isolates (WARCUP & TALBOLT, 1967; WARCUP, 1981; MILLIGAN & WILLIAMS, 1987; MUIR, 1987; ANDERSEN, 1990, ZETTLER & MCINNIS, 1992, 1993). The former are typified by wider hyphae and faster growing, coarse colonies that vary in color from cream to dark brown. Members of the latter group have finer hyphae, are slower growing and produce white or pink colonies (WARCUP, 1981). Unless isolates appeared morphologically distinct, only stock cultures of one isolate from each orchid host species were maintained.

Fungi were compared within and between host *Disa* species by plotting fungal association onto the morphology cladograms of LINDER & KURZWEIL (1999; Appendix One). Cladograms were not available for *D. nervosa*, *D. stachyoides* (section *Emarginatae*, LINDER & KURZWEIL, 1999) and *D. pulchra* (section *Stenocarpa*, LINDER & KURZWEIL, 1999). In the absence of these cladograms, the parsimonious evolution of fungal association remains relatively uninformative and has not been illustrated. The molecular phylogeny of BELLSTEDT *ET AL.* (2001; Appendix Two) was not used because it reveals the relationships of comparatively fewer of the small-seeded *Disa* species examined here and is reliant on the sectional classification of LINDER & KURZWEIL (1999).

5.3.2 SYMBIOTIC SEED CULTURE *IN VITRO*

Seed was collected from wild plants of the summer-rainfall grasslands of South Africa (Table 5.2). To initiate symbiotic cultures, mature decontaminated seed (Chapter Three; page 69) was evenly distributed over Whatman No. 1 filter paper discs and placed onto a symbiotic germination medium. Four media have proved successful in the symbiotic germination of a

wide range of Australian and Holarctic terrestrial orchids (CLEMENTS *ET AL.*, 1986; RASMUSSEN *ET AL.*, 1990, 1991; RASMUSSEN, 1995). These media (outlined below) were investigated in order to establish their value in the context of mycotrophic *Disa* seed culture. Media were solidified in 65 mm plastic petri dishes.

i) ZAK medium (BORRIS, 1969)

Sucrose	10 g L^{-1}
Coconut milk	20 ml L^{-1}
Agar	15 g L^{-1}
	*p*H adjusted to 5.8

ii) Oats medium (CLEMENTS *ET AL.*, 1986)

Whole oats	25 g L^{-1} H_2O
Agar	12 g L^{-1} H_2O
	*p*H adjusted to 5.5

iii) WAR MI medium (WARCUP, 1973)

$NaNO_3$	0.3 g L^{-1}
KH_2PO_4	0.2 g L^{-1}
$MgSO_4.7H_2O$	0.1 g L^{-1}
KCl	0.1 g L^{-1}
Cellulose powder	10 g L^{-1}
Yeast extract (Difco)	0.1 g L^{-1}
Agar	12 g L^{-1}
	*p*H adjusted to 5.8

iv) H_1 Oats medium (RASMUSSEN *ET AL.*, 1990 after CLEMENTS *ET AL.*, 1986)

$Ca(NO_3)_2.4H_2O$	0.2 g L^{-1}
KCl	0.1 g L^{-1}
KH_2PO_4	0.2 g L^{-1}
$MgSO_4.7H_2O$	0.1 g L^{-1}
Yeast extract (Difco)	0.1 g L^{-1}
Sucrose	2.0 g L^{-1}
Finely ground oats	3.0 g L^{-1}
Agar	12 g L^{-1}
	*p*H adjusted to 5.8

A small inoculum comprising ± 3 mm^3 of solid isolation media, containing hyphae of the appropriate fungal stock culture, was placed on the filter paper edge and the culture vessel sealed with Parafilm. The seeds of all species (Table 5.2) were sown in intraspecific association – that is, with the fungal strain isolated from host plants of the same species. However in *D. chrysostachya*, *D. cooperi*, *D. nervosa* and *D. versicolor*, where seeds were plentiful, these were also sown with fungal strains isolated from each of the other three *Disa* species in interspecific association according to an incomplete matrix (Table 5.3). Specific and sectional comparisons were made. Symbiotic cultures were initiated with fungi isolated the previous spring.

Incubation was carried out at ± 25 °C in the dark for the first eight weeks, after which cultures were maintained under a low-light intensity of 8.5 μmol m^{-2} s^{-1} (RASMUSSEN, 1995; ZETTLER & HOFER, 1998). Two control plates, comprising symbiotic germination media and decontaminated seeds in the absence of the fungal stock inoculum and three experimental plates were prepared in all cases. Germination, protocorm and seedling development were recorded fortnightly up to a maximum of 24 months (ZETTLER & HOFER, 1998).

Table 5.3. Interspecific association matrix established for select South African *Disa* species. Fungi were isolated in spring 2000, cultures initiated in 2001. All seed was sown in intraspecific association, which are not included. Sectional classification (LINDER & KURZWEIL, 1999) is indicated in parenthesis.

Seed parent	Host orchid			
	D. chrysostachya	*D. cooperi*	*D. nervosa*	*D. versicolor*
D. chrysostachya (*Micranthae*)		✓	X	✓
D. cooperi (*Hircicornes*)	✓		✓	X
D. nervosa (*Emarginatae*)	✓	X		✓
D. versicolor (*Hircicornes*)	✓	✓	✓	

✓ indicates associations that were initiated *in vitro*; X denotes associations that were not initiated due to limited seed availability.

Percentages represent mean counts from a minimum of five plates, with approximately 150 – 250 seeds per culture. The developmental responses were graded according to modifications of the broad categories (Chapter Three; page 67) employed by WARCUP (1973), OLIVA & ARDITTI (1984), SMRECIU & CURRAH (1989) and LEROUX *ET AL.* (1997).

Cultures which proceeded to stage iv (differentiation of the apical meristem) were transferred to, and maintained under a light intensity of 35 μmol m^{-2} s^{-1} for the remainder of the observation period.

5.3.3 ENDOPHYTE ACTIVITY AND NUTRITION

Liquid cultures (500 ml) of modified H_1 Oats medium, excluding yeast and oats and supplemented with 4 g L^{-1} sucrose, were inoculated with endophyte hyphae and maintained at ± 25 °C in darkness. Liquid cultures of full

and half-strength MS salts (MURASHIGE & SKOOG, 1962), supplemented with 4 g L^{-1} sucrose, were established for comparative purposes. An endophyte (isolate *cra*2; Table 5.5 and Table 5.6) was shown to elicit stage v^{+} germination when sown symbiotically with seed of *D. crassicornis*. This endophyte was used to initiate liquid cultures in order to assess the ability of an endophyte to hydrolyze complex carbohydrates and synthesize novel compounds over and above their ability to simply absorb and translocate free nutrients. Cultures were continuously agitated by means of an orbital shaker operating at 50 cycles min^{-1} (CHU & MUDGE, 1994).

Four weeks after culture initiation the resultant fungal mass was removed by filtration (ARDITTI *ET AL.*, 1981) and sonicated in 500 ml sterile dH_2O to ensure hyphal rupture. In order to assess the effect of endophyte activity on carbohydrate form and its ability to modify the surrounding environment the following samples were analyzed by means of Thin Layer (TLC; Figure 5.1) and Gas Chromatography (GC; Figure 5.2).

- Sample 1: Liquid media prior to inoculation with fungal stock
- Sample 2: Liquid media after a four week period of fungal growth
- Sample 3: Cytoplasm composition.

Samples 1, 2 and 3 (250 ml) were dried under nitrogen to minimize the effects of carbohydrate breakdown and micro-organism mediated hydrolysis. The residue was re-suspended to a final concentration of 5 mg ml^{-1}. Fructose, glucose and sucrose reference samples of 5 mg ml^{-1} were prepared for comparison. Ten μl of each sample and the standards were applied to a TLC plate (0.25 mm, 20 x 5 cm with a stationary phase of silica gel on pre-coated plastic sheets) as discrete 5 mm bands. Compounds were separated using the solvent system ethyl acetate : acetic acid : MeOH : H_2O in the ratio 12:3:3:2 (JÄGER, 2001, PERS. COMM.). Glass chromatography chambers were pre-washed with the mobile phase and allowed

to equilibrate for approximately 2 min. The plates were developed over 15 cm (approximately 1.5 h). Plates were removed from the tank and the solvent front marked, before being air-dried. Any bands visible under visible (VIS) and UV-light (254 nm and 366 nm) were marked.

The chromatograms were stained with an anisaldehyde / sulphuric acid reagent (WAGNER & BLADT, 1995), re-evaluated and new bands marked under VIS and UV light. The reagent (10 ml) was poured over the TLC plate and heated for 5 – 10 min at 100 °C. Color development under VIS reveals carbohydrates as black. The reagent aniline-diphenylamine was used to distinguish different carbohydrates through sugar-specific color development under VIS, in combination with their relative flow rates (DAFNI, 1992; Table 5.4). The reagent was sprayed over the TLC plate and incubated at 85 °C for 10 min.

Table 5.4. Comparative color and R_f data for the Thin Layer Chromatography of carbohydrates following treatment with aniline-diphenylamine.

Carbohydrate	Color (under VIS)	Flow rate (R_f = compound front / solvent front)
fructose	pink / brown	~ 0.7
glucose	dark blue	~ 0.7
sucrose	black	~ 0.5 – 0.6

Adapted from DAFNI (1992).

For GC analysis, 5 x 50 ml of samples 1 – 3 were dried under nitrogen. Thereafter they were treated as outlined in Chapter Four (page 91). Results represent the mean of five aliquots for each sample. Soluble sugars were identified according to co-chromatography with standard sugars run under identical GC conditions. Retention times are presented in Table 4.3 (page 103).

The effect of the endophyte on seed testa integrity was investigated by observing the process of seed infection in sacrificed symbiotic cultures. Infected

Disa seed was not air-dried, but subjected to an ethanol dehydration series prior to critical point desiccation. Subsequent preparation for scanning electron microscopy as outlined in Chapter Two (page 29).

5.4 RESULTS AND DISCUSSION

Tubers from all *Disa* species (Table 5.2) were investigated but contained no fungal pelotons. This corroborates the findings of JACKSON & MASON (1984), MUIR (1989) and RASMUSSEN (1995). JACKSON & MASON (1984) reported on the presence of orchinol in the orchid tuber, a di-hydroxyphenanthrine phytoalexin common in analogous form in many plants, which is produced in response to potential pathogens. Cortical root tissue of all 11 *Disa* species investigated (Table 5.2) contained fungal coils. Infected cortical cells were generally located towards the root epidermis and were recognized by an opaque appearance or the identification of a discrete hyphal coil (RASMUSSEN, 1995). As expected, the isolation of pelotons from flowering hosts proved difficult.

5.4.1 ENDOPHYTE CULTURE *IN VITRO*: IMPLICATIONS FOR HOST SPECIFICITY

CM_1 medium (CLEMENTS *ET AL.*, 1986; RASMUSSEN *ET AL.*, 1990), modified to exclude Novobiocin, supported vigorous endophyte growth, facilitating the establishment of a maximum of 18 isolates *in vitro* over the three year period (Table 5.5). On average, a peloton-forming fungus was obtained from every fourth isolation. Isolates were assumed to be mycorrhizal, probably members of the *Rhizoctonia* form-genus (CLEMENTS, 1982; MOORE, 1987; RASMUSSEN, 1995), based on the formation of peloton-like structures in pure culture (Plate 5.1A). The mycelia were submerged and non-sporulating, as is common for *Rhizoctonia* or *Rhizoctonia* – like fungi (ZETTLER & MCINNIS, 1992, 1993; HAWKSWORTH *ET AL.*, 1995). CLEMENT'S isolation medium (CLEMENTS, 1982) did not prove suitable for

South African mycorrhizal fungi, at best supporting slow and inconsistent colony establishment. CM_1 medium (CLEMENTS *ET AL.*, 1986; RASMUSSEN *ET AL.*, 1990) was therefore utilized for the maintenance of all fungal isolates. Pathogen free isolates were typically achieved after the fourth or fifth subculture.

Table 5.5. *Disa* species from which fungal pelotons were isolated and maintained as pure strains *in vitro*. Each isolate was ascribed an epithet referring to the host species. Sequential numbering denotes distinct fungal morphs isolated from the same host species.

Host *Disa* species	Fungal isolate identification epithet		
	Year		
	1999	2000	2001
chrysostachya	*chr1; chr2*	*chr2*	-
cooperi	*coo1*	*coo1*	*coo1*
cornuta	*cor1; cor2*	-	-
crassicornis	*cra1*	-	*cra2*
fragrans	*fra1*	-	-
nervosa	*ner1*	*ner1*	*ner1*
pulchra	*pul1; pul2*	*pul1*	*pul2*
sagittalis	*sag1*	-	-
stachyoides	*sta1*	*sta1*	*sta2*
versicolor	*ver1; ver2*	*ver2*	*ver1*
woodii	*woo1*	*woo1*	*woo1; woo2*

In the absence of the precise classification of the anamorphic states of the isolates, fungi were grouped according to morphological similarity. Consequently morphologically indistinguishable fungi isolated from the same host species in different years were ascribed the same epithet (Table 5.5). This is probable, but was not confirmed. ZETTLER & HOFER (1998) reported endophyte congruency within populations of *Platanthera* Rich. spp. over five consecutive seasons. The sequential numbering of isolates from specific *Disa* hosts denoted morphological

separation (Table 5.5).

The morphological grouping of fungi isolates correlated to Australian rhizoctonia (WARCUP, 1981). It is probable that the comparative morphology of 11 of the South African isolates confirms their position within either the Ceratobasidiaceae or the Tulasnellaceae (Table 5.6), specifically because of the suspected cosmopolitan distribution (WARCUP & TALBOT, 1967; WARCUP, 1981; MUIR, 1987; ANDERSEN, 1990) and regular isolation of members of these families (DOWNIE, 1959; WARCUP & TALBOT, 1966, 1971; CLEMENTS & ELLYARD, 1979; HAWKSWORTH *ET AL.*, 1995; RASMUSSEN, 1995). However, not all isolates from the *Disa* hosts were decisively placed into these categories. In order to accommodate the remaining South African isolates two additional categories were proposed (Table 5.6).

Fungi belonging to the same morph, but isolated from different host *Disa* species, were not necessarily distinct (Table 5.5 and Table 5.6). Subsequent comparisons in this study relate only to fungal morphs. Fungi belonging to morph 1 are subsequently referred to the Ceratobasidiaceae, with morph 2 isolates being members of the Tulasnellaceae. It is acknowledged that the lack of precise classification of the anamorphic states of the fungi isolated was a major impediment in the interpretation of these data.

The largest number of *Disa* isolates (seven) was observed to belong to the Tulasnellaceae. These fungi (Table 5.6), isolated from multiple (seven) host *Disa* species, could not be distinguished visually. The prevalence of Tulasnellaceae as potential symbionts supports research by DOWNIE (1959), CLEMENTS & ELLYARD (1979), MUIR (1989), ZETTLER & MCINNIS (1992, 1993) and RASMUSSEN (1995), who reported that members of the Tulasnellaceae, specifically *T. calospora*, are the most common Australian and European endophytes. Similarly, the occurrence of members of the Ceratobasidiaceae (Table 5.6) was expected due to the regular isolation of *C. cornigerum* and related species (WARCUP & TALBOT, 1967;

ANDERSEN, 1990). Ceratobasidiaceae were isolated from four host species.

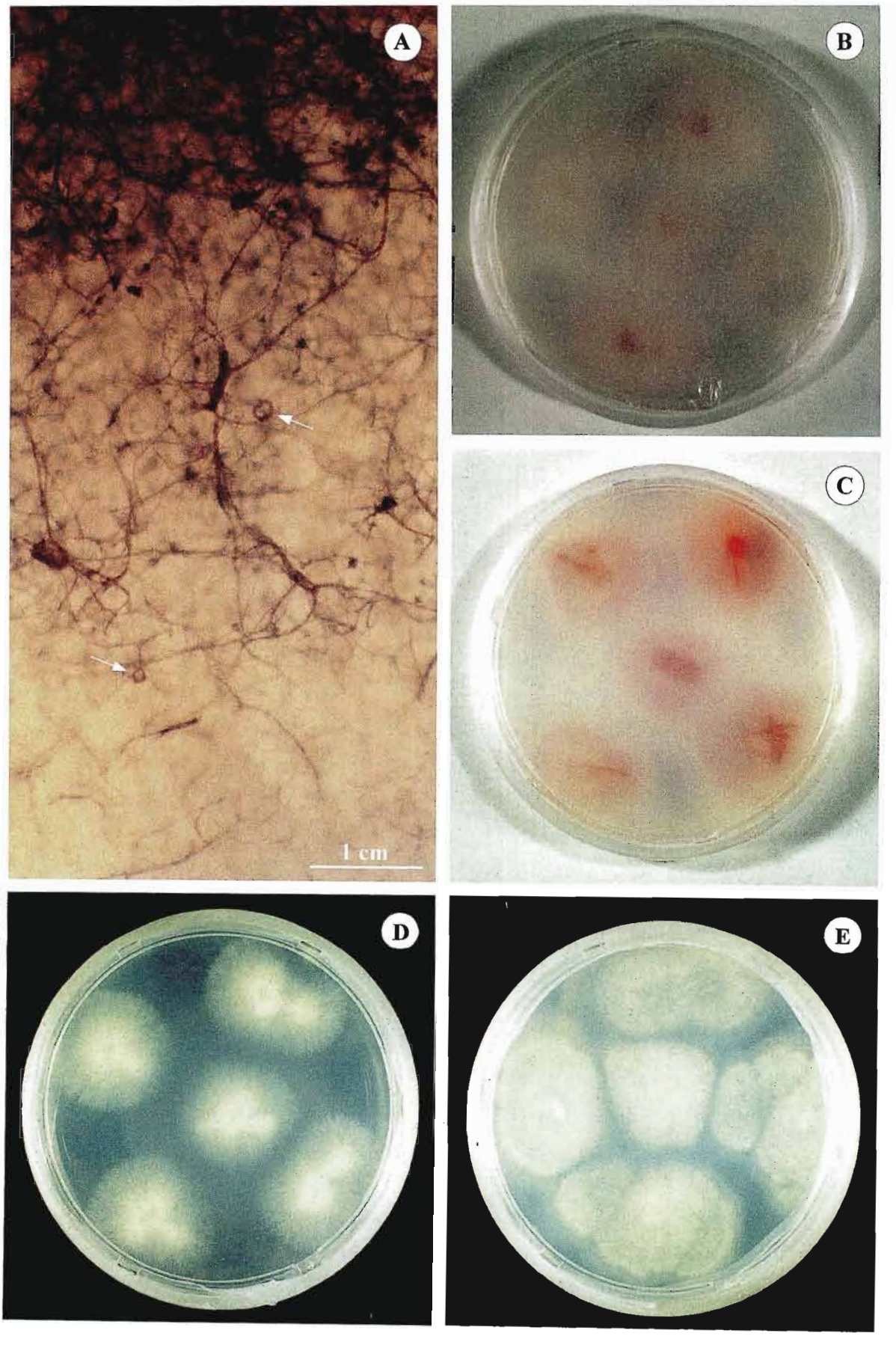
Table 5.6. *Disa* endophytes defined by hyphal and colony morphology *in vitro*. Morphs 1 and 2 after WARCUP (1981).

Morph	Isolate and colony morphology	Isolate epithet
Morph 1 (Ceratobasidiaceae)	Fast-growing, coarse colonies with wide hyphae. Never white – cream or brown in color (Plate 5.1B)	*chr*1; *cor*2; *ner*1; *pul*2
Morph 2 (Tulasnellaceae)	Slow-growing, fine hyphae that produce white or pink colonies (Plate 5.1C)	*coo*1; *cra*2; *pul*1; *sag*1; *sta*2; *ver*1; *woo*1
Morph3 (tulasnella – like)	Fast-growing, fine colonies. Always white in color (Plate 5.1D)	*cor*1; *cra*1; *fra*1; *ver*2; *woo*2
Morph 4 (ceratobasidium – like)	Slow-growing, coarse colonies. White or cream in color (Plate 5.1E)	*chr*2; *sta*1

The individual status of morphs 3 and 4 are retained on the basis of colony growth rate, since shared associations and colony morphology (fine or coarse hyphae and color; Table 5.6) are not sufficient to exclude these fungi from the true Tulasnellaceae and Ceratobasidiaceae. Morph categories 3 and 4 contained five and two *Disa* isolates respectively (Table 5.6).

Up to two fungal endophytes were isolated from individual *Disa* species. CLEMENTS *ET AL.* (1986) reported that, from 36 Australian terrestrials in 11 genera, the modal number of isolates per species was also two, with a maximum of nine from *Orchis militaris* Poir. Where multiple morphs were isolated within a single *year (D. chrysostachya*, *D. cornuta*, *D. pulchra*, *D. versicolor* and *D. woodii*; Table 5.6), this was almost exclusively as a consequence of different host plants of the same species housing separate fungal morphs.

Plate 5.1. *Disa* endophyte culture *in vitro*. Isolates were assumed to be mycorrhizal, possibly members of the *Rhizoctonia* form-genus, based on the formation of peloton-like coils (indicated) in pure culture (A). Mycelia were submerged and non-sporulating and were maintained on CM_1 medium solidified in 65 mm (⌀) plastic petri dishes. Four fungal morphs were defined according to hyphal and colony morphology and colony growth rate. Morph 1 (isolate *ner*1, a Ceratobasidiaceae from *D. nervosa*; B); Morph 2 (isolate *coo*1, a Tulasnellaceae from *D. cooperi*; C); Morph 3 (isolate *woo*1, a tulasnella-like fungi from *D. woodii*; D) and Morph 4 (isolate *chr*2, a ceratobasidium-like fungi from *D. chrysostachya*; E). The largest number of *Disa* endophytes were Tulasnellaceae.


A
1 cm
B
C
D
E

Very rarely were multiple fungal morphs isolated from a single plant (*D. woodii*; Table 5.6). WARCUP (1981) showed that, in cases of multiple fungal isolates from Australian orchids, all were the same or closely related genera. Additionally, WARCUP (1981) noted that those orchids associated with Tulasnellaceae were generally free from other orchid endophytes.

Adult *D. cooperi*, *D. crassicornis*, *D. fragrans*, *D. sagittalis*, *D. versicolor* and *D. woodii* hosts consistently revealed associations with the Tulasnellaceae (including tulasnella – like fungi). Alternatively, *D. chrysostachya* and *D. nervosa* were associated exclusively with the Ceratobasidiaceae (including ceratobasidium – like fungi). Such data confirms the separation of *Disa* endophytes into two broad categories, based on host specificity. However, *D. cornuta* and *D. pulchra* yielded inconsistent endophyte associations within a single year. *Disa stachyoides* was inconsistently infected with Ceratobasidiaceae and Tulasnellaceae between years.

Disa chrysostachya and *D. versicolor*, which were collected from the same geographic vicinity, consistently revealed associations with Ceratobasidiaceae and Tulasnellaceae respectively. Infection was consistent in these hosts, even though alternative *Rhizoctonia* species were available in the immediate environment. This contradicts patterns of several European orchids that establish mycorrhizae on available fungi (RASMUSSEN & WHIGHAM, 1998; ZETTLER & HOFER, 1998). *Disa pulchra*, from the same locality, was infected by both types of fungi.

The genus *Disa* was therefore associated with Ceratobasidiaceae and Tulasnellaceae. Individual host species however, employed either – or both – groups of fungi as their adult mycorrhizal partner. No relationships were identified between endophyte and the taxonomic affiliation of the host *Disa*. Associations with the various rhizoctonias were distributed randomly in *Disa* sections *Coryphaea*, *Hircicornes*, *Micranthae* and *Repandra* (Appendix One). The sparse sectional representation meant that such interpretation is tentative. This was

compounded by the lack of morphological cladograms for sections *Emarginatae* and *Stenocarpa*. No correlation was found between the type of endophyte and the seed germinability index (GI; Table 5.2) achieved *in vitro* on modified media.

The restriction of the *Disa* endophytes to limited groups of fungi is plausible as RASMUSSEN (1995) and RASMUSSEN & WHIGHAM (1998) reported that the association of orchids with fungi becomes increasingly, but not absolutely, specific following germination. The suggestion of such a notion is that germination can be brought about by a relatively simple, non-specific and brief stimulus – with little or no specificity between seed and fungus. However, MASUHARA & KATSUYA (1994), PERKINS & MCGEE (1995) and BATTY *ET AL.* (2001a) reported that under field conditions some orchids exhibited a greater specificity for fungal partners (their 'ecological specificity') than under laboratory conditions (their 'potential specificity').

5.4.2 SYMBIOTIC SEED CULTURE: ACHIEVING GERMINATION *IN VITRO*

At 25 °C and in darkness, ZAK and WAR MI media did not consistently support fungal isolate growth. Alternatively, Oats medium saw fungi preferentially utilizing the constituent components of the growth medium and pathogenically infecting the *Disa* seed in the absence of embryo enlargement or testa rupture. H_1 Oats medium proved to be the most successful symbiotic medium in terms of the mycotrophic culture of *Disa* seed. This medium was used in all subsequent trials.

Of the 30 intraspecific association trials initiated (Table 5.5), where seed was sown with fungi isolated from the same species, 27 were ineffective. This lack of germination was graded as zero. These failed *in vitro* associations were placed towards either side of the continuum outlined by BURGEFF (1959), where

seeds were either not infected (association i), infected without germination (association ii) or over-infected (parasitized) without germination (association vi – vii) during the 24 month observation period (Table 5.7). Seeds were considered uninfected if isolate hyphae were not observed to penetrate the testa. Over-infection typically took place within two months (Table 5.7), with these cultures being discarded after six months.

Table 5.7. Unsuccessful intraspecific association symbiotic germination trials initiated for *Disa*. Germination response = 0 (WARCUP, 1973; OLIVA & ARDITTI, 1984; SMRECIU & CURRAH, 1989 and LEROUX *ET AL.*, 1997). The nature of the association after BURGEFF (1959). Thirty trials were initiated, incorporating 11 *Disa* species and 18 potential mycorrhizal fungi. Isolates were allocated to a particular morph, based on shared associations and hyphal and colony morphology.

Seed parent	Isolate morph	Nature of association	Seed parent	Isolate morph	Nature of association
D. chrysostachya	1	ii	*D. pulchra*	2	vi
"	4	vi	*D. sagittalis*	2	ii
D. cooperi	2	vi	*D. stachyoides*	4	vii
D. cornuta	3	i	"	2	vi
"	1	vii	*D. versicolor*	3	i
D. crassicornis	3	i	*D. woodii*	2	ii
D. fragrans	3	i	"	3	i
D. nervosa	1	ii			

Morph 1 – Ceratobasidiaceae, Morph 2 – Tulasnellaceae, Morph 3 – tulasnella – like fungi and morph 4 – ceratobasidium – like endophytes (after WARCUP, 1981). i – no infection, ii – infected within two weeks, without germination, vi – parasitized over approximately two months, without germination, vii – parasitized within two weeks, without germination. The number of seeds per culture vessel varied from 150 – 250.

Disa chrysostachya, *D. cooperi*, *D. cornuta*, *D. fragrans*, *D. nervosa*, *D. sagittalis*, *D. stachyoides* and *D. woodii* were not germinated symbiotically in this

study (Table 5.7). The nature of the failed association was independent of the seed parent. In addition, Ceratobasidiaceae (including ceratobasidium – like fungi) and Tulasnellaceae were involved in unsuccessful mycorrhizae without pattern. However, seed cultured in association with tulasnella – like fungi were never infected. Mycelia remained submerged, as was typical for all *Disa* isolates when cultured as pure strains *in vitro*. For the remainder, mycelia parasitized the seeds without stimulating germination (Table 5.7).

Nine interspecific association trials were established (Table 5.3), incorporating four seed parent species and a single representative of each of four rhizoctonia morphs identified during this study. All fungal morphs were associated with multiple host *Disa* species *in situ*. None of these resulted in germination. It must be noted that the intraspecific associations (included in Table 5.7) initiated as control cultures in the interspecific matrix also failed to induce germination.

These data on *Disa* mycorrhizae confirm that fungi isolated from a specific host do not necessarily stimulate the germination of seeds of the same species *in vitro*, corroborating other research (HARVAIS & HADLEY, 1967; CLEMENTS *ET AL.*, 1986; SMRECIU & CURRAH, 1989; ZETTLER & MCINNIS, 1992). Additionally, the same fungi were unable to elicit germination in other *Disa* species. The chance of successful *in situ* infection and germination would be enhanced if the association were comparatively non-specific (RASMUSSEN, 1995), as the *Disa* seed is both widely dispersed and is not mycorrhizal at the time of capsule dehiscence. However, these data provide evidence for a certain degree of specificity between endophyte and seed, as suggested by RAMSAY, SIVASITHAMPARAM & DIXON (1987) and MASUHARA & KATSUYA (1994). Such a result is unexpected since *in vitro* germination data for other orchid taxa typically confirms the lack of specificity between seed and endophyte (HADLEY, 1970; WARCUP, 1973; ARDITTI *ET AL.*, 1981; CLEMENTS, 1982, 1988; OLIVA & ARDITTI, 1984; SMRECIU & CURRAH, 1989; RASMUSSEN *ET AL.*, 1990; ZETTLER & MCINNIS, 1992, 1993; ZETTLER *ET AL.*, 1999).

The ability of an endophyte to stimulate germination in the seeds of the orchid species from which the fungus was isolated is the accepted test for the existence of a symbiotic relationship in the mature mycorrhizal system (WARCUP, 1971; RAMSAY, DIXON & SIVASITHAMPARAM, 1986). However, the lack of successful association, as revealed by a negative result in the vast majority of *Disa* germination trials, does not necessarily indicate that the fungi are not mycorrhizal. Rather, this only provides evidence that the isolates are not effective mycorrhizal partners during germination and early seedling maturation, despite being isolated from mature *Disa* plants.

Three *Disa* species (*D. crassicornis*, *D. pulchra* and *D. versicolor*) were successfully germinated using symbiotic techniques (Table 5.8). *Disa crassicornis* and *D. versicolor* seed germinated only when in association with a member of the Tulasnellaceae. Both species were intractable *in vitro* on modified media, recording germinability indices (GI) of 3 (Table 5.2). Alternatively, *D. pulchra* seed germinated only when associated with a member of the Ceratobasidiaceae. This species was also germinable (GI = 2) under asymbiotic conditions (Table 5.2). Attempts to reproduce successful associations in subsequent years were unsuccessful. It is possible that the ability of the *Disa* isolates to form mycorrhizal associations decreased with storage time and repeated sub-culturing. Similar effects were documented by MARX & DANIEL (1976), ALEXANDER & HADLEY (1983) and RASMUSSEN (1995). Limited seed prevented interspecific trials involving *D. crassicornis, D. pulchra* and *D. versicolor*.

The symbiotic germination of *D. pulchra* was successful in approximately 80 % of seeds (Table 5.8), but only as far as the formation of rhizoids and the enlargement of the protocorms (stages ii and iii; Plate 5.2 A, B). Rupture of the testa and protrusion of the protocorm were observed two weeks after sowing. Similar germination times were reported by ZETTLER *ET AL.* (1999) for *Spiranthes odorata* Lindl. Germination was higher (80 % vs. 30 %) and quicker (2 weeks vs. 13 weeks) under symbiotic rather than asymbiotic conditions for this small-seeded

species (Table 5.2). These data corroborate findings for other orchid taxa by STOUTAMIRE (1963, 1974), HADLEY & WILLIAMSON (1971), WARCUP (1973), CLEMENTS *ET AL.* (1986), ZETTLER & MCINNS (1992) and RASMUSSEN (1995), who argued the merits of symbiotic culture over asymbiotic techniques.

Table 5.8. Successful intraspecific association symbiotic germination trials initiated for *Disa*. Thirty trials were initiated, incorporating 11 *Disa* species and 18 potential mycorrhizal fungi. Isolates were allocated to a particular morph, based on shared associations and hyphal and colony morphology. Germination response after WARCUP (1973), OLIVA & ARDITTI (1984), SMRECIU & CURRAH (1989) and LEROUX *ET AL.* (1997), with the nature of the association after BURGEFF (1959). Shooting as a percentage of the protocorms produced.

Seed parent	Isolate morph	Germination response	Percentage germination	Percentage shooting
D. crassicornis	2	5	40	5
	Protocorms after 10 – 24 weeks. Shoots at 6 months, tubers at seven months. Without roots. Green seedlings, 2 – 3 cm tall, did not survive past 20 months *in vitro*. 100 % mortality. (Plate 5.3)			
D. pulchra	1	3	80	0
	Protocorms after two weeks. Enlarged but without primordia. Brown eight weeks post germination. (Plate 5.2)			
D. versicolor	2	?	10	0
	Germination after four weeks. Embryo atypically enlarged and undifferentiated at 16 weeks. (Plate 5.2)			

Morph 1 – Ceratobasidiaceae, Morph 2 – Tulasnellaceae, Morph 3 – tulasnella – like fungi and morph 4 – ceratobasidium – like endophytes (after WARCUP, 1981). ? – atypical germination response. Sowing density varied from 150 – 250 seed per culture vessel.

Protocorms of *D. pulchra* became brown and deteriorated approximately eight weeks after germination (Plate 5.2C). None developed apical meristems.

Protocorm browning was accompanied by endophyte proliferation, which overgrew the culture vessel. Consequently the association of *D. pulchra* with a member of the Ceratobasidiaceae, although allowing for germination, did not support seedling development as a result of the plant tissue being parasitized by the fungus. When interpreting similar data, SMRECIU & CURRAH (1989) and RASMUSSEN & WHIGHAM (1998) reported that the pelotons were not lysed by the orchid tissue. The lack of germination for *D. pulchra* when sown with a member of the Tulasnellaceae was surprising, since these fungi were shown to enter into successful mycorrhizal partnerships with *D. crassicornis* and *D. versicolor*.

The symbiotic germination response in *D. versicolor* was observed four weeks from initiation. Approximately 10 % of seeds germinated (Table 5.8). Seeds showed signs of rupture, which was followed closely by the protrusion of a protocorm-like structure. The term protocorm was not used here since under symbiotic conditions several abnormalities of this initial development were observed. Protocorm-like bodies protruded from the ruptured testa as white masses of tissue (Plate 5.2D). Spherical protocorms and rhizoids never developed. After four months tissue had enlarged and developed into an amorphous mass (Plate 5.2E). Differentiation did not occur, although the protocorm-like body was reminiscent of the mature, poly-embryogenic protocorms observed in other species (ARDITTI, 1982; ARDITTI & ERNST, 1984; RASMUSSEN, 1995). Tissue remained achlorophyllous and undifferentiated for the remainder of the 24-month observation period.

Disa crassicornis germinated beyond stage v (greening of primary leaf tissue) when sown in association with an isolated Tulasnellaceae. The initial response occurred at 10 weeks from culture initiation, substantially longer than the symbiotic germination times reported for other small-seeded *Disa* species in this study and other terrestrial orchid taxa germinated globally (STOUTAMIRE, 1963, 1974; HADLEY & WILLIAMSON, 1971; WARCUP, 1973; CLEMENTS *ET AL.*, 1986; ZETTLER & MCINNS, 1992; RASMUSSEN, 1995). Germination was not synchronous,

with protocorms forming over 14 weeks (Plate 5.3A). Although 40 % of seeds produced achlorophyllous protocorms, only one quarter of these survived to produce apical meristems. The remainder turned brown, apparently unable to lyse the internal pelotons and resist uncontrolled infection. Similarly, only a small proportion of protocorms with meristems produced primary leaves, resulting in only 5 % of the total number of protocorms shooting (Table 5.8).

During the asynchronous 14-week germination period, the initial protocorms developed beyond stage v, producing primary leaf tissue. Shooting therefore occurred at approximately six months, twice as long as asymbiotically germinated small-seeded *Disa* species (Chapter Three). Thereafter development was rapid, with seedlings producing multiple tubers in an additional four weeks (Plate 5.3B). Asymbiotically produced seedlings of small-seeded *Disa* species developed tubers six months after germination. Rooting was not observed throughout the 24-month observation period in the symbiotically germinated *D. crassicornis* seedlings (Plate 5.3C). Atypical development is not unique to symbiotically germinated *Disa* species and was reported from several Australian terrestrials by CLEMENTS & ELLYARD (1979). In the absence of roots, mortality of the 2 – 3 cm tall *D. crassicornis* seedlings at 20 months was 100 % (Plate 5.4D). Similarly high levels of mortality (> 97 %) are consistent with several North American orchid species germinated symbiotically (ANDERSEN, 1990; ZETTLER & MCINNIS, 1992; ANDERSON, 1996; ZETTLER & HOFER; 1998).

Several species (*D. cooperi*, *D. cornuta*, *D. nervosa*, *D. sagittalis* and *D. woodii*) which were involved in unsuccessful fungal association responded *in vitro* asymbiotically, achieving *in vitro* germinability indices of two on modified media (Table 5.2). Such data, specifically in the absence of infection, makes their lack of germination under mycotrophic conditions puzzling. Similar data were reported by ZETTLER *ET AL.* (1999), who concluded that for *Encyclia tampensis* (Lindl.), the carbohydrate source must be metabolized by the fungus before germination can be initiated.

Plate 5.2. Symbiotic germination of mature seed of *D. pulchra* (A – C) and *D. versicolor* (D, E), two small-seeded, summer-rainfall species. *Disa pulchra* seed germinated in association with a member of the Ceratobasidiaceae (isolate *pul*2), with protocorm emergence two weeks after culture initiation. Rhizoids (r) were present from testa rupture. Over the subsequent six weeks protocorms become brown (b) and never developed apical meristems. Necrosis was accompanied by proliferation of the endophyte (*). Testa rupture for *D. versicolor* seed was observed four weeks after culture initiation. Seed was sown in association with a member of the Tulasnellaceae (isolate *ver*1). Development was atypical, without spherical protocorms. Protocorm-like bodies (pb) formed, which enlarged over the subsequent four months. These remained achlorophyllous and undifferentiated for the duration of the 24-month observation period. Mycelia were not visible externally.

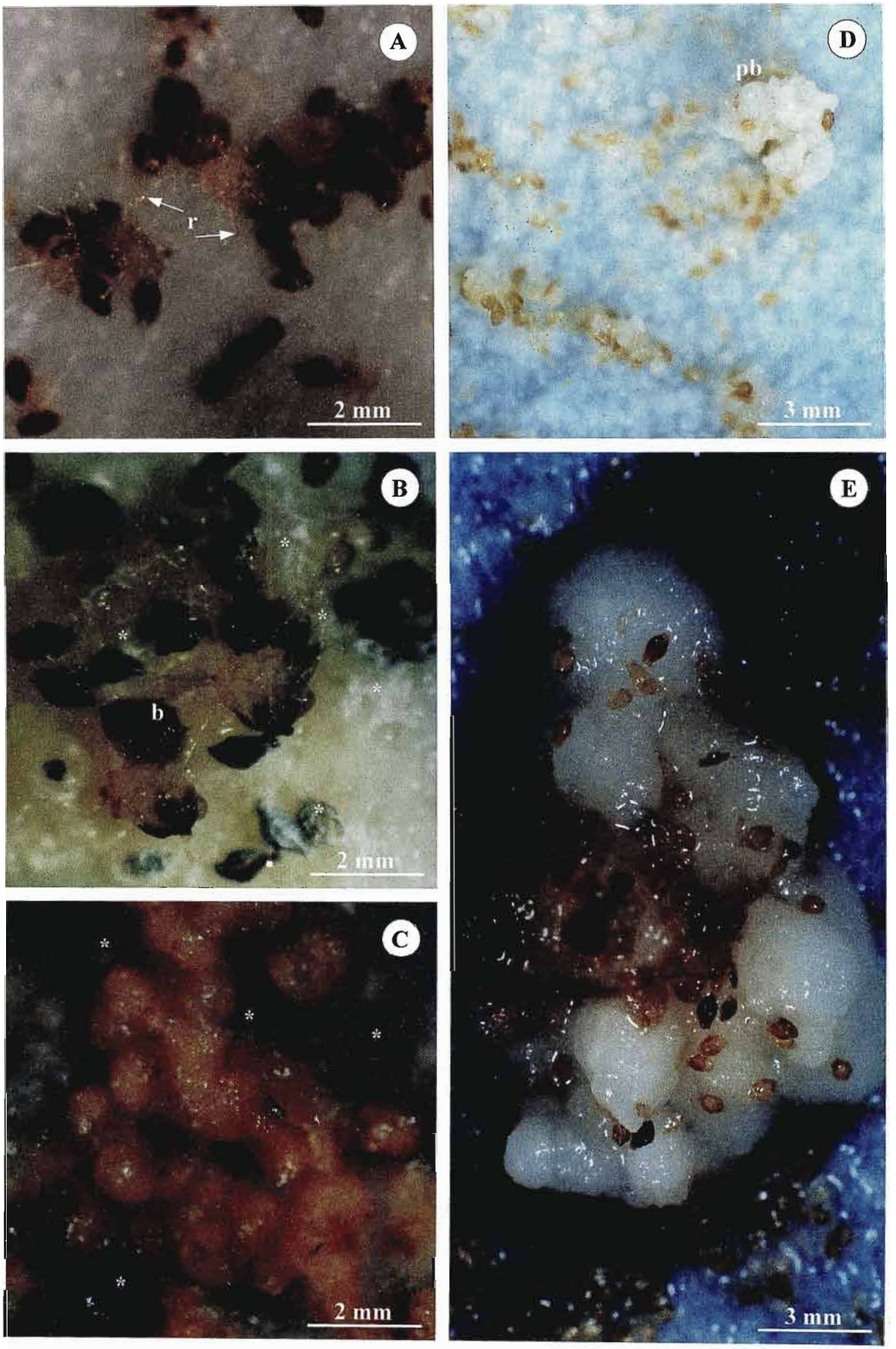
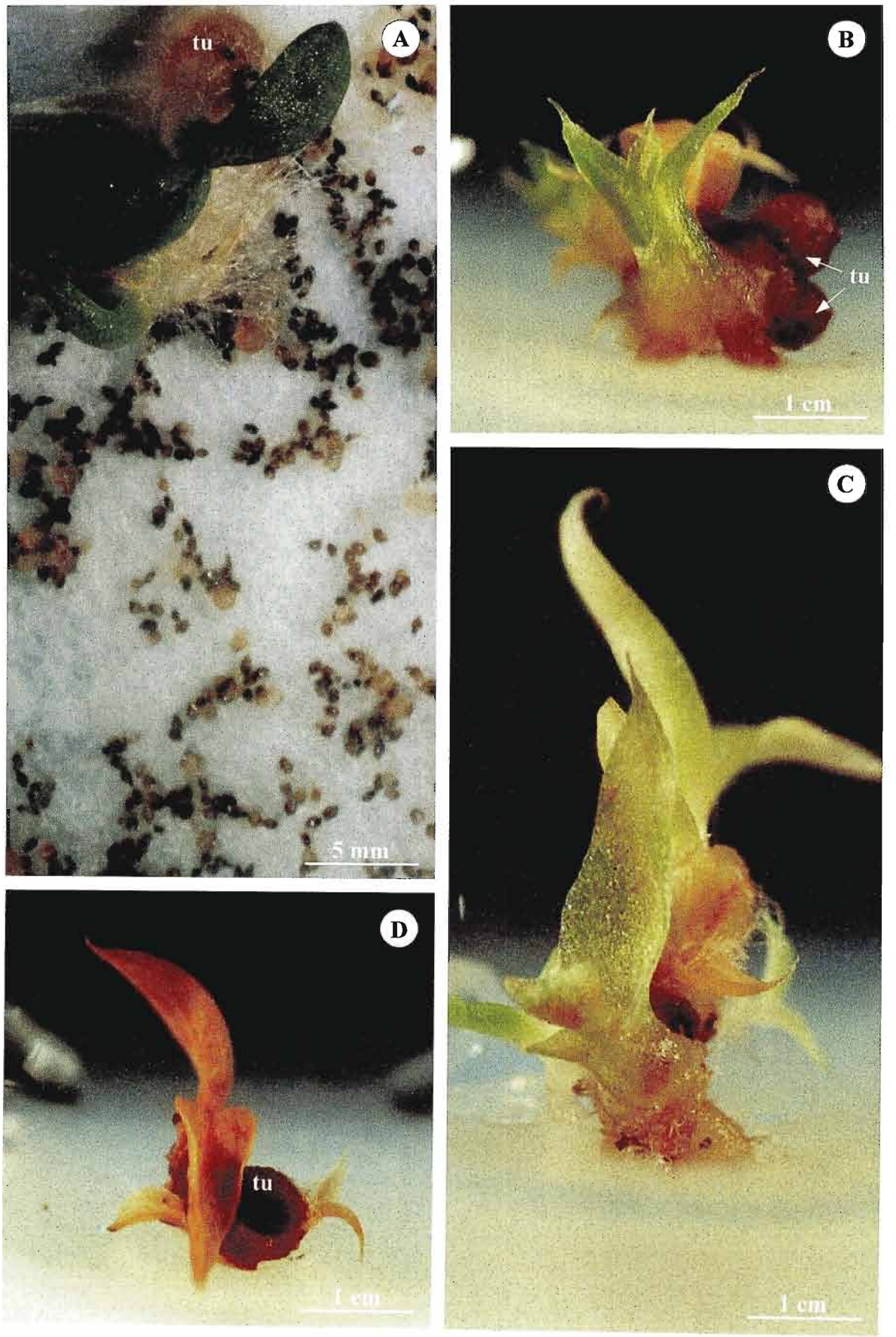

A
r
2 mm
D
pb
3 mm
B
b
2 mm
E
3 mm
C
2 mm

Plate 5.3. Symbiotic germination of mature *D. crassicornis* seed, a summer-rainfall species. Seed was sown in association with a Tulasnellaceae endophyte (isolate *cra*2). Germination was asynchronous, occurring initially at 10 weeks from culture initiation and being spread over an additional 14 weeks (A). Shooting occurred at six months from germination, after which multiple tubers (tu) developed (B). Rooting was not observed during the 24-month observation period (C). Consequently, seedling mortality was 100 % (D).


A
tu
5 mm
B
tu
1 cm
C
1 cm
D
tu
1 cm

Consequently, it may be that essential nutrients were unavailable to the *Disa* embryos as a result of the composition of the symbiotic media and that the addition of simple sugars and other factors, such as activated charcoal, would have promoted asymbiotic germination. The lack of germination in control cultures, specifically for *D. crassicornis*, *D. pulchra* and *D. versicolor*, supports such a notion. Additionally, the implication is that infection of the *Disa* embryo was preceded by penetration of the testa, ruling out rhizoids as the possible site of infection (RASMUSSEN, 1995). These data corroborate the findings of JACKSON & MASON (1984).

From this study it was apparent that the production of rhizoids and the rupture of the *Disa* seed testa were not accurate indicators of forthcoming development. High germination rates did not necessarily correspond with successful seedling development. Clearly an additional stimulus is required for continued growth in symbiotically germinated seeds of *D. crassicornis*, *D. pulchra* and *D. versicolor*.

This study stands as the first report that describes the cultivation of any *Disa* from seed in association with fungi. However, the genus seems incapable of yielding a large number of successful mycorrhizae, particularly with advanced stages of germination (stages iv – v), for reasons that are not fully understood. SMRECIU & CURRAH (1989) used seed dormancy characteristics to explain why symbiotic germination was not observed in *Epipactis purpurata* Sm., since the embryos of many orchid species are surrounded by an impenetrable, sclerous testa which represents a barrier to imbibition and germination (VAN WAES & DEBERGH, 1986a, b; BEWLEY & BLACK, 1994; RASMUSSEN, 1995; ARDITTI & GHANI, 2000). Additionally, embryos may be physiologically dormant when the seeds are mature (ARDITTI *ET AL.*, 1981; RASMUSSEN, 1995). Furthermore, ZETTLER & MCINNIS (1992) observed a correlation between species that were difficult to propagate asymbiotically and their response in fungal association. THOMPSON *ET AL.* (2001) reported on the presence of impervious and phenol rich seed coats in *Disa*, which

similarly could contribute towards the failure of establishing symbiosis *in vitro*.

5.4.3 ENDOPHYTE ACTIVITY AND NUTRITION

H_1 Oats media (RASMUSSEN *ET AL.*, 1990) and half-strength MURASHIGE & SKOOG salts (1962) did not support endophyte growth over a four week period. Consequently full strength MS liquid cultures, supplemented with 4g L^{-1} sucrose, were inoculated with mycelia stock (Figure 5.1 inset).

The TLC chromatographs for sucrose, fructose and glucose developed as expected. Following development with the reagent anisaldehyde these carbohydrates stained black with R_f values of 0.5, 0.7 and 0.7 respectively (Figure 5.1A) using the solvent system ethyl acetate : acetic acid : MeOH : H_20 (12:3:3:2). Glucose (blue) and fructose (pink) were distinguished after incubation with aniline-diphenylamine (Figure 5.1B).

Thin layer chromatographs for the sterile media revealed sucrose (R_f = 0.5; stained black under both developing conditions), which was expected for sample 1 since analysis occurred prior to inoculation with fungal stock. An additional carbohydrate band (R_f = 0.7; Figure 5.1A) detected in sample 1 was shown to comprise a combined fructose – glucose fraction (stained blue-pink; Figure 5.1B), the result of disaccharide hydrolysis during autoclaving. Gas chromatography of the defined culture medium confirmed these data (Figure 5.2A and Table 5.9) and revealed that approximately 30 % of the sucrose added to the media underwent hydrolysis. Percentage contributions were recorded as sucrose (70.0 %), fructose (15.4 %) and glucose (14.6 %).

GC revealed the proficiency of the endophyte to catabolize complex carbohydrates, with 8.6 % of the provisioned sucrose remaining (Figure 5.2B) in the post-incubation medium. This sucrose was not detected in the TLC fingerprints for sample 2 (Figure 5.1).

Table 5.9. Comparative percentage data for the chromatographic detection of specific carbohydrate species as a result of heat-induced hydrolysis and endophyte metabolism. Standard sugar retention times (min) are presented in parenthesis.

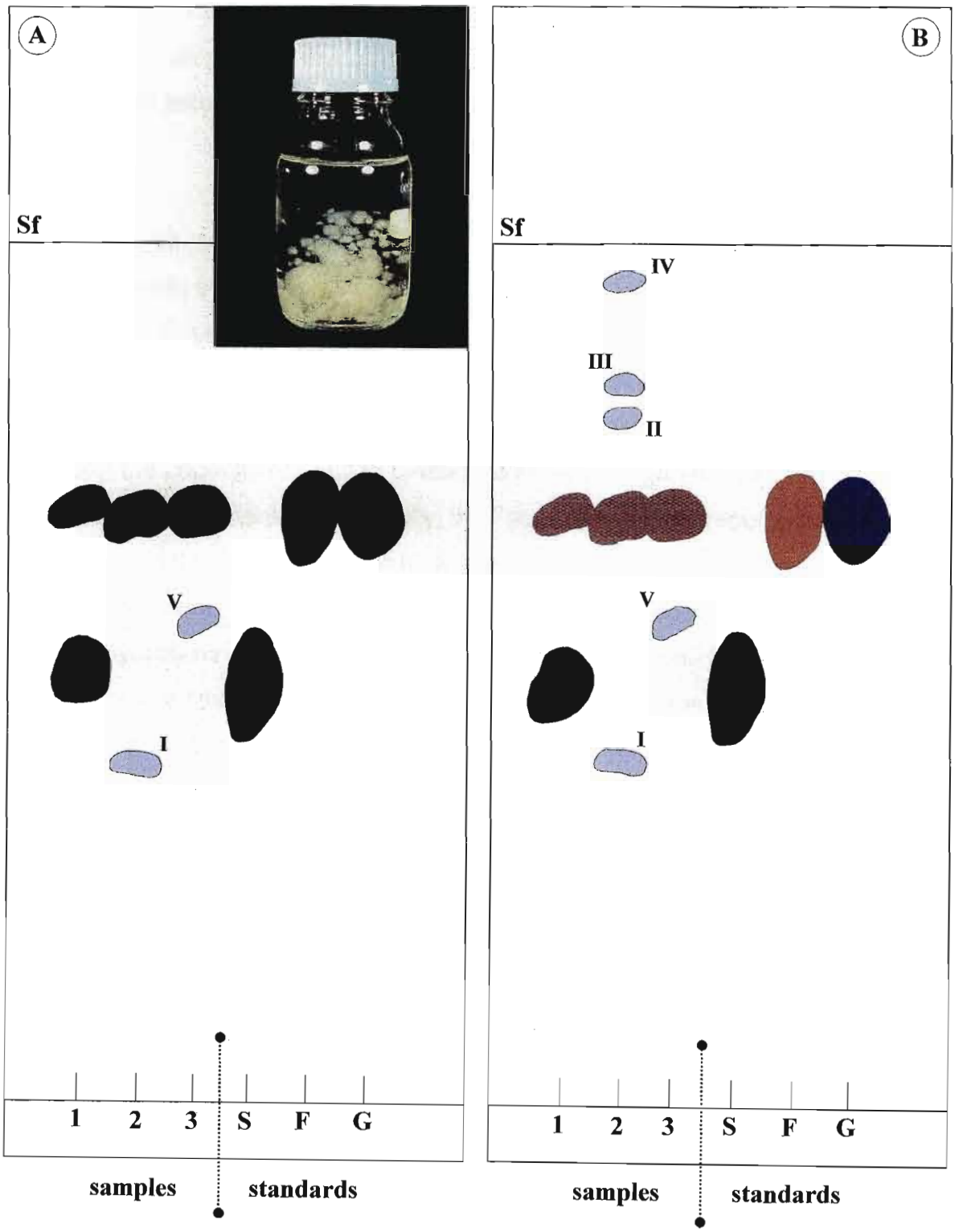
Sugar	Sample 1	Sample 2	Sample 3
rhamnose (11.5)	–	3.48	9.89
fructose (14.5)	15.42	38.46	33.6
glucose (16)	14.55	43.69	26.90
inositol (18)	–	–	4.79
CHO_2 (24)	–	–	2.54
sucrose (30)	70.03	8.58	–
trehalose (32.5)	–	5.91	14.62
maltose (33.5)	–	5.44	4.21
Percentage sugar incorporation post-incubation		55 %	45 %

1 – MS medium prior to fungal inoculation, 2 – medium after fungal culture, 3 – endophyte cellular component.

By the end of the four-week incubation period the carbohydrate substrate (sucrose) was almost completely hydrolyzed to its constituents – fructose and glucose. These were visualized as a combined blue-pink fraction with a $R_f = 0.7$ (Figure 5.1B). GC revealed that sucrose hydrolysis was accompanied by increased amounts of fructose (38.5 %) and glucose (43.7 %) in the culture medium. Novel carbohydrates rhamnose, trehalose and maltose were also detected in the bathing medium, albeit in low percentages (< 6 %; Figure 5.2B and Table 5.3). Both trehalose and maltose were recorded previously as being of fungal origin (GLAZIOU & GAYLER, 1969; SMITH, 1973), synthesized by the endophyte from glucose monomers (Figure 5.2B).

During the incubation period, 45 % of the carbohydrates provisioned in the media were incorporated into the endophyte. This includes carbohydrates used in the production of the hyphae walls, typically polysaccharide mannans bound as glucoproteins, β-glucans and chitin (RUIZ-HERRERA, 1992).

Figure 5.1. Thin layer chromatograms for samples 1 – 3, originating from the *in vitro* culture of *cra*2, the Tulasnellaceae that promoted germination in *D. crassicornis* seed. Full-strength, liquid MS cultures were inoculated with mycelia stock and maintained in 500 ml glass bottles (inset). Compounds were separated using ethyl acetate : acetic acid : MeOH : H_2O (12:3:3:2). Sf = solvent front. Bands under visible (VIS) and UV-light (254 nm and 366 nm) were marked. Carbohydrate species were identified following staining with anisaldehyde / sulphuric acid (A) and aniline-diphenylamine (B), and through co-chromatography with reference standards sucrose (S), fructose (F) and glucose (G). Sample 1 = media prior to inoculation; sample 2 = media after four weeks of hyphal growth; sample 3 = endophyte cytoplasm.


A
Sf
V
I
1 2 3 S F G
samples
standards
B
Sf
IV
III
II
V
I
1 2 3 S F G
samples
standards

TLC revealed that the endophyte cytoplasm (sample 3) contained the carbohydrate monomers fructose and glucose (Figure 5.1). GC confirmed the complete absence of sucrose and the dominance of glucose and fructose (Table 5.9). In addition, 5 novel mycorrhizal carbohydrate species were identified viz. rhamnose, inositol, an unidentified sugar CHO_2, trehalose and maltose (Figure 5.2C).

Sucrose hydrolysis presumably occurred extracellularly since no sucrose was detected in the endophyte cytosol. The dominance of fructose and glucose, both in the medium and the cytosol, implicate these monomers as the carbohydrate forms preferentially absorbed and translocated by the fungus. These data corroborate previous findings (BRADY, 1984; BEYRLE & SMITH, 1993) by revealing the capacity of fungi to catabolize complex organic molecules and utilize the resultant monomers. Additionally, they augment the few reports that have demonstrated the ability of mycorrhizal fungi to alter their surroundings. The increased presence of fructose and glucose in the incubation media reflects sucrose hydrolysis by-products that have yet to be absorbed by the hyphae. Alternatively, a small percentage may arise from the leakage of internal carbohydrates. Evidence for limited leakage was presented by JACKSON & MASON (1984) and was supported in this study by the lower occurrence of rhamnose and trehalose in the media than in the cytoplasm.

The extracellular cleavage of sucrose by the endophyte dictates that at least one compound with this capacity be synthesized and secreted by the mycelia. Hydrolytic enzymes have been reported from orchid endophytes (BURGEFF, 1959; HADLEY & PEROMBELON, 1963; BARROSO *ET AL.*, 1986; RASMUSSEN, 1995). Four non-carbohydrate fractions (I – IV) were detected in sample 2, which represent relatively complex organic compounds not present in the defined medium (sample 1) nor the cytosol (sample 3). Compound I (R_f = 0.4) was visible on both anisaldehyde (Figure 5.1A) and aniline-diphenylamine treated plates, whilst novel compounds II – IV were detected only following the latter treatment

(Figure 5.1B).

R_f values were recorded as 0.8, 0.85 and 0.97 respectively. A single non-carbohydrate compound (V; R_f = 0.57) was detected exclusively in sample 3 under UV-light (366 nm) following both spray reagent treatments (Figure 5.1). All synthesized compounds (I – V) were visible under UV-light (366 nm) as lilac fluorescence. These complex non-carbohydrates provisioned by the *Disa* endophyte, if shown to possess enzymatic or growth regulatory capabilities, have the potential to facilitate gluconeogenesis or other, as yet, undetermined catabolic or anabolic nutritional pathways within the embryo.

The lysis of internal hyphae by *D. crassicornis* embryos therefore liberated a minimum of seven soluble carbohydrates (Table 5.9) and one non-carbohydrate, making these compounds available to the embryonic tissues. HARVAIS & RAITSAKAS (1975) showed that the digestion of hyphal walls resulted in products which orchid embryos were not able to assimilate.

This research (Chapter Four) has shown that *Disa* species become increasingly difficult to germinate when the embryos are depauperate in sucrose and rich in glucose and fructose. Consequently, the ability of the endophyte to liberate these sugar monomers seems of little value to the intractable *D. crassicornis* embryo as elevated glucose and fructose levels, in the absence of sucrose, were correlated with the inability of this species to germinate.

The importance of previously undetected sugars such as rhamnose, inositol, CHO_2 and the typical fungal sugars trehalose and maltose remains unclear. These sugars were detected in the embryos of many of the *Disa* species investigated (Table 4.3; page 103), but no relationships were identified between their occurrence and the ability of the seeds to germinate. The overall augmentation of the free sugar status of the embryo may therefore be required to facilitate germination in *D. crassicornis*.

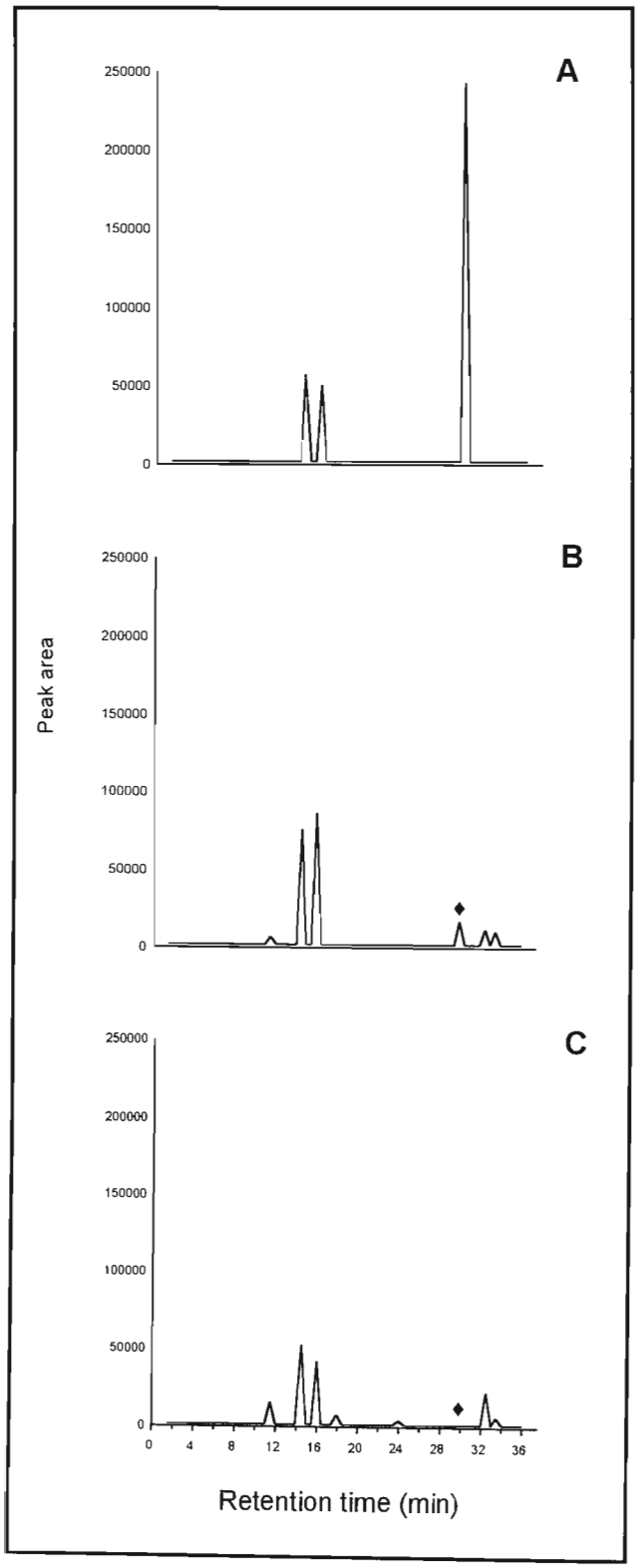


Figure 5.2.
Representative sugar chromatographs for samples 1 – 3, originating from the *in vitro* culture of *cra*2, the Tulasnellaceae that promoted germination in *D. crassicornis* seed.

A) **Sample 1 – MS media prior to inoculation**
B) **Sample 2 – media after four weeks of hyphal growth**
C) **Sample 3 – endophyte cytoplasm**

Attention is drawn to the partial and complete absence of sucrose (♦) from the post-incubation medium (B) **and the cytosol** (C) **respectively.**

Alternatively, the provision of one or several key sugars by the endophyte may dictate fungal association. These data on the *Disa* endophyte suggest that those sugars that are crucial in germination extend beyond sucrose, fructose and glucose – the dominant sugars identified in *Disa* embryos (Chapter Four). Trehalose may be one such key carbohydrate and has been shown to support germination and seedling establishment in species from several orchid genera (ERNST, 1967; ERNST *ET AL.*, 1971; SMITH, 1973). SMITH (1973) speculated on the presence of trehalase in orchids, but this has never been shown conclusively. The occurrence of trehalose in the *Disa* endophyte confirms the reputation of this carbohydrate as a common fungal sugar – the implication being that it may play a critical role in *Disa* germination. As an endogenous sugar, trehalose was only detected in four of the *Disa* species investigated, but its occurrence was not linked to seed germinability.

Light and scanning electron microscopy (Plate 5.4A) confirmed the site of infection in symbiotically germinated *D. crassicornis* seeds as being via the suspensor, which is micropyle-adjacent. Congruent results were recorded for *D. pulchra* and *D. versicolor*. Those *Disa* seeds that did not germinate when sown in fungal association were infected randomly, with hyphae commonly penetrating between the testa cells along their fused anticlinal walls (Plate 5.4B). Successful association between *Disa* and endophyte is therefore reliant on infection via the micropyle. This corroborates previous reports on other orchid taxa by FAST (1982), ALEXANDER & HADLEY (1983), MUIR (1987) and HADLEY & PEGG (1989). Since compatible infection of the *Disa* seed does not take place via the rhizoids of an already protruding protocorm (RASMUSSEN, 1995), the initial germination response is dependent on mycorrhizal association. These data provide evidence that the role of the *Disa* endophyte is not necessarily exclusively nutritive. JACKSON & MASON (1984) reported that the germination of several *Cypripedium* species was dependent firstly on the physical penetration of endophyte hyphae through the testa and that the nutritive role of the fungus was secondary.

Where coat-imposed dormancy is implied (BATTY *ET AL.*, 2001a, b; THOMPSON *ET AL.*, 2001), increased testa permeability probably facilitates germination. Chemical scarification of the testa and the success of 'green-podding' in asymbiotic germination trials for the small-seeded *Disa* species provide evidence for this (Chapter Three). In *D. crassicornis* and other members of the genus, the phenol-rich testa and continuous carapace effectively isolate the embryo from the external environment, preventing imbibition. Embryonic tissues must be imbibed before non-pathogenic infection can occur (RASMUSSEN, 1995). However, if the physiological and / or morphological state of the seed allows imbibition, then there is no explanation as to why symbiosis must be obligate. Seeds which imbibe in the absence of mycorrhizal infection should also be able to take up exogenously supplied nutrient sources, enzymes and growth regulators, making their asymbiotic germination possible in theory. In the intractable small-seeded *Disa* species, embryo imbibition under natural germination conditions may therefore be dependent on mycorrhizal penetration.

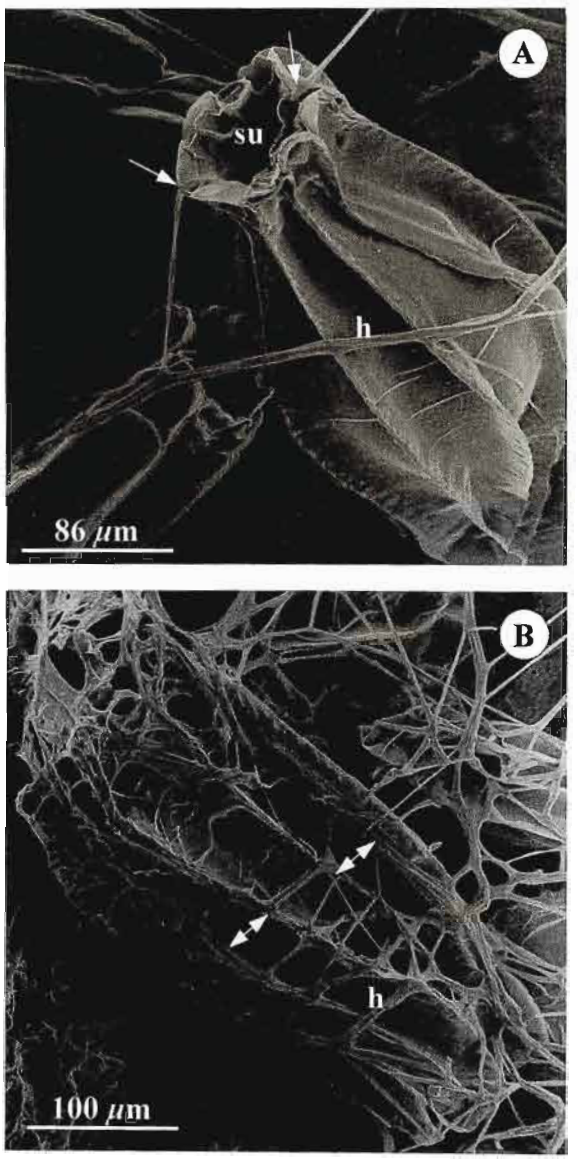
Two equally likely and not necessarily mutually exclusive scenarios involving fungal association are proposed;

i. seed testa is penetrated by mycelia via the suspensor and micropyle and the passive uptake of water, exogenous nutrients and compounds from the environment by the embryo occurs. Modification of the seed's immediate environment by the fungus dictates the type and abundance of compounds available; and
ii. seed testa is penetrated by hyphae via the suspensor and micropyle and following embryo imbibition, the active translocation of nutrients from fungus to host tissue occurs.

The germination-promoting effects of endophytes on the *Disa* seed have been established. Previously, the precise effects of mycorrhizae on germination

in other orchid taxa have not been sufficiently investigated and are often overlooked. MASUHARA & KATSUYA (1989, 1994) and PERKINS, MASUHARA & MCGEE (1995) warned against a direct interpretation of *in vitro* compatibility and cautioned that it is difficult to evaluate the involvement of fungi in germination *in situ* since the responses that are obtained *in vitro* may not be representative of the natural process. An understanding of mycotrophy within *Disa* will benefit from continued research into the nutritional status of both seed and fungus, since the results presented in this regard allow for very few firm conclusions. Symbiotic germination has been shown to be an invaluable propagation technique that has aided conservation in many Holarctic and Australian orchids. Until considerably more research is directed at understanding the biology of *Disa* mycotrophy, it will be difficult to assess the role of fungi and to harness this *in vitro*. However, if the role of *Disa* endophytes is as simple as the physical breaking of coat-imposed dormancy and the provision of auxiliary compounds, as suggested by this study, then continued research should allow for replication *in vitro* without fungal association.

Plate 5.4. Controlled (A) and pathogenic (B) infection of *D. crassicornis* seed by fungal isolates *cra*2 (Tulasnellaceae) and *cra*1 (a tulasnella – like endophyte) respectively, two weeks after culture initiation. Electron microscopy confirmed the site of infection in symbiotically germinated seeds as being via the seed suspensor (su), which is micropyle-adjacent. Germination occurred at 10 weeks. Unsuccessful association resulted in random infection, with hyphae (h) penetrating between and separating testa cells along their anticlinal walls (indicated).


A
su
h
86 μm
B
h
100 μm

CHAPTER SIX

VIABILITY TESTING AND EVIDENCE OF WATER-IMPERMEABLE SEED DORMANCY IN *DISA*[*]

Water-impermeable dormancy is suspected to account for poor germination in temperate terrestrial orchids – an issue compounded by the lack of accurate viability data. Restricted permeability to aqueous EVANS' blue was linked to the integrity and the leachable phenolic content (LPC) of the testae of 11 South African *Disa* species – with phenols being both hydrophobic and phytotoxic. Large-seeded, easily germinable species were highly permeable at dehiscence, displaying disrupted testae and LPC below 1 mg L^{-1}. Alternatively small-seeded, often intractable *Disa* species were impermeable, with significantly higher LPC. Germinability was ultimately defined by a significant linear regression with LPC. Phenolic deposition increased exponentially with increasing seed maturity and was linked to decreased permeability to aqueous EVANS' blue and the development of testa colouration. These data emphasize the potential for 'green-pod' germination in *Disa*. Light and transmission electron microscopy revealed a stratified embryo carapace in poorly germinable and intractable species, elaborate extensions of which serve to obstruct the suspensor and prevent imbibition. NaOCl treatment rendered the testae of the small-seeded species permeable, with the duration of scarification needed to visualize viability using TTC correlating with LPC. Scanning electron microscopy illustrated the corrosive effect of NaOCl on testa integrity. Viability was 50 – 100 % higher than the percentage germination reported for small-seeded species under modified *in vitro* conditions, indicating that i) the methods used to break dormancy are not adequate or ii) an additional factor may be acting in concert with the testa to regulate germination. Dormancy in *Disa* is discussed with reference to strongly fluctuating annual temperatures and rainfall cycles and annual and perennial water sources.

[*] The contents of this chapter are reproduced, in part, from THOMPSON, D.I., EDWARDS, T.J. & VAN STADEN, J. 2001. *In vitro* germination of several South African summer-rainfall *Disa* (Orchidaceae) species: Is seed testa structure a function of habitat and a determinant of germinability? *Systematics and Geography of Plants* 71(2): 597-606.

6.1 INTRODUCTION

Many terrestrial orchid species have been germinated readily *in vitro* (ARDITTI, 1982; RASMUSSEN, 1995; MICHEL, 2002) and display a loss of germinability when stored under laboratory conditions (KOOPOWITZ & WARD, 1984; PRITCHARD, 1984, 1985, 1986; SEATON & HAILES, 1989; ARDITTI, 1993; RASMUSSEN, 1995; PRITCHARD, POYNTER & SEATON, 1999). Seeds of these species are presumably short-lived and germinate immediately post-dispersal. Unfortunately the majority of terrestrial species, including *Disa*, have never been recorded as protocorms *in situ* due to their small seed size, subterranean development and considerable dispersal distances (RASMUSSEN & WHIGHAM, 1993; RASMUSSEN, 1995; BATTY, DIXON & SIVASITHAMPARAM, 2000).

Limited field data suggests that most Holarctic orchids germinate primarily in spring, with less in summer, autumn and winter (Table 6.1). RASMUSSEN & WHIGHAM (1993) and VAN DER KINDEREN (1995) reported *in situ* germination of *Goodyera pubescens* R.Br., *Corallorhiza odontorhiza* Nutt. and *Epipactis helleborine* Crantz seven months after their autumn dispersal. Utilizing controlled seed sowing and retrieval methods RASMUSSEN & WHIGHAM (1998) corroborated previous findings, in addition to recording spring or summer germination for members of the genera *Microtis* R.Br. and *Spiranthes* L.C. Rich. Similar data exist for *Cypripedium reginae* (VUJANOVIC *ET AL.*, 2000). Alternatively, BATTY *ET AL.* (2000, 2001a, b) reported summer seed dispersal and winter germination for terrestrials that experienced the Mediterranean climate of western Australia (Table 6.1). These data imply dormancy mechanisms immediately post-dispersal (ARDITTI *ET AL.*, 1981, 1982a, b; BRADBEER, 1988). Germination proceeds when conditions are conductive for growth, which for orchids has been linked to temperature, soil moisture and appropriate fungal activity (SIVASITHAMPARAM, 1993; BASKIN & BASKIN, 1998; BATTY *ET AL.*, 2000). However, ARDITTI (1982) demonstrated that the season of germination was inconsistent within *Dactylorhiza* spp., varying with provenance.

The lack of a correlation between reported storage times and field observations of germination has further confused the issue of seed dormancy in orchids (KANO, 1968; STOUTAMIRE, 1974; ANDERSEN, 1990; VAN WAES, 1987; JOHANSEN & RASMUSSEN, 1992; ZETTLER & MCINNIS, 1993). Typically, little or no data exists for the survival of orchid seed under field conditions (RASMUSSEN & WHIGHAM, 1993, 1998; BATTY *ET AL.*, 2000).

Table 6.1. Season of germination for Holarctic and Australian* terrestrial orchids. Data are mostly estimated from the time of year when seedlings have been located *in situ*, since field observations of germination are rare.

	Season			
Orchid	**Spring**	**Summer**	**Autumn**	**Winter**
**Caladenia arenicola* Hopper & A.P.Br.			✓	✓
Coeloglossum viride Hartm.	✓			
Corallorhiza odontorhiza		✓		
C. trifida Chatelain			✓	
Cypripedium acuale Linn.	✓	✓		
C. calceolus Linn.				✓
C. calceolus var. *parviflorum* Fernald	✓	✓		
C. reginae	✓	✓		
Dactylorhiza fuchsii (Druce) Soó			✓	✓
D. maculata (L.) Soó			✓	
D. sambucina (L.) Soó	✓			
D. spp.	✓	✓	✓	✓
Epipactis helleborine	✓			
Epipogium aphyllum R.Br.			✓	
Galearis spectabilis (L.) Rafin.		✓		
Goodyera pubscens	✓			

Table 6.1. (continued)

Orchid	Season			
	Spring	Summer	Autumn	Winter
Himantoglossum hircinum Spreng.			✓	
Liparis loeselii (L.) Rich.	✓			
Listera ovata R.Br.	✓			
Microtis spp.	✓	✓		
Neottia nidus-avis (L.) L.C.Rich.	✓			
Ophrys apifera Huds.	✓			
Orchis mascula Crantz		✓		
O. militaris			✓	
O. pallens Linn.		✓		
Platanthera bifolia (L.) L.C.Rich.	✓			
P. chlorantha Cust. Ex. Reichb.	✓			
**Pterostylis sanguinea* D.Jones & M.A.Clem.			✓	✓
Spiranthes cernua (L.) L.C.Rich.			✓	
S. spp.	✓	✓		

Adapted from ARDITTI (1982), VAN WAES (1987), RASMUSSEN & WHIGHAM (1993, 1998), RASMUSSEN (1995), VAN DER KINDEREN (1995), BATTY *ET AL.* (2000) & VUJANOVIC *ET AL.* (2000).

RASMUSSEN (1995) and BATTY *ET AL.* (2000) reported that seed dormancy, although common in many other families (TAYLORSON & HENDRICKS, 1977; ROLSTON, 1978; BEWLEY & BLACK, 1994), is unexpected in the Orchidaceae since mycotrophy should favor autumn germination when the abundance of dead biomass stimulates increased fungal activity in the soil. However, environmental conditions during this period are often unfavorable for germination and growth. Despite mycotrophy, increasing evidence exists for the presence of dormancy mechanisms in temperate orchid seeds. BASKIN & BASKIN (1998) and VUJANOVIC *ET AL.* (2000) suggest that several types of dormancy exist in terrestrial orchid

seed based on their difficult germination and variable requirements *in vitro* (ARDITTI, 1979, 1982; RASMUSSEN, 1995) and their specific requirements for nutrients (ARDITTI & ERNST, 1984), environmental stimuli (OLIVA & ARDITTI, 1984; VAN WAES & DEBERGH, 1986a) and fungal association (ARDITTI, 1982; PRITCHARD, 1984; SMRECIU & CURRAH, 1989; ARDITTI *ET AL.*, 1990; RASMUSSEN, 1995; MIYOSHII & MII, 1998).

Evidence suggests that impermeability to water is commonly the physical dormancy that must be overcome if germination is to occur in terrestrial orchids (VAN WAES & DEBERGH, 1986a; RASMUSSEN, 1995). Additionally, KANO (1968), ARDITTI *ET AL.* (1981) and RASMUSSEN (1992, 1995) reported the possible occurrence of physiological (cold stratification) and morphological (after-ripening) secondary dormancy mechanisms, one or both of which may act in conjunction with water deprivation.

Water impermeability characterizes coat-imposed dormancy in non-orchid seeds (ROLSTON, 1978; BEWLEY & BLACK, 1994) – yet researchers have been loath to ascribe dormancy in the minute orchid seed to the transparent and seemingly delicate testa. However, BURGEFF (1959) and ARDITTI *ET AL.* (1982a) reported that the testa of terrestrial orchid seed was difficult to wet due to the outer walls being lignified and covered by a cuticle. Additionally, PRUTSCH, SCHARDT & SCHILL (2000) demonstrated that the seeds of *Sobralia dichotoma* Ruiz & Rav. did not imbibe for several weeks despite contact with water. VAN WAES & DEBERGH (1986a, b) provided evidence that definitively correlated testa impermeability with dormancy in several European terrestrials. This was attributed to the presence of suberin in the integuments, based on a previous report by HARVAIS (1980).

Testa cells are derived from the outer integument and are dead in mature orchid seeds (KURZWEIL, 1993; MOLVRAY, 2002). They contain a wide range of polyphenolic deposits, including lignins, suberins and tannins (HARVAIS, 1980; VAN WAES & DEBERGH, 1986a; VAN DER KINDEREN, 1987). Phenols and their

derivatives, which are characterized by a benzene ring displaying a hydroxyl substitution (GOODWIN & MERCER, 1983; WATERMAN & MOLE, 1994), are hydrophobic (SWAIN & GOLDSTEIN, 1964; HARBORNE, 1982). Additionally, phenols augment oxygen deprivation of embryos (WATERMAN & MOLE, 1994). Colouring of mature seed is often due to phenolic quinones, where a darker testa implicates phenol deposition (HARBORNE, 1982, 1989). Ontogenetic studies have revealed that water impermeability and colour development occur late in the development of seeds (ROLSTON, 1978) – primarily during desiccation. A relationship between seed colour and water impermeability was reported by GUTTERMAN & HEYDECKER (1973). However, comparable studies on orchids are lacking. The variously brown or black, sclerous testae typically reported for terrestrial orchids (WITHNER, 1959, 1974; ARDITTI *ET AL.*, 1979, 1980; RASMUSSEN, 1995; ARDITTI & GHANI, 2000; MOLVRAY, 2002) – including members of *Disa* (KURZWEIL, 1993, 2000; MICHEL, 2002) – are assumed to stem from phenolic deposition. VAN WAES & DEBERGH (1986a) and RASMUSSEN (1995) proposed that the varying levels of phenolic compounds within the orchid testa represent adaptation to unpredictable environmental conditions. Similarly, the increased occurrence of intensely coloured sclerous testae in terrestrial rather than epiphytic orchid species (STOUTAMIRE, 1974; ARDITTI *ET AL.*, 1979, 1980; HEALEY *ET AL.*, 1980; RASMUSSEN, 1995; ARDITTI & GHANI, 2000) makes ecological sense where phenol-rich seed coats represent a barrier to germination (BEWLEY & BLACK, 1994). The correlation between phenolic content and dormancy in mature orchid seeds has been alluded to by several authors (SINGH, 1981; SHOUSHTARI *ET AL.*, 1994) and the commonplace practice of 'green-podding' (ARDITTI, 1982; FAST, 1982; PRITCHARD, 1989; RASMUSSEN, 1995) suggests that seeds of certain species become increasingly dormant as they mature (ARDITTI *ET AL.* 1981; RASMUSSEN *ET AL.*, 1991). The theory surrounding phenolic chemistry supports the link between testa composition and germinability, but this has never been quantified in the Orchidaceae. Additionally, VEYRET (1969) demonstrated that germinability between species was negatively correlated to the thickness of the testa.

The cells of the inner integument are almost completely resorbed during orchid seed maturation, leaving only a persistent carapace (VEYRET, 1969; ARDITTI & GHANI, 2000, MORRIS, TIEU & DIXON, 2000). This sheaths the embryo and is interrupted only at the micropyle. VEYRET (1969) and FAST (1982) noted that representatives from orchid genera with well-developed carapaces germinated with difficulty, whilst in some easily germinated species the carapace is incomplete. Additionally, LUCKE (1981) reported the lack of a carapace in germinable tropical orchid seed. These data suggest that the carapace is an additional barrier against imbibition. STOUTAMIRE (1974), PRITCHARD (1984) and MIYOSHI & MII (1987) demonstrated increased germinability as a consequence of an interrupted carapace. Impermeable orchid carapaces are stratified, comprising both hydrophobic and hydrophilic strata that may play a role in the retention of viability through seed de- and re-hydration (PRUTSCH *ET AL.*, 2000).

Testa-imposed dormancy also includes chemical inhibition and mechanical restriction (BEWLEY & BLACK, 1994; MORRIS *ET AL.*, 2000). The former has been reported for orchids by VAN DER KINDEREN (1987) and VAN WAES (1987), who observed the inhibitory effect of abscisic acid (ABA) on European terrestrials. Phenolics are also documented inhibitors (SWAIN & GOLDSTEIN, 1964; MUELLER & GREENE, 1978; GOODWIN & MERCER, 1983; BEWLEY & BLACK, 1994). Mechanical restriction is not suspected for orchid seed, since complete removal of the testa is typically not needed to induce germination – rather, increased permeability to water and / or oxygen allows development to proceed.

Various mechanical and chemical treatments of the testa break dormancy in orchids (RASMUSSEN, 1995; BATTY *ET AL.*, 2000). Dormancy in orchids has often been overcome indirectly with caustic sterilants (HARVAIS & HADLEY, 1967; HARVAIS, 1980; LINDÉN, 1980; VAN WAES & DEBERGH, 1986a, b; RASMUSSEN, 1995; THOMPSON *ET AL.*, 2001, 2002). A positive germination response to scarification is a feature common to seeds with coat-imposed dormancy (MORRIS *ET AL.*, 2000). BURGEFF (1959) and ARDITTI (1982) have noted the advantage of soaking or using

liquid media as a germination substrate.

Apart from increasing the permeability of the testa through the leaching of phenols, it is also conceivable that soaking leaches out other inhibitory substances such as ABA (HARBORNE, 1989). Long term soaking produces an effect similar to that of removing or partially decomposing the seed testa (VAN DER KINDEREN, 1987). HARVAIS (1972) reported that germinability was promoted in *Dactylorhiza purpurella* (T. & T.A.Stephenson) Soó if decontamination was continued until seeds lost their dark colouration. VAN WAES & DEBERGH (1986a) and RASMUSSEN (1992) showed that germinability was correlated with the duration of decontamination, until an optimum is reached above which germinability declines.

In natural habitats testa decomposition is primarily related to fluctuations in temperature (HARVAIS, 1980; KOOPOWITZ & WARD, 1984; SEATON & PRITCHARD, 1990) and variable periods of soaking (THORNHILL & KOOPOWITZ, 1992). Chilling may also initiate physiological changes in the embryo (VAN DER KINDEREN, 1987). Interactions with bacteria and fungi and predation by microfauna have been proposed as secondary mechanisms that aid in the breaking of testa-imposed dormancy (VEYRET, 1969; RASMUSSEN, 1995). Orchid seed size mostly precludes mechanical scarification *ex situ*, although STOUTAMIRE (1974), PRITCHARD (1985) and MIYOSHI & MII (1988, 1998) have documented grinding, physically crushing and ultrasonic seed treatments respectively.

The requirement of lengthy soaking or scarification means that *in situ* germination is i) delayed and ii) spread over an extended period, since increments of a polymorphic seed population become permeable to water and germinate at successive intervals (WILLIAMS & ELLIOT, 1960). In one of few studies, *in situ* testa decay in *Goodyera pubescens*, *Corallorhiza odontorhiza* and *Galearis spectabilis* was spread over 6 – 12 months in the soil (RASMUSSEN & WHIGHAM, 1993). Such seeds retain viability longer than a few weeks and seeds in the soil seed-bank are

probably not imbibed (BATTY *ET AL.*, 2000).

Determining seed viability is a prerequisite for studying seed dormancy and optimizing the conditions required for germination. The only direct measure of viability was to record percentage germination *in vitro* (seed germination counts – SGC; FAST, 1982). However, SGC are time-consuming and give an indication of germinability only when performed under optimal conditions that allow all viable seeds to germinate. MICHEL (2002) noted that stored seed from the seasonally dry-growing *Disa* species revealed low percentage germinability, whilst *D. uniflora* seed was ungerminable after 60 days. Seasonally dry-growing *Disa* species are assumed to be analogous to the small-seeded species. Using germinability data to infer viability in dormant species is problematic since ideal germination conditions are unknown and comparatively few have been germinated (ERNST *ET AL.*, 1970, 1971; STOUTAMIRE, 1974; PURVES & HADLEY, 1976; ARDITTI, 1982; RASMUSSEN, 1995). HARVAIS (1980) and PRITCHARD & PRENDERGAST (1990) reported that, in the absence of a viability test for orchid seeds, germination data were meaningless.

Chemical staining was proposed as being more appropriate than SGC as a measure of seed viability (FAST, 1982). Two staining procedures involving 2,3,5 - triphenyltetrazolium chloride (TTC) and fluorescein diacetate (FDA) have been used to determine viability in orchid seed (VAN WAES & DEBERGH, 1986a; PRITCHARD, 1986; BATTY *ET AL.*, 2001b), with TTC carrying official recognition from the International Seed Testing Association (HARTMAN & KESTER, 1983). TTC is reduced to a red, insoluble compound within living protoplasts whilst FDA is hydrolyzed to yield fluorescein, both of which are accumulated within the cells of the embryo (WIDHOLM, 1972).

VAN WAES & DEBERGH (1986a) revealed that very few (< 12 %) western European terrestrial orchids responded to 'classic' TTC staining (after LÄKON, 1949), with percentage staining below 2.5 %. These data were attributed to

water-impermeable testae, which in terrestrials are typically entire or with few superficial cracks (VAN WAES & DEBERGH, 1986b; ARDITTI & GHANI, 2000; MOLVRAY, 2002). *Disa uniflora* and *D. tripetaloides* are notable exceptions (KURZWEIL, 1993). Soaking and chemical scarification pretreatments, which disrupt the testa, were therefore prescribed by VAN WAES & DEBERGH (1986a, b) and VAN WAES (1987) to increase percentage staining. Embryo staining therefore reflects the highest level of germination that can be achieved in seeds after similar pretreatment.

However, two assumptions are coupled with viability staining:

i. the maximum percentage coloured embryos, under ideal staining conditions, represents the maximum percentage viability; and
ii. the percentage viability, under ideal germination conditions, equals the percentage germination achieved.

Thus in all germination studies it is essential that the optimum pretreatment regime that gives the highest possible reaction to the stain be determined (PRITCHARD, 1986; VAN WAES & DEBERGH, 1986a; RASMUSSEN, 1995), with the ultimate goal of obtaining germination percentages that approximate the maximum percentage of viable (coloured) embryos after modified TTC staining (PRITCHARD & PRENDEGAST, 1990; VUJANOVIC *ET AL.*, 2000). LAUZER, ST-ARNAUD & BARABÉ (1994) and SHOUSHTARI *ET AL.* (1994) showed that modified TTC staining was applicable to germination studies on North American terrestrials, specifically *Cypripedium acaule*. MICHEL (2002) reported the use of modified TTC staining in tropical terrestrial species, including several *Disa* species.

Brief reviews by VOGELPOEL (1980) and ARDITTI (1982) suggest that *Disa* seed should be sown within one month of dehiscence to avoid the loss of viability. These data were limited to the large-seeded *Disa* species and were determined using SGC. THORNHILL & KOOPOWITZ (1992) concluded that *D. uniflora* was a

useful candidate for viability studies since unlike most terrestrial species, viable seeds were germinated readily and rapidly. Viability data were again inferred from SGC, and were only meaningful, as *D. uniflora* is easily germinable. The percentage germination for mature, fresh *D. uniflora* seed has been recorded as 80 % (ARDITTI, 1982), with the implication of at least 80 % viability. CROUS (1999, PERS. COMM.) reported 70 – 80 % germinability for this species, indicating specific consistency. MICHEL (2002), despite having reported the use of modified TTC staining in *Disa*, did not present viability data for mature seed.

The conservation of species and the proposed establishment of seed banks and *in vitro* propagation techniques demand information regarding germinability, dormancy and viability (SINGH, 1981; FAST, 1982; HARVAIS, 1982; PRITCHARD, 1984, 1985, 1986, 1989; VAN WAES & DEBERGH, 1986a, b; SEATON & HAILES, 1989). Few studies have addressed these issues and consequently it remains difficult to distinguish non-viable and dormant orchid seed (RASMUSSEN, 1995). No data exists on mature seed dormancy and viability for African terrestrials.

6.2 AIMS

- To document the leachable phenolic content (LPC) of mature and developing *Disa* seed.
- To elucidate testa and carapace ultra-structure for mature, small-seeded *Disa* species.
- To correlate seed type (Chapter Two), *in vitro* germination performance (Chapter Three), phytogeography and LPC for 10 South African *Disa* species.
- To establish a viability staining protocol for and to report percentage viability for the mature, small-seeded *Disa* seed.

- To examine the effect of the viability staining pretreatment on the topography of the small-seeded *Disa* testa.
- To correlate *in vitro* germination performance (Chapter Three) and percentage viability for four small-seeded South African *Disa* species.
- To define the role of the *Disa* seed testa in regulating germination.

6.3 MATERIALS AND METHODS

Leachable phenolic content (LPC) and testa permeability of mature seed of 11 *Disa* species (Table 6.2) was investigated. Total phenolic content cannot be quantified since exhaustive treatments with alcoholic solvents leave residual compounds bound to the cell wall (HARBORNE, 1982; WATERMAN & MOLE, 1994). Unless otherwise acknowledged, seed was collected from wild populations of *Disa*. Where possible fresh seed was analysed, although the variable timing of capsule dehiscence within and between species meant that for the most part, seeds experienced a maximum of three weeks in cold storage at ± 4 °C with 0 % relative humidity. Representatives of both the large (*D. uniflora* – type *sensu* KURZWEIL, 1993) and small (*Satyrium* – type *sensu* KURZWEIL, 1993) seeds were included, which form an aggregated continuum from easily germinable to intractable on modified media *in vitro* (Chapter Three; Figure 3.2; page 77).

Additionally, the LPC and testa permeability was determined for *D. cooperi*, *D. pulchra* (GI = 2; Table 6.2), *D. chrysostachya* and *D. versicolor* (GI = 3; Table 6.2) seed across four broad maturity classes (Chapter Three; page 61). Mature seed viability was assessed for these species in accordance with the recommendations of VAN WAES & DEBERGH (1986a).

Table 6.2. *Disa* species in which leachable phenolic content was assessed. Seed type is included in parentheses. A germinability index (GI), based on time taken to and percentage germination *in vitro* on modified media, is included for each species.

D. chrysostachya (small)	3	*D. stachyoides* (small)	3
D. cooperi (small)	2	*D. tripetaloides* (large)[1]	1
D. cornuta (small)	2	*D. uniflora* (large)[2]	1
D. crassicornis (small)	3	*D. versicolor* (small)	3
D. nervosa (small)	2	*D. woodii* (small)	2
D. pulchra (small)	2		

In vitro germinability (GI) indices: 1 – easily germinated with > 80 % success in less than 8 weeks; 2 – germinated with < 30 % success in a minimum of 12 weeks; 3 – intractable / ungerminated *in vitro* on modified media. [1] Plant located in the University of Natal Botanical Gardens, Pietermaritzburg Campus, South Africa. [2] Mature seed supplied by DR. L. VOGELPOEL, Cape Town, South Africa.

6.3.1 LEACHABLE PHENOLIC CONTENT AND TESTA PERMEABILITY

Phenols were extracted from seed batches (100 mg) using aqueous MeOH (H_2O : MeOH; 1:1). Seeds were extracted three times in 10 ml of the solvent at 25 °C, with each extraction lasting 24 h. Combined extracts were dried at 25 °C in Eppendorf tubes, sealed and stored at -10 ± 3 °C (WATERMAN & MOLE, 1994). Three seed lots were extracted for each species in each year of analysis.

The LPC was quantified using the FOLIN-DENIS method as outlined by SWAIN & GOLDSTEIN (1964) and WATERMAN & MOLE (1994). Stored samples were resuspended in 1 ml MeOH and mixed into 60 ml sterile dH_2O. FOLIN-DENIS reagent (5 ml) was added and mixed. After 4 min 10 ml of saturated sodium carbonate solution were added.

i) FOLIN-DENIS reagent (WATERMAN & MOLE, 1994)

Sterile dH_2O	75 ml
Sodium tungstate	10 g
Phosphomolybdic acid	2 g
Orthophosphoric acid	5 ml

The solution was refluxed for 2 h, cooled and diluted to a final volume of 100 ml with sterile dH_2O. Stored at 5 ± 2 °C.

ii) Saturated sodium carbonate solution (WATERMAN & MOLE, 1994)

Anhydrous sodium carbonate	3.5 g
Sterile dH_2O	10 ml

The solids were dissolved at 75 °C and the solution cooled overnight. The solution was seeded with sodium carbonate decahydrate to promote crystallization and filtered through glass wool. Stored at 5 ± 2 °C.

The total volume was made up to 100 ml with sterile dH_2O and mixed thoroughly. Following 30 min (colour development time) the absorbance of the reactants at 760 nm was determined using a Varian Cary WinUV 51 spectrophotometer.

Quantitative assays, such as spectrophotometry, require calibration for the interpretation of absorbance data. Since the nature of the phenols associated with *Disa* is unknown, a standard curve was not constructed. Phenolic content data were therefore calibrated according to tannic acid – a commonly used reference standard – and are represented as tannic acid equivalents. The significant linear regression ($y = 0.0722x + 0.0087$, $r^2 = 0.993$) which defines the relationship between absorbance at 760 nm and tannic acid concentration (mg L^{-1}) was adopted from WATERMAN & MOLE (1994).

Species mean absorbance and LPC data were plotted for mature seeds. Linear and exponential regressions were fitted to the data using Statistica (STATSOFT INC., 1998), where r^2 represents the proportion of variation accounted for by the equation. An r^2 value that approaches one indicates the best fit (ZAR, 1996). Species were grouped according to germinability. Assumptions of normality and variance homogeneity were tested with KOLMOGOROV -SMIRNOV and LEVENES' tests respectively. Data were shown to be non-parametric, consequently the between group differences of mean LPC were tested using a KRUSKAL-WALLIS ANOVA, where the response variable was the seed germinability index. All analyses were performed using Statistica (STATSOFT INC., 1998).

The LPC of developing seeds was plotted as a function of seed maturation for *D. chrysostachya*, *D. cooperi*, *D. pulchra* and *D. versicolor*. Similarly, linear and exponential regressions were fitted to these data.

Seed sections of representative small-seeded *Disa* species were viewed under an Olympus BH-2 light microscope. Tissue was prepared and stained with haemotoxylin and eosin *Y* (CULLING *ET AL.*, 1985; DAMJANOV, 1996) as outlined in Chapter Four (page 89). Only median longitudinal sections were used to illustrate i) the entirety and ii) the connectivity of the seed testa and the embryo carapace. These data were augmented by a TEM investigation into the ultra-structure of the testa and carapace. Seed was prepared according to Chapter Four (page 88).

EVANS' blue dye is excluded from cells by functional plasma membranes (BAKER & MOCK, 1994) and was prevented from reaching the embryo by an impermeable testa. This dye, prepared as a 1 % (w/v) aqueous solution (WIDHOLM, 1972) and stored in darkness at 10 ± 2 °C, was used to illustrate i) the site of water penetration through the testa and ii) the percentage permeability of the mature seed testa. Equal volumes (1 ml) of seed solution (seed suspended in sterile dH_2O) and stain were mixed and viewed after 1 and 24 h under an Olympus BH-2 light microscope. A minimum of 100 seeds were observed for

each of the 11 species investigated. Only complete colouration was considered when determining the number of stained seeds.

6.3.2 MODIFIED VIABILITY STAINING

In order to determine the percentage embryo staining for each species, 3 x 50 mg batches of seed were prepared by exposure to 30 ml of 1.75 % NaOCl (w/v) + 1 % Tween-20 (v/v). The use of NaOCl was reported by MICHEL (2002). After 0, 1, 2, 4, 6, 8 and 10 h a seed sample was removed from the scarifying solution and triple rinsed (5 min each) and bathed in sterile dH_2O for 24 h. Drained seed was mixed with 10 ml 1 % (w/v) aqueous TTC solution (*p*H adjusted to 7 with 1*M* NaOH and stored in darkness at 10 ± 2 °C) and sealed in small glass bottles. Staining took place in the dark at 30 ± 2 °C for 24 h.

Following staining the seeds were triple rinsed in sterile dH_2O. Two ml of the final seed solution was evenly dispersed on a Whatman No. 1 filter paper disc, which was divided into quarters. Twenty-five randomly chosen seeds per disc quarter for each of the three replicates were allocated to one of two categories based on testa and embryo colouration (after VAN WAES & DEBERGH, 1986a). Seeds were viewed under a Wild Heerbrugg M400 photomicroscope. Categories were:

i. seed with a brown to colourless testa and a white or opaque embryo; and
ii. seed with a colourless testa and a completely pink or red embryo.

The mean ± SD number of coloured embryos per disc quarter was determined according to the methods of the International Seed Testing Association for biochemical tests for viability (HARTMAN & KESTER, 1983). Data are reported as the percentage coloured embryos per seed sample per treatment.

Controls were either mature seed subjected directly to TTC (LÄKON, 1949) or seed soaked for 24 h in sterile dH_2O without prior exposure to NaOCl (exposure duration = 0 h). Optimal duration (the time elapsed to obtain maximum staining percentage) was determined for each species. EVANS' blue staining was also carried out on seed batches which were independently subjected to the pre-treatment which yielded maximum TTC embryo staining, where percentage permeability was compared to percentage coloured embryos.

The viability stain FDA was also investigated. Two ml of the rinsed seed solution was mixed with 2 ml 0.5 % (w/v) FDA in absolute acetone (stored in darkness at -10 ± 3 °C; PRITCHARD, 1986). The percentage seed viability (as revealed by green-yellow fluorescence under a Zeiss IM 35 inverted microscope equipped with an ultraviolet lighting facility) was recorded after 10 min. At least 100 seeds were observed for each treatment.

A scanning electron microscopy study was performed to investigate the influence of NaOCl pretreatment on testa topography in the small-seeded *Disa* species. Seed was prepared according to Chapter Two (page 29). Twenty-five seeds were examined for each treatment.

6.4 RESULTS AND DISCUSSION

With the exception of the large-seeded *Disa* species, the permeability of mature testae to aqueous EVANS' blue was shown to be variously restricted, with percentage permeability after 1 h ranging from only 3 % in *D. versicolor* to 10 % in *D. stachyoides* (Table 6.3 and Plate 6.1A). Alternatively, permeability of the large-seeded *Disa* testae were comparatively high after 1 h of staining, reaching a maximum of 73 % in *D. uniflora* (Table 6.3). In the small-seeded species, increased time of exposure to EVANS' blue (up to 24 h) did not increase the percentage permeability. These data corroborate the findings of BURGEFF (1959) and ARDITTI *ET AL.* (1982a) who reported difficulty in wetting

terrestrial orchid seed and recommended soaking for several weeks or months in order to achieve increased permeability to water. In contrast, the permeability of *D. uniflora* and *D. tripetaloides* seed increased with increased staining (Table 6.3), with in excess of 90 % of the former species seeds being stained after 24 h.

Table 6.3. The percentage permeability of mature seed for 11 South African *Disa* species, as visualized through staining with aqueous EVANS' blue. Germinability indices (GI) are included in parentheses and species with highly permeable seed are indicated in grey, *n* = 100.

	Duration of pretreatment			Duration of staining (h)	
	1	24		1	24
	% permeability			% permeability	
D. chrysostachya (3)	3		*D. stachyoides* (3)	10	
D. cooperi (2)	6		*D. tripetaloides* (1)	48	81
D. cornuta (2)	11		*D. uniflora* (1)	73	94
D. crassicornis (3)	5		*D. versicolor* (3)	3	
D. nervosa (2)	4		*D. woodii* (2)	6	
D. pulchra (2)	7				

EVANS' blue staining after WIDHOLM (1972).

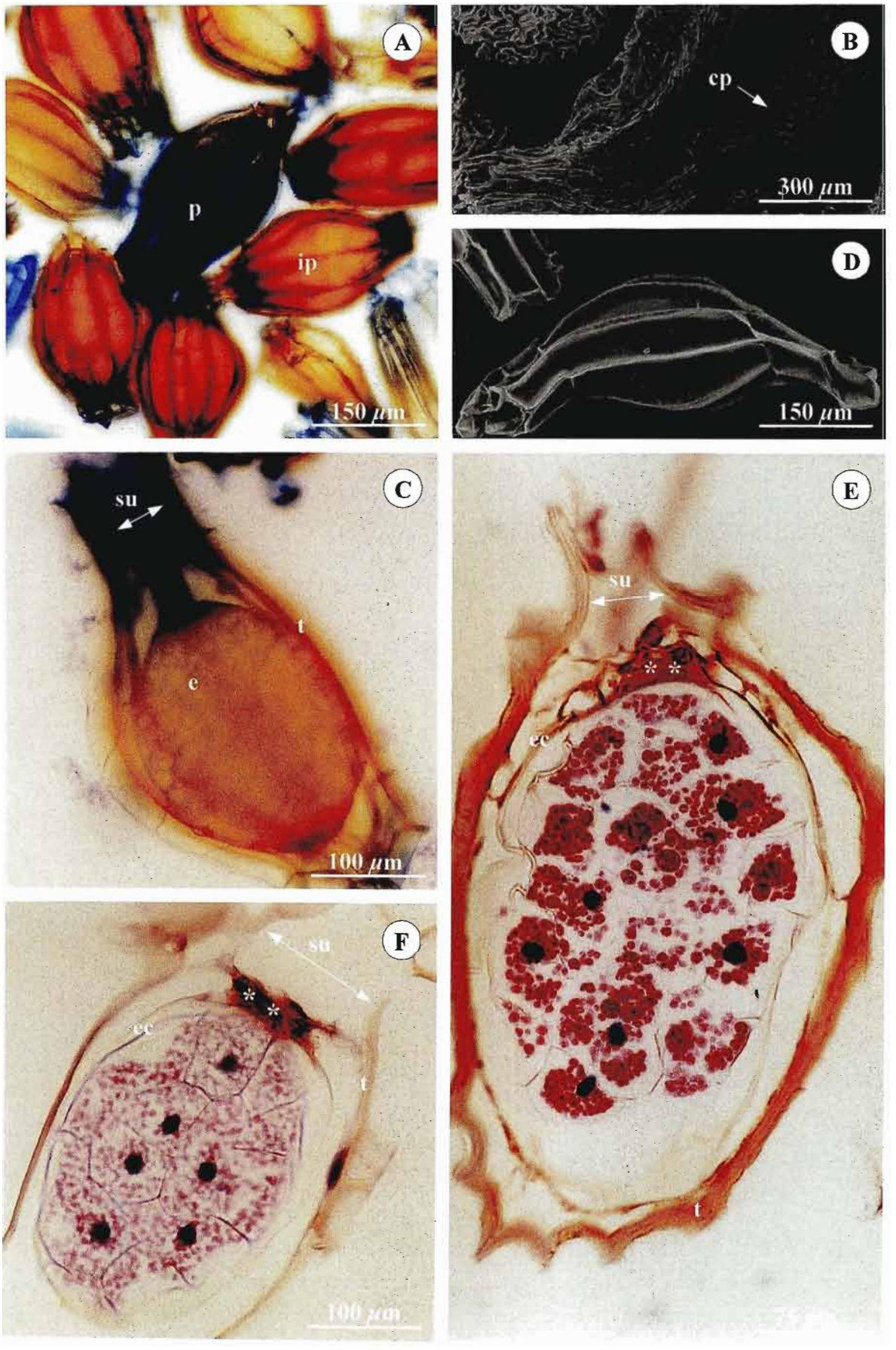
The passage of water through the testa to the embryo carapace was reported by RASMUSSEN (1995) as being via the suspensor and micropyle, but this was not observed in the large-seeded *Disa* species. In these species water saturated the seed via the cracks and perforations that disrupt the testa (Plate 6.1B). Such data supports the notion that rapid waterlogging facilitates hydrochorous dispersal in these streamside species. The unrestricted availability of water to the embryo of the large-seeded *Disa* species is in keeping with their rapid germination (GI = 1). STOUTAMIRE (1974) reported a lack of dormancy in several terrestrials, including *D. uniflora*.

Aqueous EVANS' blue entered the suspensor of most of the small-seeded

species, although this stain was prevented from reaching the sub-testa lumen occupied by the embryo (Plate 6.1C). Most seeds remained unstained. However, in the rare stained seeds the site of entry was between adjacent testa cells. Small-seeded *Disa* species were intractable *in vitro* on unmodified media, as would be expected for seeds with testa-imposed dormancy. However, the rare permeable seeds were also incapable of germination – suggesting either i) a loss of germination capacity associated with the atypical testa condition of the mature seed or ii) more than water-impermeable dormancy is operative in the small-seeded *Disa* species. Several authors (STOUTAMIRE, 1974; RASMUSSEN, 1995) reported testa permeability but no germination in several orchids, which was interpreted by BRADBEER (1988) and RASMUSSEN (1995) as intrinsic embryonic dormancy from the failure to mobilize nutrient reserves. This study revealed ubiquitous storage of lipids and proteins in small-seeded *Disa* species. In addition, MANNING & VAN STADEN (1987) revealed that *D. polygonoides* lacked the ability to mobilize such reserves. These data imply endogenous dormancy, which may act in concert with an impermeable testa.

Impermeability in the small-seeded *Disa* species was expected as a consequence of testa integrity (Plate 6.1D). In addition, TEM revealed the testa cell walls of *D. cooperi* and *D. chrysostachya* as comprising a series of convoluted layers surrounded by heavily stained deposits (Plate 6.2A, B). Similar deposits, in *Sobralia dichotoma*, were interpreted by PRUTSCH *ET AL.* (2000) as hydrophobic phenols. In addition, embryos of the small-seeded *Disa* species were surrounded by entire, stratified carapaces, which were separated from the testae by air-filled interstices (Plate 6.2C, D). Transmission electron microscopy revealed a thin, outer hydrophobic layer to the carapace that was subtended by two to three layers of dead, collapsed cells. These were proposed by PRUTSCH *ET AL.* (2000) as a water sink due to the relatively high matric potential of pectin in the cell walls.

Plate 6.1. Permeability of mature *Disa* seed testa to aqueous EVANS' blue dye. Typically, < 10 % of the seed of small-seeded species were stained at dehiscence (A), with impermeable testae excluding the stain. Permeable (p) and impermeable seed (ip) of *D. crassicornis* are illustrated. Dye entered the suspensor (su), but did not reach the sub-testa lumen surrounding the embryo (e; C). Impermeably of these seeds was expected as a consequence of testa integrity (D). In excess of 45 % of large seeds stained, attributable to the porous nature of their testae (B). In these species circular perforations (cp) disrupted testa integrity. Light microscopy confirmed the integrity of the testa and the embryo carapace (ec) in *D. cooperi* (E) and *D. cornuta* (F), two-small seeded species. Elaborate extensions (*) of the carapace were identified, which linked the testa (t) and the carapace and served to obstruct the suspensor in the small-seeded *Disa* species.


A
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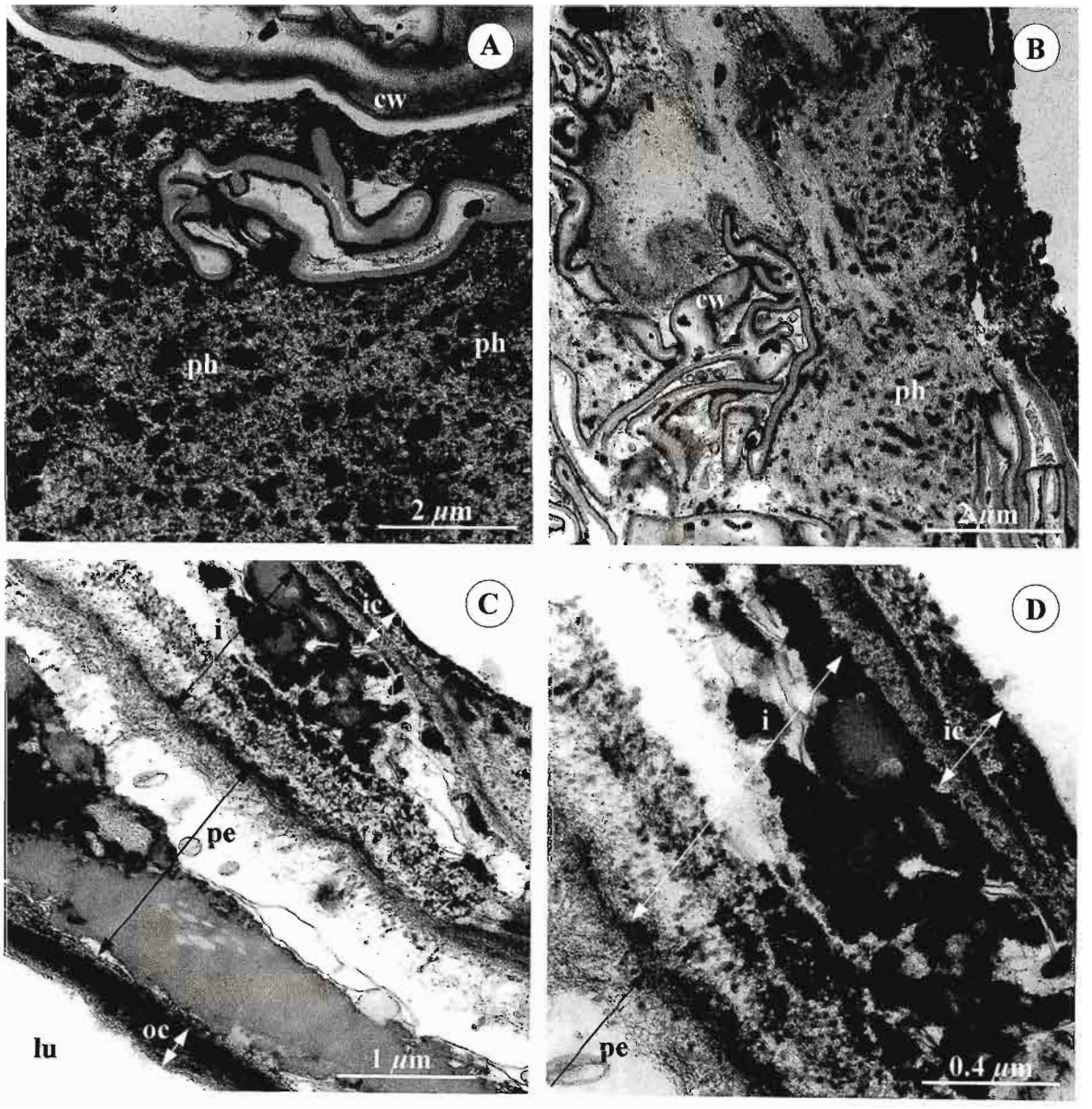
The innermost layer of the *Disa* carapace formed a broad, hydrophobic cuticle. A partial interstice with hydrophilic deposits was visible under TEM between the inner carapace envelope and the layer of crushed cells to the outside. A similar carapace documented for *S. dichotoma* was interpreted as crucial in regulating embryo hydration (PRUTSCH *ET AL.*, 2000) by ensuring sufficient desiccation to permit embryos to enter into dormancy, whilst retaining a film of water within the carapace to protect the embryo against fatal osmotic stresses during de- and re-hydration.

Difficulties experienced in sectioning seed of *D. uniflora* and *D. tripetaloides* meant that the ultra-structure of the carapace, if present, was not illustrated for these large-seeded species. However, their rapid saturation and apparent lack of dormancy makes the presence of an entire carapace unlikely.

Light microscopy confirmed the integrity of both the testa and the carapace in the small-seeded *Disa* species (Plate 6.1E, F). These observations corroborate data which suggested a correlation between carapace integrity and difficult germination (FAST, 1982). Elaborate extensions of the carapace were identified in this study, at the micropylar and less commonly at the chalazal pole (Plate 6.1E, F). This was without specific affiliation. The comparatively large masses, derived from the inner integument, linked the two integuments and served to obstruct the suspensor in the small-seeded *Disa* species. Alternatively, CARLSON (1940) and VEYRET (1969) reported only a few connective strands between testa and carapace in mature orchid seed. On the basis of colouration, which was similar to that of the testa, it was assumed that these obstructions were rich in hydrophobic phenols.

Plate 6.2. Testa and carapace ultra-structure for ***D. cooperi*** (A, C) and ***D. chrysostachya*** (B, D); two small-seeded, summer-rainfall species. Cell walls (cw) comprised convoluted layers surrounded by vast deposits of heavily stained, hydrophobic phenols (ph). The embryo carapace was stratified and separated from the testa by an air-filled lumen (lu). The outer, hydrophobic cuticle (oc) was subtended by two to three layers of collapsed cells, assumed to be pectin rich (pe). The inner cuticle (ic) was surrounded to the outside by a partial interstice (i) with hydrophilic deposits. These deposits, together with the high matric potential of pectin, ensure that a film of water persists within the carapace throughout dormancy and during embryo de- and re-hydration.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The mature seed of all *Disa* species revealed leachable phenolics, expressed as mg L^{-1} tannic acid equivalents (Figure 6.1 and Plate 6.3A). The data range (0.308 – 6.943 mg L^{-1} equivalents) was expected given the variable colour of the mature seed across the species investigated (Plate 6.3B), providing quantitative support of the link between testa colouration and phenolic content. Tan seeds occurred only in species with low LPC, such as *D. uniflora* and *D. tripetaloides*. Alternatively, intensely brown seeds were reported for *D. versicolor* – with an LPC of approximately 7 mg L^{-1} equivalents. Phenolics impart testa impermeability (GUTTERMAN & HEYDECKER, 1973; ROLSTON, 1978; BEWLEY & BLACK, 1994) and are a definitive feature of water-impermeable seed dormancy in non-orchids.

The inclusion of germinability rank data (Figure 6.1) – based on a combination of germination rate and total percentage germination, revealed a significant linear regression ($y = 0.6231x - 1.2249$, $r^2 = 0.895$) between LPC and the ability of a species to germinate *in vitro* on modified media. This regression confirms the relationship between phenolic content and germinability that was hinted at by SINGH (1981), VAN WAES & DEBERGH (1986a) and SHOUSHTARI *ET AL.* (1994).

The benefit of defining this relationship is that levels of dormancy and germinability can be predicted based on visual assessment of a seeds' external topography and colouration. Exponential regression of the same data was not significant ($y = 0.2633e^{0.3042x}$, $r^2 = 0.7471$). The juxtaposition of species germinability indices with phenolic content (Figure 6.1) revealed the easily germinable, large-seeded *Disa* species (GI = 1) as collectively having a LPC below 1 mg L^{-1} equivalents. The LPC of germinable, small-seeded species (GI = 2) was between 1 – 2.5 mg L^{-1} equivalents. The germination of these species *in vitro*, albeit at levels below 30 %, nullifies the notion that endogenous dormancy resides within the embryos of at least some seeds of these species. However, FAST (1982) reported consistency within species with regard to their germination

response – making the occurrence of endogenous dormancy unlikely in these *Disa* species. These data are in keeping with the findings of MORRIS *ET AL.* (2000), who concluded that further stimuli, over imbibition, were not needed to induce germination in dormant, water-impermeable seeds. By comparison the small-seeded, intractable species (GI = 3) varied in their phenolic content from 2.6 to almost 7 mg L^{-1} equivalents.

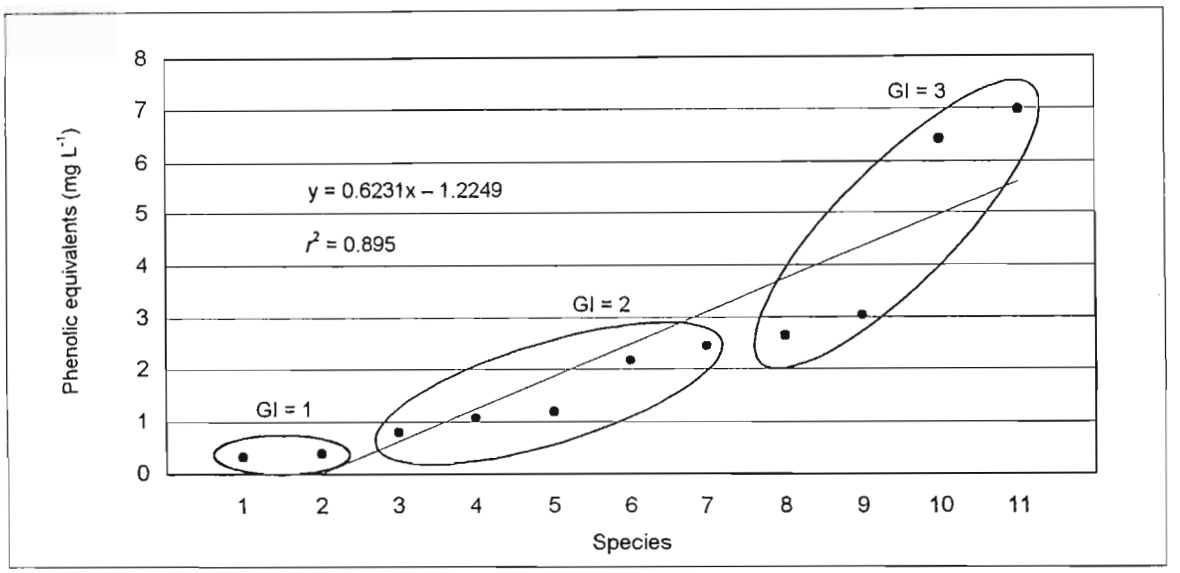
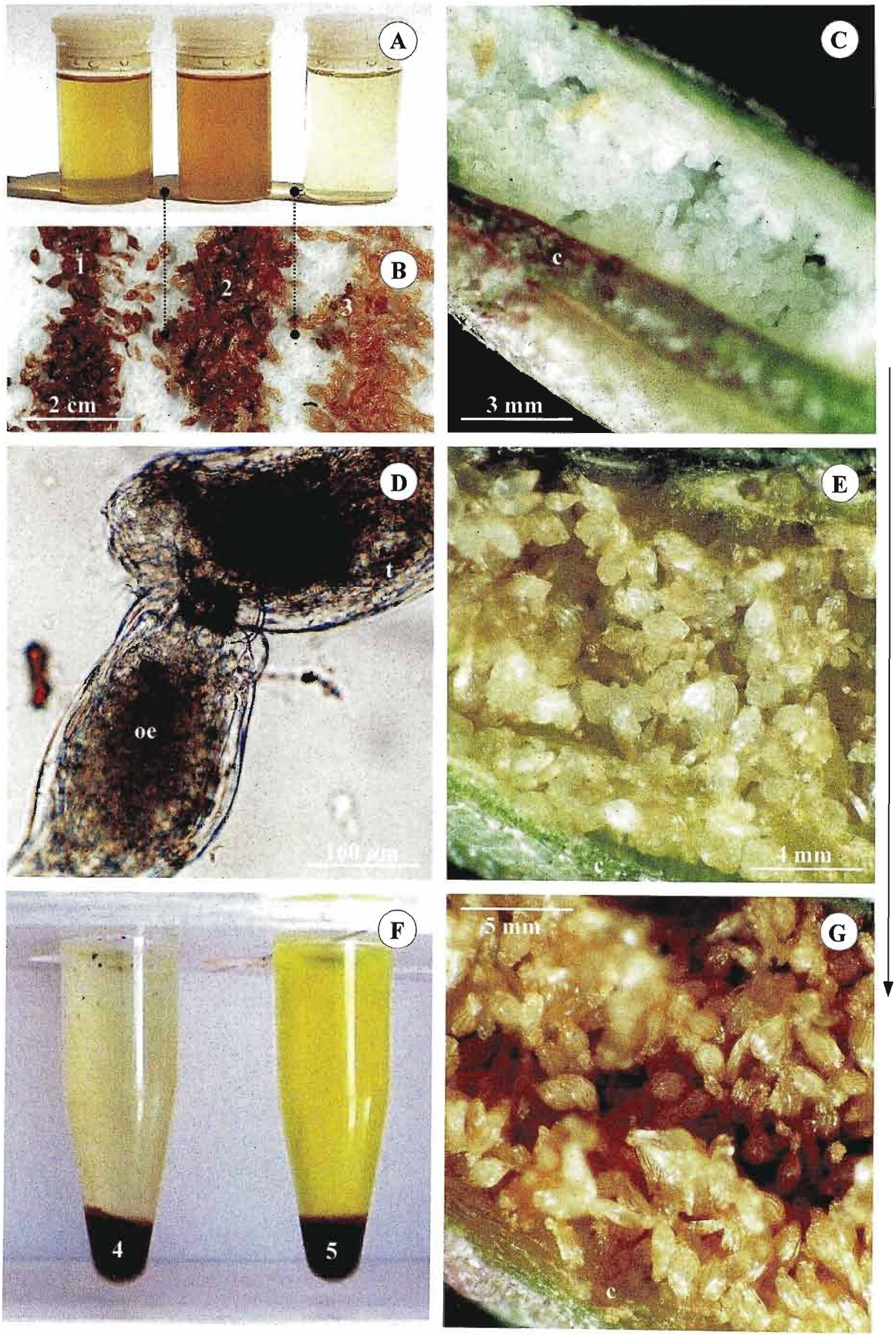


Figure 6.1. The relationship between seed phenolic content and the *in vitro* germinability of 11 *Disa* species on unmodified media, as defined by a significant linear regression. Germinability indices (GI) are included and species ranked according to the combination of their germination rate and total percentage germination *in vitro*: 1 – *D. uniflora*; 2 – *D. tripetaloides*; 3 – *D. cornuta*; 4 – *D. pulchra*; 5 – *D. woodii*; 6 – *D. cooperi*; 7 – *D. nervosa*; 8 – *D. stachyoides*; 9 – *D. chrysostachya*; 10 – *D. crassicornis*; 11 – *D. versicolor*. A KRUSKAL-WALLIS ANOVA revealed highly significant differences between the mean phenolic content of the groups established according to germinability indices ($H_{2, 66} = 52.23$, $P < 0.001$).

A KRUSKAL-WALLIS ANOVA revealed highly significant differences between the mean phenolic content of the groups established according to seed germinability indices ($H_{2, 66} = 52.23$, $P < 0.001$). Consequently, the large-seeded *Disa* species were associated with comparatively low levels of phenolic deposition within the seed, inferring negligible impermeability and uninhibited seed imbibition.

Plate 6.3. Testa phenolics of mature and developing seeds, and embryo viability in summer-rainfall *Disa* species. Specific levels of phenolics, expressed as mg L^{-1} tannic acid equivalents, were observed after seed leaching in aqueous MeOH (20 mm ø glass pill vials; A). These data were expected due to the variable colour of the seed across the species investigated (B); *D. chrysostachya* (1); *D. nervosa* (2) and *D. pulchra* (3). Phenolic deposition increased exponentially with increasing seed maturity in *D. cooperi* and was related to decreasing testa permeability and an increase in testa colouration (indicated). Two weeks after pollination (C), four weeks after pollination (E) and six weeks after pollination (G). Dehiscence of the capsule (c) took place eight weeks after pollination. Four-week-old seeds were presumably germinable as they contained opaque embryos (oe) and translucent testae (t; D). Embryo staining with 0.5 % FDA was unsuccessful in this study, with false positive results being obtained (F). The combination of FDA and NaOCl produced a yellow-green solution under VIS that masked embryo fluorescence under UV. Increased colour intensity stemmed from increased pre-treatment duration, despite a 24 h sterile dH_2O rinse; 2 h (4) and 6 h (5) exposure to NaOCl.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5 mm
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Large-seededness is restricted to five closely related streamside species (KURZWEIL, 1993; LINDER & KURZWEIL, 1999). However, only *D. uniflora* and *D. tripetaloides* possess an interrupted testa. A permeable testa as a result of limited phenol deposition facilitates rapid seed saturation and germination in the remainder of this sub-clade (Appendix One).

Since small, mid-brown seeds with entire testae represent the primitive condition in *Disa* (KURZWEIL, 1993), it can be assumed that the ancestral *Disa* species exhibited moderate water-impermeable dormancy. Phylogenetic releases from this dormancy through decreased phenol deposition, or alternatively increased phenolic deposition to deepen dormancy, are therefore both derived conditions. Not surprisingly, *Disa* seed colour was a convergent character; being distributed without phylogenetic constraint across all sections examined (Chapter Two).

The broad relationship between testa permeability and phenolic content was confirmed through the assessment of these parameters in the developing seeds of four representative small-seeded species. Phenolic deposition increased exponentially with increasing maturity in these seeds (Figure 6.2A), being defined by the regression equation $y = 0.0667e^{0.8989x}$ ($r^2 = 0.8081$). Linear regression of the same data was not significant ($y = 1.0078x - 1.2875$, $r^2 = 0.4335$). Alternatively, testa permeability decreased with increased seed age (Figure 6.2B). First developmental quarter seeds (two weeks after pollination) of all species were 100 % permeable and revealed negligible phenol accumulation (≤ 0.3 mg L^{-1}; Plate 6.3C). Seeds of the second developmental quarter (four weeks after pollination) were between 79 – 94 % permeable to aqueous EVANS' blue, depending on the species. The same seeds contained well below 1 mg L^{-1} equivalent phenols (Plate 6.3E). The exponential nature of the regression indicates that the bulk of phenol deposition occurred during the third (Plate 6.3G), but primarily the fourth developmental quarter. During this four-week period testa permeability became highly reduced, being less than 8 % in mature seeds.

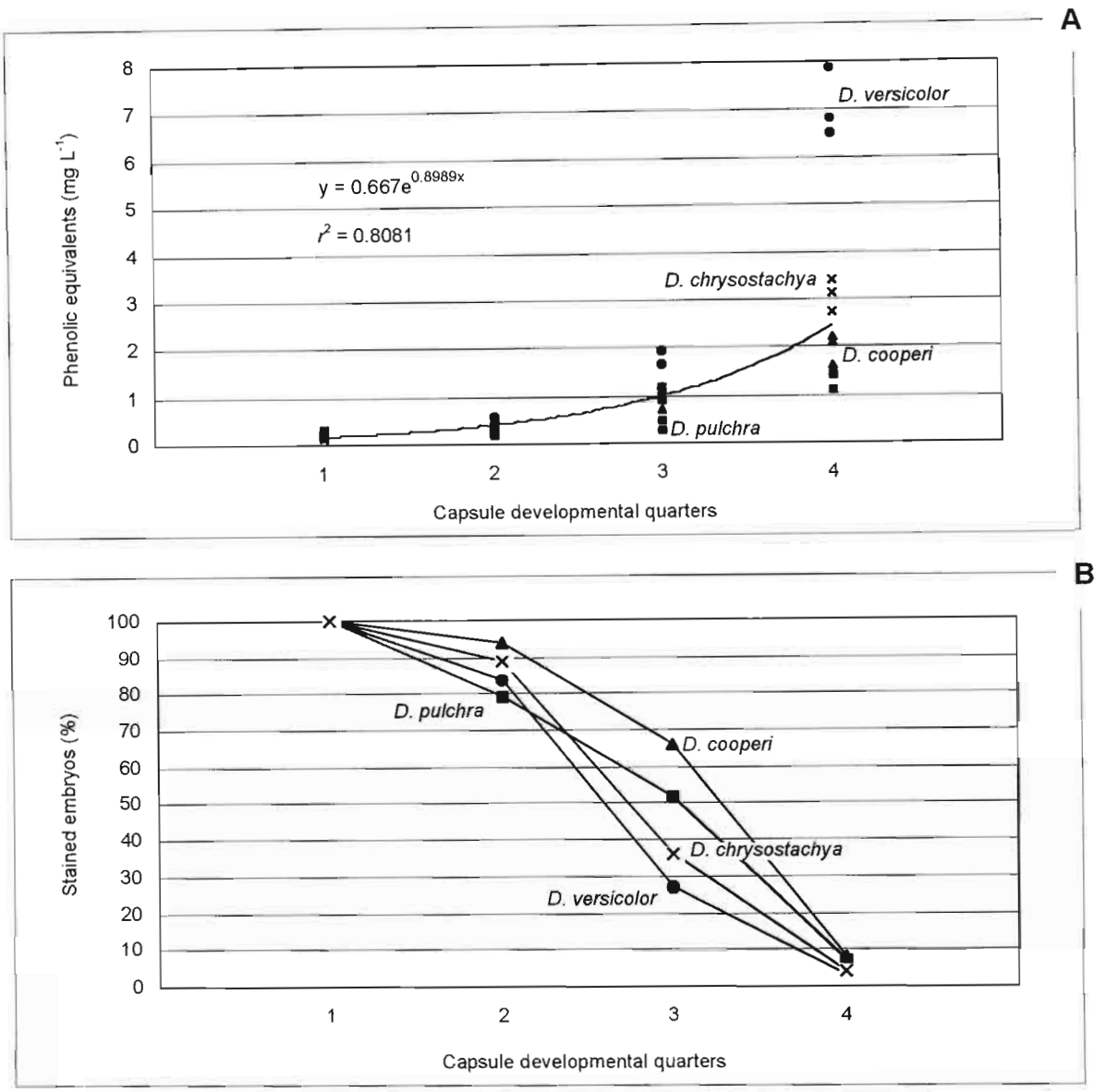


Figure 6.2. Seed phenolic content (A) **and the percentage testa permeability to aqueous EVANS' blue** (B) **as a function of seed maturity in four small-seeded *Disa* species. Phenolic deposition over time occurred exponentially and a significant regression equation defined this relationship. *Disa cooperi* and *D. pulchra* were germinated *in vitro* on modified media (GI = 2). *Disa chrysostachya* and *D. versicolor* remain intractable (GI = 3). For EVANS' blue staining *n* = 100 for each species. Seed originated from four capsule developmental quarters: first quarter – two weeks after pollination; second quarter – four weeks after pollination; third quarter – six weeks after pollination and fourth quarter - dehiscent capsules, eight weeks after pollination.**

Seeds originating from the third quarter therefore displayed a comparatively low phenolic content and remained variously permeable. Germinable species (*D. cooperi* and *D. pulchra*) revealed that 66 % and 52 % of their seeds were permeable two weeks before dehiscence respectively, whilst intractable species (*D. chrysostachya* and *D. versicolor*) were only 36 % and 27 % permeable.

These data emphasize the potential of 'green-pod' germination in *Disa*. 'Green-podding' times for *D. uniflora* and *D. tripetaloides* were approximately 6 weeks after pollination (MICHEL, 2002), which corresponds to the third developmental quarter defined in this study. The success of third quarter immature seed culture on unmodified media (to a maximum of 28 %) was comparable to charcoal supplementation or media with decreased viscosity or reduced inorganic salts (to a maximum of 30 %). 'Green-podding' is reliant on immature seeds with permeable testae having embryos that are capable of germination. Although the viability of immature embryos was not addressed here, light microcopy revealed the presence of opaque embryos midway through the eight-week maturation period (Plate 6.3D). Whilst permeability was negatively correlated to seed maturity, maximum percentage germination was recorded for seeds originating from the third developmental quarter – indicating that embryos younger than four weeks were incapable of germination. *Disa* species that displayed moderate levels of dormancy commonly occur in marshes or seepage areas. In contrast, small-seeded species with phenols in excess of 2.5 mg L^{-1} equivalents, remained intractable (GI = 3), despite media and method manipulation. These species typically occur in seasonal, high altitude grasslands with severe winter conditions.

For *D. cooperi* and *D. pulchra* (GI = 2), germination percentages *in vitro* never exceeded 30 %. However, such data are meaningless in the absence of embryo viability data, which reflects the germination potential (GP) of a seed population. The embryos of the small-seeded *Disa* species were not coloured by

TTC staining (0 h exposure to NaOCl; Figure 6.3). These data are comparable to European terrestrials (VAN WAES & DEBERGH, 1986a, b), with the lack of embryo colouration being attributed to testae impermeability. In addition, TTC staining confirmed that the seed of *D. chrysostachya, D. cooperi, D. pulchra* and *D. versicolor* that was permeable at the time of dispersal was not viable. Soaking seeds for 24 h in sterile dH_2O without chemical scarification increased the percentage embryo colouration for *D. cooperi* to 4 %. The percentage staining in the remainder of the species following similar treatment remained negligible (below 0.5 %; Figure 6.3). Modified TTC staining revealed viability data for these representative small-seeded South African *Disa* species, with the duration of the pretreatment in NaOCl + Tween-20 having an important influence over the percentage coloured embryos (Figure 6.3).

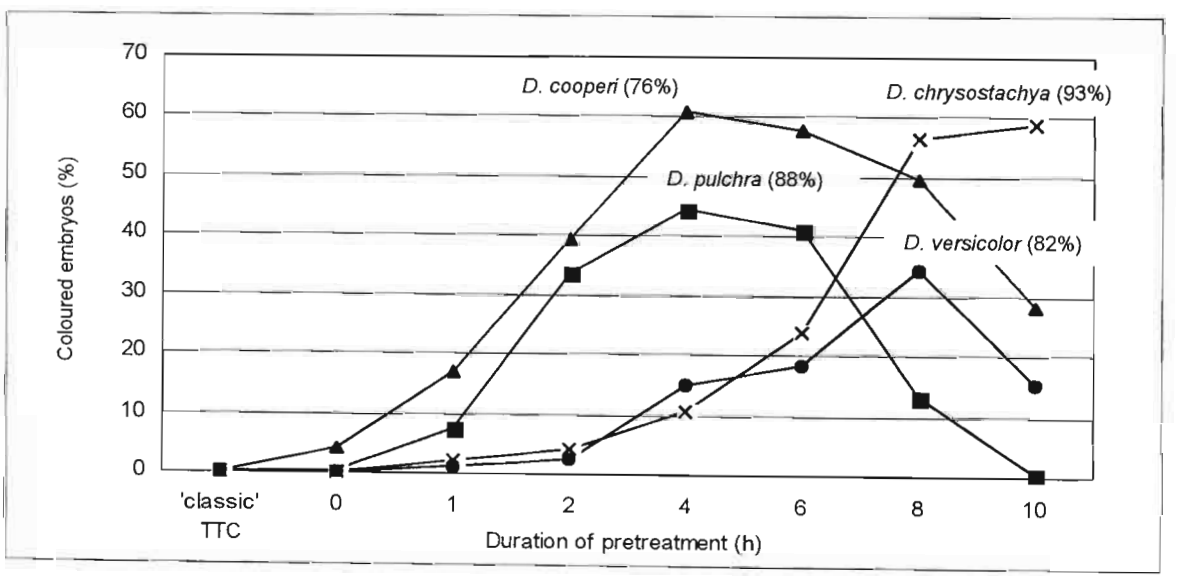


Figure 6.3. Relation between coloured embryos (TTC) and the duration of pretreatment with 1.75 % NaOCl (w/v) + 1 % Tween-20 (v/v) in four small-seeded *Disa* species. Controls were either mature seed subjected directly to TTC ('classic' TTC) or seed soaked for 24 h in sterile dH_2O without prior exposure to NaOCl (exposure duration = 0 h). Each value is the mean of three replicates with 100 seeds in each. Values reflecting the percentage permeability of the testa to aqueous EVANS' blue under optimal pretreatment conditions for each species are included in parentheses, *n* = 100.

After a pretreatment of 1 h in the scarifying solution, all four species revealed coloured embryos, ranging from 1 % in *D. versicolor* to 17 % in *D. cooperi*. Relatively short-term scarification permitted limited testae permeability in *Disa*. Other authors have reported that seed decontamination protocols promote germination (HARVAIS & HADLEY, 1967; HARVAIS, 1980; LINDÉN, 1980; VAN WAES & DEBERGH, 1986a, b; RASMUSSEN, 1995). The optimal duration for embryo staining in the small-seeded *Disa* species (Table 6.4) was considerably shorter than the minimal time taken to achieve seed decontamination (Chapter Three).

Table 6.4. Optimal pretreatment in 1.75 % NaOCl + 1 % Tween-20 (v/v) and the mean ± SD percentage of coloured embryos for the mature seed of four small-seeded *Disa* species after modified TTC staining. Percentage permeability after staining with aqueous EVANS' blue. Germinability indices (GI) are included in parentheses.

	Optimal duration of pretreatment (h)	Mean ± SD percentage of coloured embryos (n = 300)	Percentage permeability (n = 100)
D. chrysostachya (3)	10*	58.67 ± 6.89	93
D. cooperi (2)	4	60.67 ± 9.16	76
D. pulchra (2)	4	44.52 ± 10.35	88
D. versicolor (3)	8	34.33 ± 6.71	82

Modified TTC staining after VAN WAES & DEBERGH (1986a), EVANS' blue staining after WIDHOLM (1972). * pretreatment duration did not exceed 10 h.

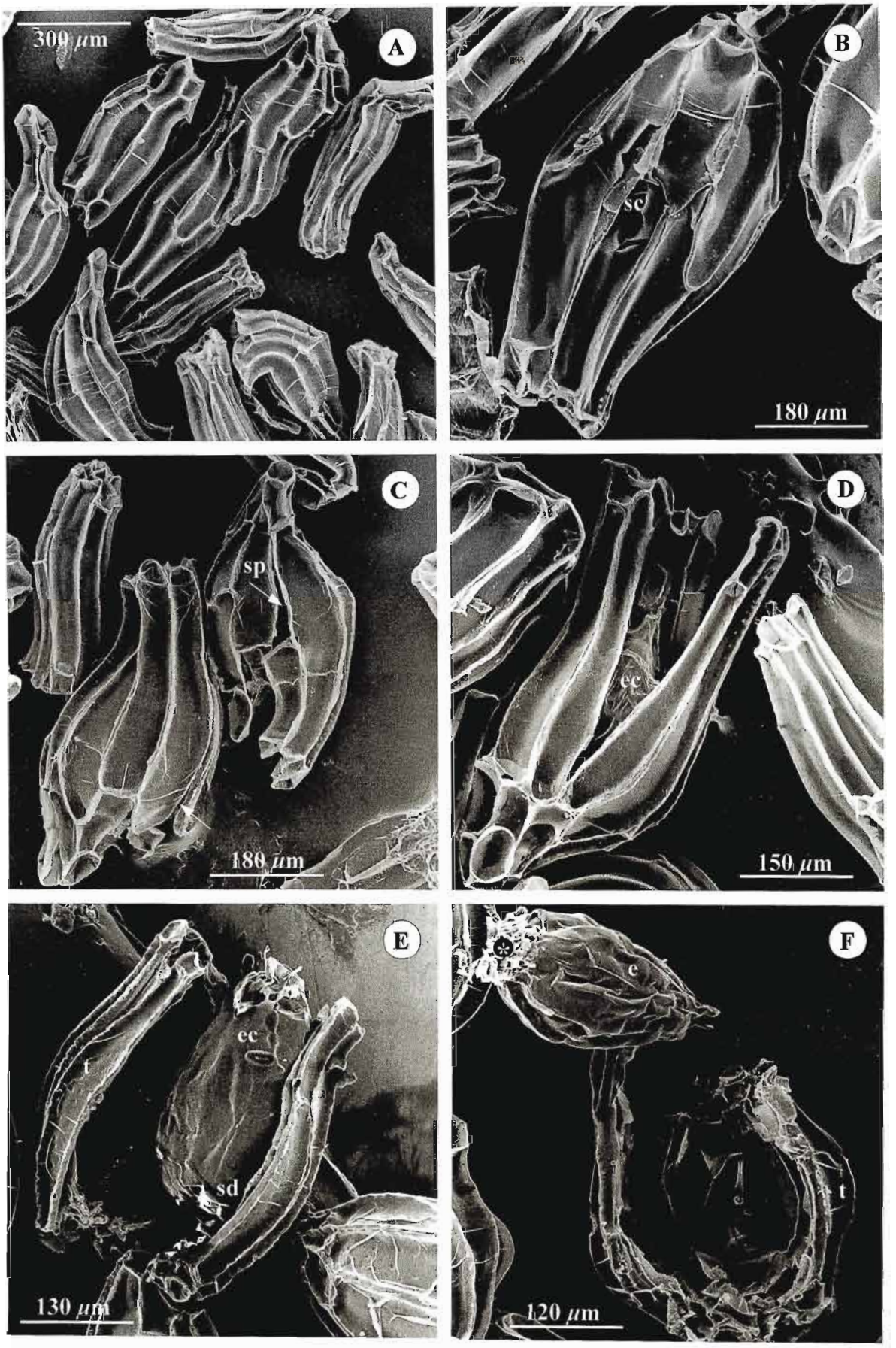
The optimal duration of the pretreatment with 1.75 % NaOCl + 1 % Tween-20, together with percentage coloured embryos, were different for the four *Disa* species investigated (Figure 6.3 and Table 6.4). In *D. chrysostachya* maximum embryo staining occurred after 10 h, the longest pretreatment investigated. These data show that the duration of the pretreatment correlated positively with the leachable phenolic content of the seed, with seeds containing higher levels of phenols (*D. chrysostachya* and *D. versicolor*; GI = 3) requiring increasingly lengthy scarification (Plate 6.4) in order to visualize their GP.

The testae of untreated seeds of *D. pulchra* were smooth and entire and the cells remained fused (Plate 6.4A). After 1 h surface cracks developed that penetrated into and between the testa cells (Plate 6.4B). Corrosion continued with the testae being disrupted after pretreatments of 2 and 4 h respectively – testa cells became separated along their anticlinal walls (Plate 6.4C, D). After 4 h, cell separation exposed the embryo carapace and produced optimum embryo colouration (Table 6.4). Continued exposure (6 – 8 h) revealed increased surface damage to the embryo carapace (Plate 6.4E). During the same period a reduction in the percentage coloured embryos in *D. chrysostachya*, *D. cooperi* and *D. pulchra* was noted – attributed to embryo damage and toxicity (Figure 6.3). After 10 h *D. pulchra* testae were completely degraded and embryos disjunct from the testae remnants (Plate 6.4F), with the percentage coloured embryos recorded being 0 % (Figure 6.3).

The optimal duration for scarification, as reflected by the highest percentage coloured embryos, followed a normal distribution for all species (Figure 6.3). Normality here suggested that seeds from each species may be variously impermeable at dehiscence since they become permeable incrementally. *In situ* germination in the small-seeded *Disa* species is therefore not only delayed, but spread over an extended period since increments of the seed population become permeable to water and germinate sequentially. Such differences in permeability would be dictated by differences in testa colouration or entirety. However, visibly polymorphic seed was only observed for *D. versicolor*. VEYRET (1969) demonstrated that small differences in testa thickness influenced the optimal duration of pretreatment, especially where the duration was relatively short (< 1 h).

In all species investigated, the percentage coloured embryos was lower than the percentage staining by EVANS' blue after similar pretreatment (Figure 6.3 and Table 6.4) – indicating that not all seeds rendered permeable through chemical scarification are viable.

Plate 6.4. The corrosive effect of prolonged 1.75 % NaOCl (decontaminating solution) exposure on *D. pulchra* seed integrity. Modified TTC staining after VAN WAES & DEBERGH (1986a, b). 0 h exposure – testae intact with few or no cracks, cells remained fused, percentage coloured (viable) embryos 0 % (A); 1 h – surface cracks (sc) developed and penetrated into and between testa cells, 7.3 % (B); 2 h – testa integrity disrupted, cells split (sp) along anticlinal walls, 33.6 % (C); 4 h - increased cell separation, embryo carapace (ec) exposed, resulting in maximum viability staining, 44.5 % (D); 6 – 8 h – continued exposure to NaOCl revealed increased surface damage (sd) to the carapace and a corresponding decrease in embryo viability, 41 and 13.3 % respectively (E); 10 h - testae (t) were completely degraded and embryos (e) disjunct from testae remains. The suspensor plug (*) – a derivative of the carapace, is highlighted, 0 % (F).


300 µm
A
B
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180 µm
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ec
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120 µm

Embryo fatality before optimal pretreatment duration was predicted for species with polymorphic seeds and this may contribute to the large disparity between percentage permeability (82 %) and percentage viability (36 %) for seed populations of *D. versicolor*. In addition, the percentage germinability for *D. cooperi* and *D. pulchra* was substantially lower than the percentage of stained embryos reported for these species. These data contrast with LAUZER *ET AL.* (1994) and SHOUSHTARI *ET AL.* (1994), who reported that germinability and viability correlated well in selected species. Low germination, despite pretreatment, was explained by the presence of an unidentified germination inhibitor (HADLEY, 1982).

The efficacy of TTC staining in mature orchid seeds is therefore questionable, specifically after chemical exposure. ST-ARNAUD, LAUZER & BARABÉ (1992) and VUJANOVIC *ET AL.* (2000) reported inconsistent staining in species from several Holarctic genera, attributed to variation in seed testa permeability which varied from year to year and between populations of the same species. Consequently VUJANOVIC *ET AL.* (2000) presented preliminary data on the biological assessment of seed viability, being reliant on the ability of mycorrhizal *Fusarium* Link ex Fr. to induce red embryo colouration prior to germination. Alternative embryo staining with FDA (PRITCHARD, 1986) proved unsuccessful in this study, although BATTY *ET AL.* (2001b) reported FDA as the more suitable predictor of actual germination for Australian terrestrials than either TTC or EVANS' blue. False positive results were obtained here, with the combination of FDA and NaOCl producing a yellow-green solution under VIS – masking any embryo fluorescence under UV. False positive data were obtained despite the 24 h sterile dH_2O rinse, with increased colour intensity under VIS stemming from increased pretreatment duration (Plate 6.3F). Such data indicates that the exposure to NaOCl may be substantially longer than the pretreatment, with residual corrosive effects resulting in non-viable embryos. The issue is further complicated since hypochlorites are reported to interfere with TTC staining (VAN WAES, 1987; RASMUSSEN, 1995).

A means of establishing the germination potential of a *Disa* seed population without being reliant on chemically induced testa permeability is therefore necessary. The highly permeable nature of immature *Disa* seed, in light of second and third quarter seeds being germinable *in vitro*, suggests that the GP of a population of immature seeds can be defined as:

$$100 \times \frac{\text{number of embryos stained with TTC}}{\text{number of seeds permeable to aqueous EVANS' blue.}}$$

The validity of such an assumption is that the unquantifiable viability of impermeable seed mirrors the quantified viability of permeable seeds within any given seed population. Estimating the GP of immature seed is critical in assessing the efficacy of the 'green-pod' culture protocol that is recommended for the *in vitro* germination of the small-seeded *Disa* species.

Despite no field data, the results presented here make it probable that germination of certain *Disa* seed is delayed until the next growth cycle as a result of water-impermeable dormancy. In regions with strongly fluctuating annual temperatures and rainfall cycles, the timing of germination is determined by several factors, including the timing of dehiscence and the period of optimal growth conditions. The seed dispersal period (late summer and autumn) is coincident with the dry, winter months. Dormancy in these species is therefore expected to be severe, lasting a minimum of six months until environmental temperature and moisture increases the following spring. Seed dormancy therefore corresponds to the phenology of vegetative dormancy of the adult plant. Similar germination delays were reported by RASMUSSEN & WHIGHAM (1993), VAN DER KINDEREN (1995) and VUJANOVIC *ET AL.* (2000) for Holarctic species.

BATTY *ET AL.* (2000) reported summer seed dispersal and winter germination for terrestrials that experience the Mediterranean climate of western Australia. Small-seeded *Disa* species that experience a Mediterranean climate in South

Africa were omitted from this study, but it is probable that they possess moderate dormancy that allows for the retention of viability from dehiscence (late summer or autumn) to the period of improved environmental conditions (winter) – approximately three months. *In vitro* germination times (approximately 12 weeks) reported for several of these species corroborate such a notion (VOGELPOEL, 1980, 1987, 1993; LACROIX & LACROIX, 1997; WODRICH, 1997; CROUS, 1999, PERS. COMM.). It is therefore proposed that seed dormancy in *Disa* accounts for the rainfall-based endemism reported for the genus.

The evergreen nature of the large-seeded *Disa* species, which was attributed to their streamside habitat, is reflected by the highly permeable, non-dormant seeds identified in these species. Reports revealed the rapid loss of viability (VOGELPOEL, 1980; ARDITTI, 1982; THORNHILL & KOOPOWITZ, 1992) that would be expected in an environment with perennial water.

These data provide evidence in support of testa-imposed dormancy in the small-seeded *Disa* and report on the viability of the mature seed for the first time. Additionally, results have indicated that dormancy relief can be achieved by manipulating permeability of the testa, although germinability levels achieved are far lower than the percentage viability. However, ARDITTI *ET AL.* (1981) and RASMUSSEN (1992) reported that in addition to water-impermeable dormancy, seeds synchronized to germinate in spring might require chilling to alleviate endogenous physiological dormancy. Nevertheless, recognising at least one of the mechanisms by which dormancy is imposed on the small-seeded *Disa* species, together with viability staining, are necessary steps in developing practical propagation methods for the conservation of members of this genus.

CHAPTER SEVEN

ASYMBIOTIC SEED CULTURE II: DEVELOPING NEW TECHNIQUES FOR THE *IN VITRO* CONSERVATION OF SOUTH AFRICAN *DISA* SPECIES

In vitro germination as a conservation aid demands propagation systems that yield increasingly larger numbers of plants as rapidly as possible. However, summer-rainfall *Disa* species are germinated infrequently under asymbiotic conditions. Attempts to bypass water-impermeable dormancy in these species resulted in the formulation of a dual-phase protocol – with the specific aim of increasing water availability to the embryo. Dual-phase cultures comprised a solid, charcoal-rich medium overlaid with a reduced strength, liquid medium fraction of the same type. The former served to negate the influence of leached phenols and allowed protocorms to establish polarity, whilst the latter increased water availability. The dual-phase protocol germinated nine *Disa* species, representing five first time reports. Furthermore, germination was comparatively rapid and percentages for most species approximated their estimated germination potential. Congruency here suggests that dual-phase conditions are amenable to germinating a number of seeds that reflects the maximum number of viable embryos – critical for conservation efforts. For the remaining species, where germination percentages were low, germination is controlled at least in part, by factors other than the testa. *In vitro* seedling maturation is reported, along with the unexpected occurrence of annual vegetative dormancy that impacted negatively on recruitment. The *ex vitro* transfer of deciduous *Disa* seedlings within one year of germination is prescribed, providing that germination percentages, seedling survival and the frequency and number of tubers produced are optimal.

7.1 INTRODUCTION

The plight of endangered orchids, when juxtaposed with continuing habitat loss and the inherent problems associated with *ex situ* conservation (THORNHILL & KOOPOWITZ, 1992; KOOPOWITZ, 2001), demands propagation

methods that yield increasingly larger numbers of plants as rapidly as possible (DRESSLER, 1981; ARDITTI, 1982; BUTCHER & MARLOW, 1989; JOHANSEN & RASMUSSEN, 1992; ZETTLER & MCINNIS, 1992, 1993; THOMPSON *ET AL.*, 2001, 2002).

Many authors, although recognizing *in vitro* seed germination as a tool for conservation, have noted the lack of reliable and repeatable procedures for germinating all the viable seeds of terrestrial species (STOUTAMIRE, 1964, 1974; ARDITTI, 1967, 1979, 1982; DALLA ROSA & LANERI, 1977; ARDITTI *ET AL.*, 1981; CLEMENTS, 1982; FAST, 1982; HADLEY, 1982; CLEMENTS *ET AL.*, 1986; JOHANSEN & RASMUSSEN, 1992; THORNHILL & KOOPOWITZ, 1992; ZETTLER & MCINNIS, 1993; RASMUSSEN, 1995; KOOPOWITZ, 2001; BATTY *ET AL.*, 2001b; MICHEL, 2002). Furthermore, reports of terrestrial orchid seed viability are rare. Most germination studies are undertaken without an understanding of life-histories and seasonal phenology – two factors essential for the success of *in vitro* seed culture and *ex situ* conservation (BUTCHER & MARLOW, 1989; RASMUSSEN & WHIGHAM, 1993; RASMUSSEN, 1995; ZETTLER & HOFER, 1998; BATTY *ET AL.*, 2000).

RASMUSSEN (1995) and BATTY *ET AL.* (2001a) reported that understanding the nature of seed dormancy in terrestrial species would allow for the formulation of practical solutions that could be applied *in vitro*. However, researchers typically apply dormancy-breaking treatments singly (RASMUSSEN, 1995; BATTY *ET AL.*, 2000; MORRIS *ET AL.*, 2000) since they share their mode of action, or resort to symbiotic culture methods (CLEMENTS *ET AL.*, 1986; WILKINSON *ET AL.*, 1994; RASMUSSEN, 1995, 2000; DIJK *ET AL.*, 1997; ZETTLER *ET AL.*, 1999; BATTY *ET AL.*, 2001a, b). In many cases the former has not solved all the problems associated with dormant seeds *in vitro*, with germination percentages being substantially lower than the percentage of viable embryos (KOHL, 1962; STOUTAMIRE, 1974; ARDITTI *ET AL.*, 1981; ARDITTI, 1982; OLIVA & ARDITTI, 1984; VAN WAES & DEBERGH, 1986b; LINDÉN, 1992; CHU & MUDGE, 1994; SHOUSHTARI *ET AL.*, 1994; MIYOSHII & MII, 1998; VUJANOVIC *ET AL.*, 2000).

There is a demand for improved systems for the *in vitro* germination of dormant *Disa* seed. Summer-rainfall species are germinated infrequently and percentage germination represented, at most, half of the viability established for a particular mature seed batch. In the absence of suitable symbiotic techniques for the genus, novel asymbiotic methods are paramount. The logical starting point here would be to build upon, or combine, previous successes for dormant *Disa* seed, which were germinated by increasing water availability to the embryo (through decreased media viscosity and decreased media additive concentrations) and decreasing phyto-inhibitor concentration (through charcoal supplementation). A single report (SEEMANN, 1953) documents the exploitation of dormancy-breaking mechanisms *in vitro* and reports the use of a double flask in which orchid seeds could be aseptically leached in dH_2O, for several months if necessary, before coming in contact with a solidified germination medium. Germination percentages were not reported by SEEMANN (1953) but were presumably low, as the technique was not adopted in orchid seed culture.

Apart from achieving germination, success *in vitro* relies on the ability to produce and maintain seedlings that will survive *ex situ* transfer (DRESSLER, 1981; RASMUSSEN, 1995; BATTY *ET AL.*, 2001a). Terrestrial orchids are typically not the subjects of large-scale production. Consequently present culture methods beyond the primary seedling stage are often specific and rely largely on personal experience (ARDITTI, 1982; CRIBB & BAILES, 1989; RASMUSSEN, 1995). The latter are rarely documented and were reviewed by FAST (1982) and CRIBB & BAILES (1989).

This chapter describes a newly formulated and highly successful dual-phase culture system developed specifically for germinating the water-impermeable seeds of the small-seeded *Disa* species. In addition, secondary seedling development *in vitro* of these previously uncultured species is reported.

7.2 AIMS

- To establish the germination potential (GP) of immature seed populations for several dormant, small-seeded *Disa* species.
- To develop practical propagation methods for dormant, small-seeded *Disa* species that allow for a germination percentage that closely approximates their GP.
- To document secondary seedling maturation for several small-seeded *Disa* species.

7.3 MATERIALS AND METHODS

For the *in vitro* asymbiotic germination of dormant *Disa* seed a dual-phase culture system was developed, in which an agar-solidified germination medium was overlaid with a liquid medium fraction (Figure 7.1). This differs from the double-phase medium used in the micropropagation of hyacinths (BACH & CECOT, 1988) in that the liquid media fractions were added prior to seed placement and not several weeks after culture initiation. The nature of the media manipulations used in the formulation of dual-phase cultures were based on earlier, single-phase modifications that promoted low-level germination in several small-seeded *Disa* species (Figure 3.1; page 75) – a result attributed to increased leaching of their phenol-rich testae.

Half-strength MS salts (MURASHIGE & SKOOG, 1962), supplemented with 2 g L^{-1} acid-rinsed, activated charcoal, 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol, 0.1 mg L^{-1} thiamine, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine and 2 mg L^{-1} glycine, were solidified (8 g L^{-1} UNILAB agar) as the basal medium fraction in glass culture tubes (ø 25 mm; 10 ml media) or glass culture jars (ø 60 mm; 25 ml media).

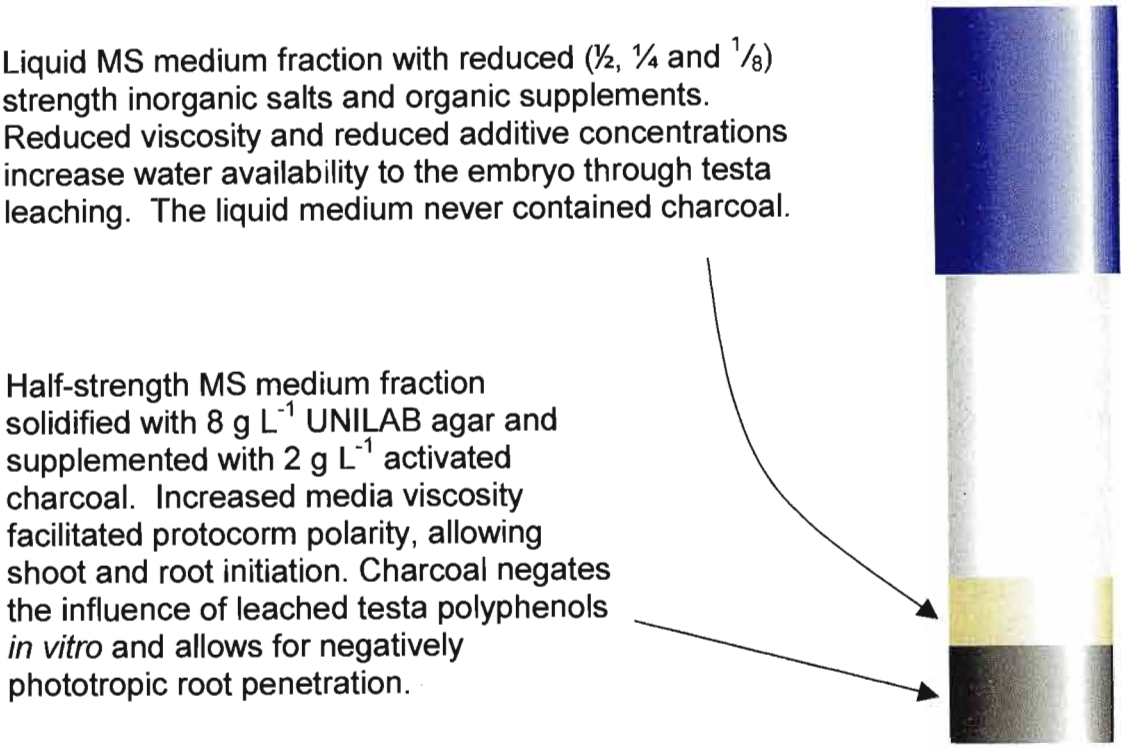


Figure 7.1. Dual-phase culture protocol established for the germination of dormant, summer-rainfall *Disa* seed, with the specific aim of relieving testa-impermeable seed dormancy. Dual-phase specifications increased water availability to the embryo and decreased phyto-inhibitor concentration.

Equal volumes of cool (~ room temp.), liquid MS media (0 g L^{-1} agar) were decanted into the culture vessels under sterile conditions. Liquid media contained half, quarter or one-eighth strength inorganic salts and organic supplements and never charcoal (Figure 7.1). The *p*H of all media was adjusted to 5.8 prior to autoclaving. Dual-phase cultures were continuously agitated by means of an orbital shaker operating at 50 cycles min^{-1}. Incubation took place at 25 ± 2 °C under continuous illumination of 8.5 μmol m^{-2} s^{-1}, supplied by cool white fluorescent tubes. Liquid media fractions were either removed using a sterile glass pipette seven days after culture initiation – which allowed the seeds to settle on the solid medium surface, or were retained for the duration of the incubation period. The pipette was customized to include a nylon filter that prevented the

drawing in of seeds that were in solution.

Cultures were initiated using immature seed sourced from six-week-old (third quarter) capsules for 10 small-seeded, summer-rainfall *Disa* species (Table 7.1). Species were either poorly germinable (GI = 2; < 30 % germination in a minimum of 12 weeks; Figure 3.1) or intractable (GI = 3; 0 % germination; Figure 3.1) on modified single-phase media. Capsules were harvested and decontaminated as outlined previously (Chapter Three; pages 59, 62 and 70). Sowing density was two transverse capsule sections per tube or four sections per jar culture. A minimum of fifty cultures were initiated for each species.

The germination responses were graded according to modifications of the broad categories (Chapter Three; page 67) employed by WARCUP (1973), OLIVA & ARDITTI (1984), SMRECIU & CURRAH (1989) and LEROUX *ET AL.* (1997). Germination parameters (viz. rate, percentage and synchrony) under dual-phase conditions were compared to i) those recorded on modified single-phase asymbiotic media and ii) those reported from symbiotic cultures for selected species.

Table 7.1. *Disa* species for which dual-phase germination cultures were established during the study period 2001 – 2002. All species are summer rainfall endemics and produce small-type seeds (*sensu* KURZWEIL, 1993). Sectional classification after LINDER & KURZWEIL (1999) is included in parentheses. An asterisk highlights those species germinated with limited success (< 30 %; GI = 2) on modified single-phase media. The remaining species were not germinated under conditions of single-phase media.

D. brevicornis (*Monadenia*)	*D. patula* var. *transvaalensis* (*Emarginatae*)
D. chrysostachya (*Micranthae*)	*D. pulchra* (*Stenocarpa*)*
D. cooperi (*Hircicornes*)*	*D. stachyoides* (*Emarginatae*)
D. crassicornis (*Hircicornes*)	*D. versicolor* (*Hircicornes*)
D. nervosa (*Emarginatae*)*	*D. woodii* (*Micranthae*)*

The mean germination percentage achieved in dual-phase culture was compared to the mean germination potential calculated from three randomly selected third quarter capsules for each species. Germination potential was defined (Chapter Six; page 197) as the ratio between the proportion of embryos stained with TTC (Chapter Six; page 179) and the proportion of seeds permeable to aqueous EVANS' blue (Chapter Six; page 178), expressed as a percentage.

Cultures where most seedlings exhibited secondary development (the attainment of the 2^+ leaf stage concomitantly with the elongation of the primary root) were transferred to a growth room with higher illumination (35 μmol m^{-2} s^{-1}). Maturation was monitored fort-nightly and transflasking took place onto solidified media, containing half-strength MS inorganic salts, vitamins and myo-inositol, at six-monthly intervals. The medium for transflasking was formulated to enhance root (0 or 2 g L^{-1} activated charcoal; UEDA & TORIKATA, 1972; PAN & VAN STADEN, 1998) and tuber formation (10, 30 or 60 g L^{-1} sucrose; RABE, 1998, PERS. COMM.).

7.4 RESULTS AND DISCUSSION: DOES *IN VITRO* SEED GERMINATION HAVE A ROLE TO PLAY IN THE CONSERVATION OF *DISA*?

With the exception of *D. chrysostachya* (GP = 78 %), all summer-rainfall *Disa* species were germinated under asymbiotic dual-phase conditions (Table 7.2) – representing first time asymbiotic reports for *D. brevicornis*, *D. crassicornis*, *D. patula*, *D. stachyoides* and *D. versicolor*. For *D. brevicornis* and *D. patula*, the germination percentages exceeded 65 % and approximated their GP's (Table 7.2). Very poor germination ($\leq$ 6 %) was recorded for *D. crassicornis* and *D. versicolor*, despite GP's of approximately 70 % (Table 7.2).

Table 7.2. ***In vitro*** **requirements for small-seeded, summer rainfall *Disa* germination in dual-phase culture[1]. Seed originated from third quarter developmental capsules. Mean percentage, time taken to first germination and the estimated germination potential[2] (GP) are presented in parentheses. Percentage germination represents the mean count of 100 randomly chosen seeds from five cultures. Species for which the percentage germination approximated the germination potential are highlighted in grey.**

Species	Liquid MS media fraction manipulation
D. brevicornis	½, ¼ and $^1/_8$ strength inorganic salts and organic additives, retained for seven days and for the duration of the incubation period (79 %; six weeks, GP = 88 %)
D. cooperi	½, ¼ and $^1/_8$ strength inorganic salts and organic additives, retained for seven days and for the duration of the incubation period (90 %; four weeks, GP = 92 %)
D. crassicornis	$^1/_8$ strength inorganic salts and organic additives, retained for the duration of incubation period (6 %; 10 weeks, GP = 72 %)
D. nervosa	½, ¼ and $^1/_8$ strength inorganic salts and organic additives, retained for seven days and for the duration of the incubation period (64 %; four weeks, GP = 86 %)
D. patula	¼ and $^1/_8$ strength inorganic salts and organic additives, retained for the duration of incubation period (65 %; seven weeks, GP = 70 %)
D. pulchra	¼ and $^1/_8$ strength inorganic salts and organic additives, retained for the duration of incubation period (82 %; five weeks, GP = 90 %)
D. stachyoides	¼ and $^1/_8$ strength inorganic salts and organic additives, retained for the duration of incubation period (28 %; 10 weeks, GP = 76 %)
D. versicolor	$^1/_8$ strength inorganic salts and organic additives, retained for the duration of incubation period (4 %; 11 weeks, GP = 67 %)

Table 7.2. (continued)

Species	Liquid MS media fraction manipulation
D. woodii	½, ¼ and [1]⁄₈ strength inorganic salts and organic additives, retained for the duration of incubation period (73 %, four weeks, GP = 85 %)

[1] Half-strength MS (MURASHIGE & SKOOG, 1962) media, solidified with agar (8 g L^{-1}) and supplemented with charcoal (2 g L^{-1}), sucrose (30 g L^{-1}), myo-inositol (0.1 g L^{-1}), thiamine (0.1 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), pyridoxine (0.5 mg L^{-1}) and glycine (2 mg L^{-1}), was the basal medium fraction in all experiments. Cultures were incubated at 25 ± 2 °C under a continuous low-light intensity of 8.5 μmol m^{-2} s^{-1} supplied by cool white fluorescent tubes. Activated charcoal was acid-rinsed, *BDH # 330324E*.

[2] Germination potential (%) was estimated as the proportion of embryos stained with TTC relative to the proportion of seeds permeable to aqueous EVANS' blue. TTC staining after LÄKON (1949), EVANS' blue staining after WIDHOLM (1972).

Disa cooperi, *D. nervosa*, *D. pulchra* and *D. woodii* were germinated in dual-phase cultures with a two to threefold increase in their germination percentages relative to the single-phase incubation of immature seed (GI = 2). The highest percentage germination for a small-seeded summer-rainfall *Disa* investigated in this study was for *D. pulchra* (90 %). With the exception of *D. nervosa*, germination percentages in these species broadly mirrored their estimated GP percentages (Table 7.2), differing by as little as 2 % for *D. pulchra*.

The retention of the liquid media fraction for the duration of incubation proved beneficial in achieving highest germination in *D. crassicornis*, *D. patula*, *D. pulchra*, *D. stachyoides*, *D. versicolor* and *D. woodii* (Figure 7.2). With the exception of *D. woodii*, increased water availability through decreased media additive concentrations also promoted germination percentage in these species. One-eighth strength media additives were obligate in achieving germination in *D. crassicornis* and *D. versicolor* (Figure 7.2). The percentage germination in *D. brevicornis*, *D. cooperi* and *D. nervosa* was not influenced by the duration of exposure to, nor the composition of the liquid medium.

Germination synchrony where the germination percentage exceeded 60 % under dual-phase conditions was similar between species (four to eight weeks

after the onset of germination) and is comparable to that reported previously for liquid, single-phase cultures. Poorly germinable species *D. crassicornis*, *D. stachyoides* and *D. versicolor* were less synchronous, with germination being spread over an additional four weeks. These data are independent of retention of the liquid fraction after seven days. Moreover, germination synchrony was unaffected by media composition. Dual-phase culture decreased the times taken to first germination (four to five weeks) relative to those recorded under single-phase conditions (> 12 weeks; Table 3.7). Furthermore, novel germination occurred within 12 weeks of culture initiation, being longest (≥ 10 weeks) in the poorly germinable species (*D. crassicornis*, *D. stachyoides* and *D. versicolor*; Table 7.2). Time taken to first germination was seven weeks or less in the remaining species and was independent of manipulation of the liquid media fraction.

Germination of the summer-rainfall *Disa* species in dual-phase culture was therefore variable, with four distinct responses being noted. These responses (i – iv below) were independent of taxonomic affiliation, distribution, macro-habitat or altitude within the summer-rainfall grasslands, nor linked to the timing and duration of flowering. For example *D. brevicornis*, *D. chrysostachya*, *D. pulchra*, *D. stachyoides* and *D. versicolor* occur in mixed populations, as do *D. cooperi* and *D. versicolor*, yet these species displayed different germination responses.

i. Rapid, synchronous and high percentage germination that was independent of the exposure period and composition of the liquid media fraction (*D. brevicornis*, *D. cooperi* and *D. nervosa*);

ii. Rapid, synchronous and high percentage germination that was dependent on a retained liquid media fraction with reduced additive concentrations (*D. patula*, *D. pulchra* and *D. woodii*);

iii. Comparatively slower, asynchronous and lower percentage germination, despite retained liquid media and reduced liquid media additives (*D. crassicornis*, *D. stachyoides* and *D. versicolor*); and

iv. No germination, despite retained liquid media and reduced liquid media additives (*D. chrysostachya*).

Leaching of the testae for a sufficient period to allow for synchronous and high (> 60 %) percentage germination therefore occurred early on during the incubation period, before or up to seven days, for *D. brevicornis*, *D. cooperi* and *D. nervosa*. Considering that the latter two species were also germinated on single-phase media, they exhibit comparatively shallow dormancy and broad germination requirements. In addition, seed populations are suspected to be relatively uniform with regard to the phenol content of their testae. *Disa patula*, *D. pulchra* and *D. woodii* required an extended leaching period to achieve similar germination percentages. Decreased concentrations of media additives and the retention of the liquid media fraction both served to increase water availability to the embryo, whilst the latter also promoted phyto-toxin dilution. Charcoal in the basal media fraction served to bind these toxins, negating their negative influence on germination (Figure 7.2). *Disa pulchra* and *D. woodii* were germinable on modified single-phase media, which suggests variable dormancy within seed populations. Only the shallowest of these was relieved in single-phase cultures (< 30 % germination), with increased leaching and water availability to the embryo needed to alleviate progressively deeper dormancy.

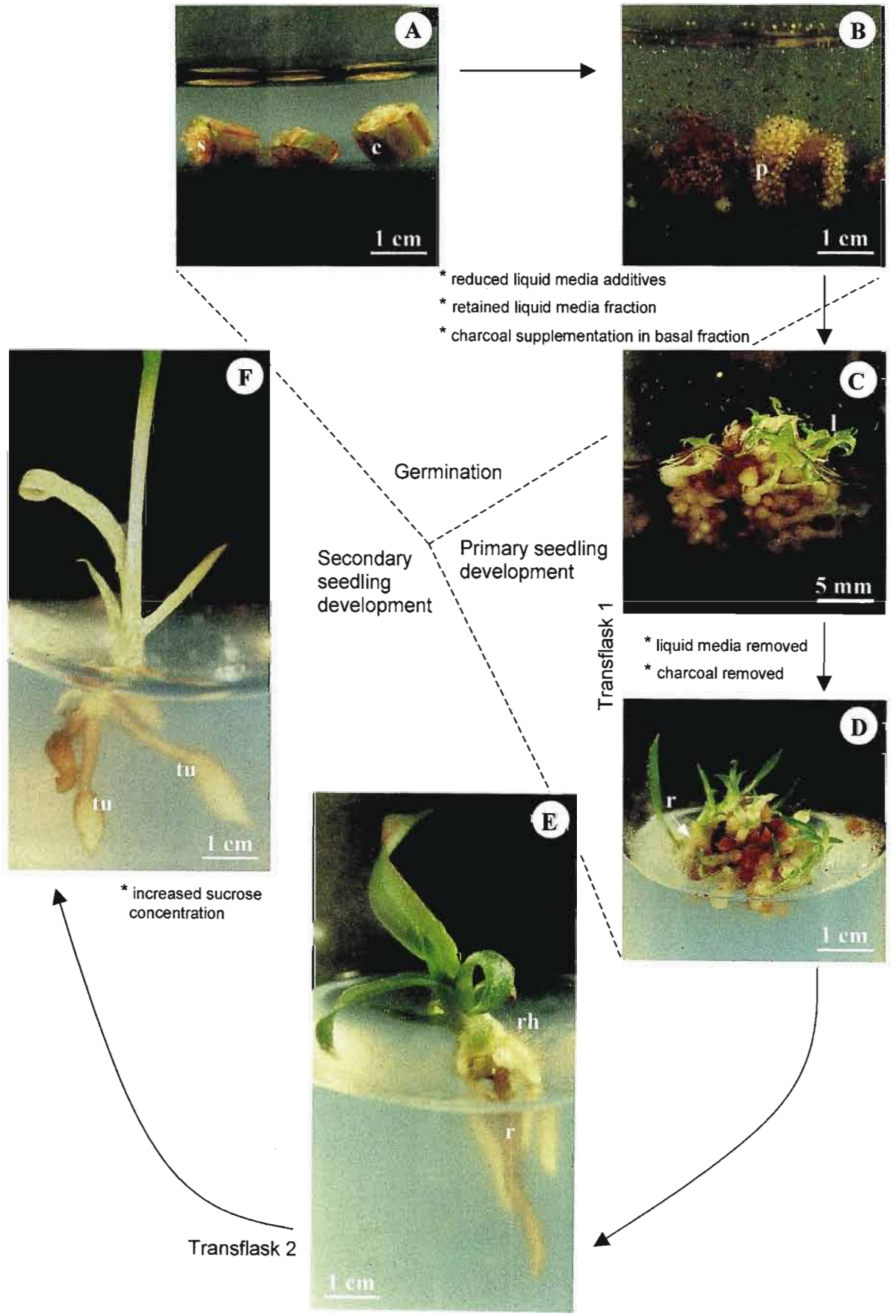
Congruency between *in vitro* germination percentage and the estimated germination potential of the immature seed of these *Disa* species indicates i) that the dual-phase conditions are amenable to germinating a number of seeds that approximates the maximum number of viable embryos and ii) that the estimated GP of immature seed may be a more accurate means of determining embryo viability than mature seed staining. For *D. cooperi* and *D. pulchra*, where mature

seed viability was also recorded (Table 6.5), GP estimates (Table 7.2) always exceeded the former. In addition, germination percentages were substantially higher than mature seed viability results for several species. Such data further questions the efficacy of embryo staining in mature terrestrial orchid seed.

Despite intensive leaching and increased water availability, low percentage and asynchronous germination was recorded for some species (*D. crassicornis*, *D. stachyoides* and *D. versicolor*; 6 %, 28 % and 4 % germination respectively; Table 7.2) and no germination was recorded for others (*D. chrysostachya*; 0 % germination; Table 7.2). The percentage germination was lower than the percentage seed permeability for these species – implying that germination is constrained by factors other than permeability of the seed testa. Sugar analysis suggested that mature seeds of these species were unable to germinate because embryos were deficient in sucrose. However, immature seed of *D. versicolor* was also rich in sucrose – the sugar status typical of germinable *D. cooperi*, *D. nervosa* and *D. pulchra* seed, and it was predicted that 'green-podding' should permit germination in all immature *Disa* seed. Data presented here contradict this, with permeable, sucrose-rich seed of these summer-rainfall *Disa* species remaining dormant. Differences in *Disa* nutrient reserves therefore reflect variation in germination strategies that are difficult to bypass *in vitro*. Nevertheless first-time asymbiotic germination for these species, although in low percentages, represents a critical step towards developing suitable *in vitro* propagation methods.

The few data on *Disa* endophytes revealed through this study suggested that the sugars that play a role in regulating germination might be more diverse than sucrose, fructose and glucose. Rather, augmentation of the overall free sugar status of the embryo, besides the provision of free water, may be required to relieve dormancy in certain summer-rainfall *Disa* species.

Figure 7.2. Germination and seedling development for small-seeded, summer-rainfall *Disa* species in dual-phase culture. Cultures were initiated using immature seed (s) from six-week-old capsules, introduced into culture as transverse capsule (c) sections (A). Beneficial media and protocol manipulations are included. Conditions of increased water potential enhanced germination and protocorm (p) survival (B), but did not support primary or secondary seedling development. Germination occurred in 4 – 11 weeks. The combination of immature seed and the dual-phase method resulted in 90 % germination for *D. pulchra*, the highest percentage recorded for a summer-rainfall *Disa* in this study. Shooting (C) and leaf maturation (l) were independent of liquid media retention. However, root (r) initiation benefited from a transient liquid media fraction (D). Secondary seedling development (E), as revealed by root elongation and the formation of root hairs (rh), occurred 12 – 16 weeks after germination. Larger plants (> 5 cm) initiated the formation of tubers (tu) 24 weeks after germination, especially under conditions of increased sucrose (F).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Secondary seedling development
Transflask 1
* liquid media removed
* charcoal removed
D
r
1 cm
E
rh
r
1 cm
Transflask 2
* increased sucrose concentration
F
tu
tu
1 cm

Several authors have reported the absence of germination *in vitro* as evidence for a reliance on mycotrophy and the need for relevant symbionts (STOUTAMIRE, 1963, 1974; HADLEY & WILLIAMSON, 1971; WARCUP 1973; CLEMENTS *ET AL.*, 1986; WILKINSON *ET AL.*, 1994; RASMUSSEN, 1995; BATTY *ET AL.*, 2001b). *Disa crassicornis* and *D. versicolor* were amongst the few *Disa* species germinated in mycorrhizal association in this study (40 % and 10 % germination percentages respectively), perhaps indicative of the obligation of fungal association in the germination of these species. However, the absolute requirement for fungal intervention *in vitro* seems unlikely, since a permeable testa allows for nutritional and physiological manipulation of the embryo. Once the exact role of fungi in germination has been defined, then specific *in vitro* manipulation could negate the need for mycotrophy. The present data suggests that this could be achieved without physical transfer of seeds or protocorms by supplementing the liquid medium *in vitro*. Similarly, CHU & MUDGE (1994) reported that in strictly liquid cultures, replacing or supplementing the liquid medium could avoid transflasking.

Protocorms originating from dual-phase germination developed as reported for single-phase cultures, attaining primary seedling status approximately 12 weeks after germination. In cultures with permanent liquid fractions, a large proportion (> 65 %) of the protocorms that developed from seeds in suspension, remained undifferentiated and displayed necrosis. Such data are interpreted as the inability of protocorms to establish polarity in a medium with little or no mechanical stability (ARDITTI, 1982; RASMUSSEN, 1995) and explains the general absence of reduced viscosity media in orchid seed research. However, the bulk of seeds remained attached to the capsule placentae (Figure 7.2A), which was in contact with the solid media surface and protocorms matured normally (Figure 7.2B). Protocorm survival was highest (> 80 %) when incubated in reduced strength (one-eighth), permanent liquid media fractions; a result attributed to a contracted water potential gradient between the medium and the protocorm tissues and the corresponding lowered osmotic stress experienced by the plant tissues. Root initiation was inhibited in single-phase liquid cultures, but not in

dual-phase cultures due to the solid basal fraction that provided the protocorms with the tactile stimulus needed to establish polarity. Rooting of protocorms maturing from attached seed was independent of the duration of exposure to the liquid media fraction and the presence of charcoal in the medium (Figure 7.2C, D).

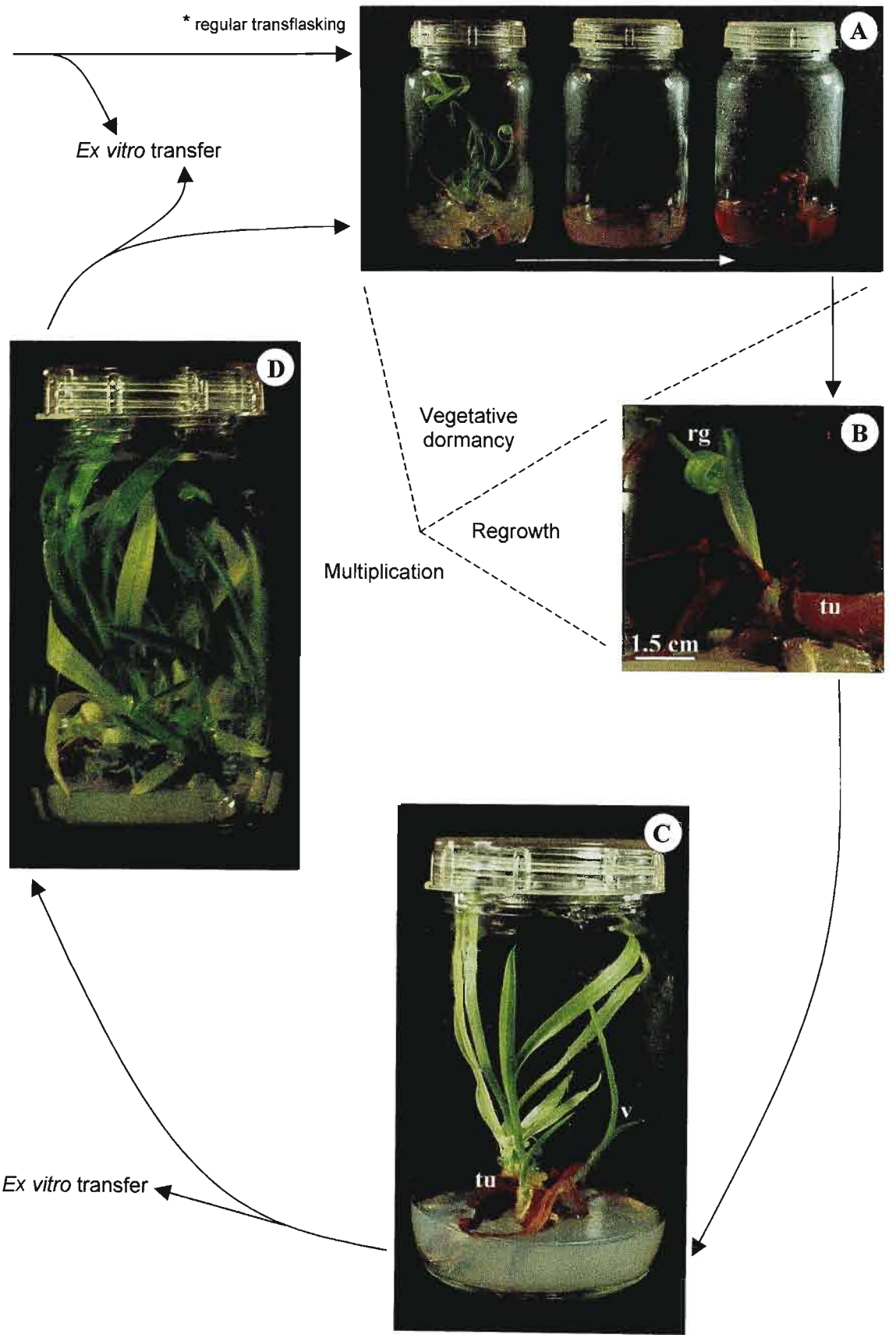
Shoot maturation and root elongation (Figure 7.2E), accompanied by the formation of root hairs, occurred 12 – 16 weeks after germination in all species, approximately 4 weeks after cultures were transferred to incubation conditions with higher light intensity. Root elongation and the negatively phototropic medium penetration of these *Disa* species in dual-phase culture was not dependent on the provision of a charcoal-rich basal medium, presumably due to the low-light intensity of the culture conditions. Manipulation of the liquid media composition did not impact on shoot or root maturation. Moreover, shooting was independent of the duration of exposure to the liquid media fraction. The mean percentage of seedlings with roots was lower on media with the permanent liquid fraction (69 % vs. 93 %). CHU & MUDGE (1994) documented a reduction in the number and length of roots and root hairs of *Cypripedium* spp. in liquid media – with the assumption that root development is negatively correlated to water availability. RASMUSSEN (1995) reported poor acclimatization from plants with a low root : shoot ratio and that *in vitro* manipulations should be carried out to maximize rooting. Consequently the retained liquid media fraction should be removed from dual-phase cultures at the primary seedling stage in order to promote root maturation (Figure 7.2D). Seedlings of *D. crassicornis*, *D. stachyoides* and *D. versicolor* were not matured beyond primary seedling status. Consequently, achieving germination does not necessarily dictate the success of *in vitro* propagation efforts.

Tuberization 24 weeks after germination was initiated by larger plants (> 5 cm) of all species that attained secondary status *in vitro*. (Figure 7.2F). Typically single tubers developed from a leaf axis on the compressed rhizome, but an

increased sucrose content (60 g L^{-1}) after transflasking resulted in plants producing multiple tubers. In addition, the number and rate of tuberization was enhanced by an increased carbohydrate supplement. However, associated osmotic stress, which was more pronounced in media with the higher sucrose content, was revealed by anthocyanin accumulation in the leaves, with eventual leaf death. The absence of charcoal after transflasking promoted tuberization but reduced the number of plants with, and their number of healthy roots. The frequency of tuberization was lower in cultures with a retained liquid fraction. Transflasking onto a solid, sucrose-rich, charcoal deficient medium is therefore recommended for the *in vitro* maturation of the summer-rainfall *Disa* species (Figure 7.2F). Ultimately, only a small fraction of summer-rainfall *Disa* protocorms (< 30 %) were matured to the stage of tuber formation, where survival over the subsequent dry season *in situ* would be expected (ANDERSON, 1990; RASMUSSEN, 1995; BATTY *ET AL.*, 2000; BATTY *ET AL.*, 2001a). These data are independent of taxonomic section. Tuberized seedlings could therefore be moved *ex vitro* (Figure 7.3), where the presence of tubers and the experience of *in vitro* osmotic stress are expected to result in an increased frequency of successful acclimatization (RABE, 1998, PERS. COMM.). Cohorts were very variable, with most seedlings remaining small (< 5 cm) and without tubers.

During the period 30 – 36 weeks after germination *Disa* plantlets entered a period of vegetative dormancy. This response was irrespective of the constant culture conditions and was not influenced by media composition. *In vitro* dormancy was associated with the stress-induced release of phenolics by the plant, the accumulation of which in the media promoted further senescence and prevented post-dormancy regrowth (Figure 7.3A). Regular transflasking during the period leading up to the dormancy period did not abort dormancy, but did yield a higher percentage of tubers that were able to resprout. Seedlings without tubers were unable to survive vegetative dormancy.

Figure 7.3. Propagation of South African, summer-rainfall *Disa* species. *In vitro* germinated seedlings were matured to secondary status 24 weeks from germination in 60 mm (ø) glass culture jars. Despite uniform culture conditions, vegetative dormancy was initiated after 30 – 36 weeks (A). Dormancy was associated with the accumulation of phyto-toxic phenolics in the medium (indicated). Dormancy lasted approximately six weeks (B), with < 50 % of tubers showing signs of regrowth (rg). The percentage regrowth was increased by regular transflasking leading up to dormancy. Seedlings without tubers did not survive dormancy. Regrowth was rapid, resulting in robust plants by the end of 52 weeks *in vitro* (C). Seedlings produced tubers (tu) and spontaneous vegetative shoots (v), permitting the asexual bulking up of *in vitro* material through division. Dormancy was also observed during the second year *in vitro*, after which daughter plantlet initiation was prolific (D) – reaching a maximum of 17 in *D. cooperi*. Due to *in vitro* dormancy, less than 3 % of germinated seeds reached two years of age. Consequently, rapid maturation and the *ex vitro* transfer of seedlings within nine months of germination are prescribed for *Disa*. However, recruitment is determined by germination percentages and the occurrence of tuberization, which must be optimized. Retaining plants *in vitro* permits the asexual bulking up of material, but genetic stock is limited by the inability of most tubers to recover from dormancy *in vitro*.


* regular transflasking
A
Ex vitro transfer
D
Vegetative dormancy
Regrowth
Multiplication
rg
B
tu
1.5 cm
C
v
tu
Ex vitro transfer

Charcoal supplementation is usually used against *in vitro* phenolics (ERNST, 1976; ARDITTI, 1982; ARDITTI & ERNST, 1984; VAN WAES, 1987; STEWART, 1989; GEORGE, 1993; RASMUSSEN, 1995; PAN & VAN STADEN, 1998), but not where tuber formation is inhibited. The provision of charcoal had no effect on *Disa* regrowth.

Regrowth (Figure 7.3B) following transflasking occurred from less than half of the tubers (47 %), with dormancy lasting a minimum of six weeks. Regrowth was rapid, resulting in > 12 cm tall plants by the end of 52 weeks post-germination. These plants displayed healthy leaf and root development and initiated the formation of new single, or rarely, multiple tubers (Figure 7.3C). During the second year *in vitro*, plants again entered a period of vegetative dormancy. By contrast, ORCHARD (1999, PERS. COMM.) reported that evergreen *Disa* species (the *D. uniflora* sub-clade) could be flowered within two years of germination.

The influence of *in vitro* dormancy on summer-rainfall *Disa* recruitment was severe, with < 3 % of germinated seeds reaching two years of age. MICHEL (2002) reported that species that died back to tubers seasonally were the most difficult terrestrial orchids to manage *in vitro*, with no data describing treatment. Few *Disa* plants (n = 14) were acclimatized during this study and although the *ex vitro* transfer of both first and second year tubers did not prevent their vegetative dormancy, all soil-transferred tubers did initiate regrowth under greenhouse conditions. Consequently, rapid maturation with the specific aim of achieving tuberization is critical for the success of *in vitro Disa* propagation. Alternatively, *in vitro* regrowth in both the first and second year resulted in robust *Disa* plants, with spontaneous vegetative shoots developing around the base of the mother-plant (Figure 7.3D). These were not observed in soil-established plants. Daughter shoots developed independent root systems. As such, they were successfully isolated from the mother-plant and matured independently on a similar medium. Daughter-plantlet production was prolific in several cultures, reaching a maximum of 17 in *D. cooperi*. Many orchids, including the evergreen *Disa* species, are

commonly propagated asexually *in vitro* by severing offshoots from the parent (VOGELPOEL, 1980, 1987, 1993; ARDITTI, 1982; SAGAWA & KUNISAKI, 1982; ARDITTI & ERNST, 1984; BUTCHER & MARLOW, 1989; STEWART, 1989; LACROIX & LACROIX, 1997; WODRICH, 1997; MICHEL, 2002).

Two less-than-ideal options are presented for summer-rainfall *Disa* propagation:

i. the *ex vitro* transfer of tubers prior to vegetative dormancy (Figure 7.3), which ensures that the maximum number of seedlings survive into their subsequent year. However, recruitment is determined by germination percentages and the occurrence of tuberization; and
ii. the indeterminate bulking up of plants asexually from genetic stock limited by the inability of most tubers to recover from dormancy *in vitro* (Figure 7.3).

Although asexual division could theoretically yield an infinite number of *Disa* plants, genetic diversity – in addition to maximum output, is a critical proviso of conservation efforts. Consequently, the *ex vitro* transfer of tubers within one year of germination is prescribed to optimize the success of summer-rainfall *Disa* propagation – providing that germination percentages, seedling survival, and the frequency and numbers of tubers produced are optimal.

Orchid seed is ideal for 'conservation through cultivation' as the seed is minute, enabling the processing of hundreds of thousands of genetically diverse seeds in a relatively small space and with comparatively little effort. If these seeds are germinated readily, then *in vitro* germination presents itself as a valid conservation aid. For *Disa*, optimum germination and maturation media and culture conditions are only now being defined – an issue hampered by the heterogeneous nature of seed and testa morphology, nutrient reserves and

ultimately the dormancy strategies of members of this genus. Consequently, the dual-phase protocol was developed and adapted to the unique requirements of individual or groups of species. A conscious effort to understand and overcome water-impermeable dormancy in the summer-rainfall *Disa* species, in the context of water availability and the seasons of dehiscence and rainfall, allowed this study to germinate several previously uncultured species, including four species (viz. *D. nervosa*, *D. pulchra*, *D. stachyoides* and *D. woodii*) reported as threatened by HILTON-TAYLOR (1996a, b; 1997) and VICTOR (2002). The links between *Disa* ecology, taxonomy and germinability are crucial, because they allow the formulation of hypotheses involving uninvestigated species.

The development of the asymbiotic dual-phase culture protocol, that resulted in rapid and synchronous germination that approximated their estimated germination potentials, is regarded as paramount in achieving maximum germination in *Disa* species where germination is controlled solely by impermeable testae. This technique is relatively easy to carry out and does not require specialized equipment, reinforcing its applicability to *in vitro* conservation. Moreover, primary seedlings can be manipulated without transflasking by supplementation or replacement of the liquid medium fraction, providing new opportunities for investigation into the effect of media additives on seedling recruitment. Previously, such studies have been precluded by the impervious nature of the seed testa and the corresponding low percentage germination.

A number of species remain for which recruitment was absent or not reliable. Mycorrhizal association is not obligate in achieving germination in *Disa in vitro*. However, data presented here suggest that nutritional provision by the endophyte is a factor that may contribute to germination control in several species. Symbiotic seed culture therefore remains as the most effective, albeit largely unsuccessful, means of germinating *D. versicolor* and *D. crassicornis*.

Nevertheless, the germination method proposed could be used more widely than was attempted in this study for the propagation of rare and threatened *Disa* species. The high percentage and predictable germination reported from this study for several summer-rainfall *Disa* species stands as the first step towards initiating research into seedling recruitment and developing programs directed at introducing artificially grown seedlings into suitable areas where populations are under threat. In principle, the implications of asymbiotic dual-phase culture for the *in vitro* conservation of *Disa* are encouraging and the outcomes of this study are likely to be relevant to the long-term preservation of other orchid taxa. However, PRITCHARD (1989) and JOHANSEN & RASMUSSEN (1992) reported that *ex situ* conservation is of little value if propagated plants cannot give rise to spontaneous seedlings and along with many other authors, motivated for the preferential use of symbiotic, rather than asymbiotic methods (CLEMENTS *ET AL.*, 1986; CLEMENTS, 1988; SMRECIU & CURRAH, 1989; RASMUSSEN *ET AL.*, 1990; ZETTLER & MCINNIS, 1992, 1993, 1994; WILKINSON *ET AL.*, 1994; ZETTLER *ET AL.*, 1999; RASMUSSEN, 2002). Consequently, there is a dire need for focussed research and improved symbiotic techniques with regard to *Disa*. Ultimately, while it is possible to propagate select *Disa* species *in vitro* using novel and specific techniques, the *ex situ* preservation of species to prevent the loss of ecological diversity is no substitute for the *in situ* conservation of existing orchid populations and their habitats.

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PERSONAL COMMUNICATIONS

BECK, SASCHA Doctor (1998)
Institute for Commercial Forestry Research, University of Natal, Pietermaritzburg, SOUTH AFRICA.

CROUS, HILDEGARD (1999)
Private orchid grower (formerly of Kirstenbosch National Botanical Gardens), Barrydale, SOUTH AFRICA.

JÄGER, ANNA Professor (2001)
Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, DENMARK.

LAST, RICK Doctor (1999)
Vetdiagnostix pathology services, Pietermaritzburg, SOUTH AFRICA.

ORCHARD, WALLY (1999)
Afrodisa commercial propagation laboratory, Tumwater, UNITED STATES OF AMERICA.

RABE, TONIA (1998)
School of Botany and Zoology, University of Natal, Pietermaritzburg, SOUTH AFRICA.

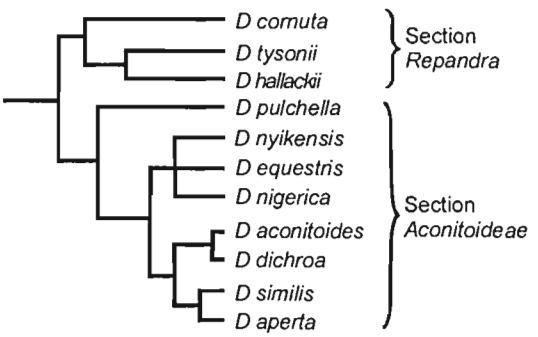
VOGELPOEL, LOUIS Doctor (2001)
Private orchid grower, Rondebosch, Cape Town, SOUTH AFRICA.

WODRICH, KARSTEN (2001)
Private orchid grower, Cresta, Johannesburg, SOUTH AFRICA.

APPENDIX ONE

Simplified cladograms showing the presumed relationships among the species of eight sections of *Disa* (after LINDER & KURZWEIL, 1999). Relationships between sections were not presented by LINDER & KURZWEIL (1999).

Sections *Aconitoideae* & *Repandra*



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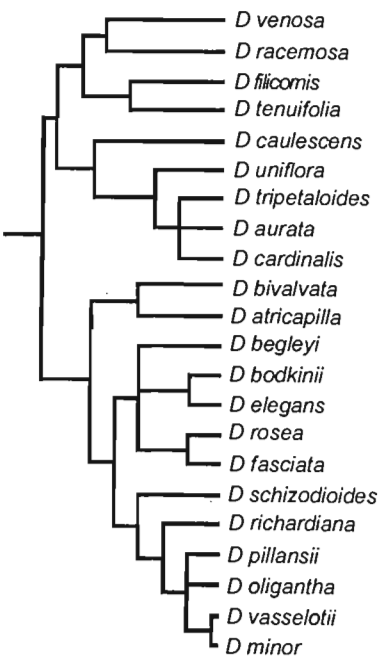

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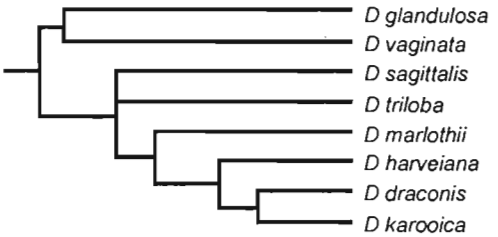

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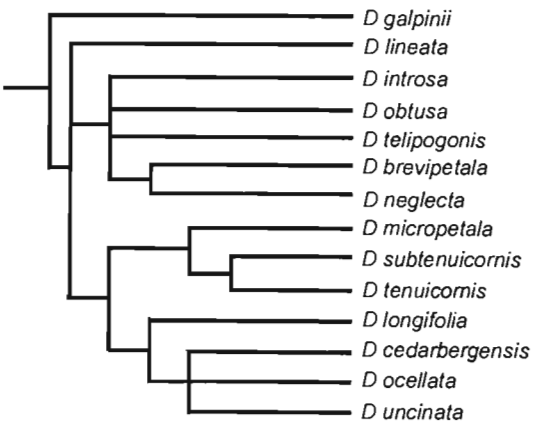
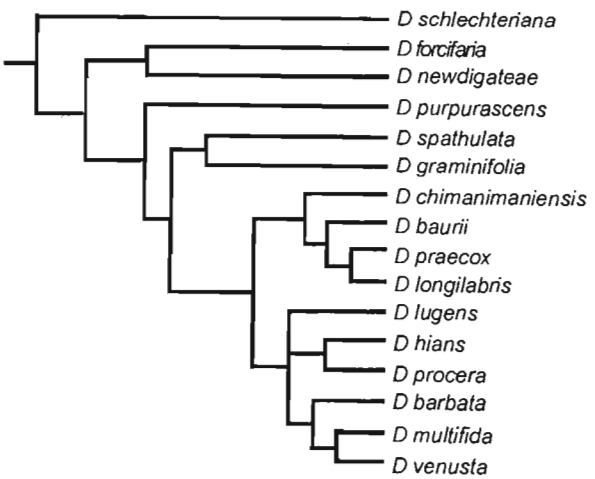
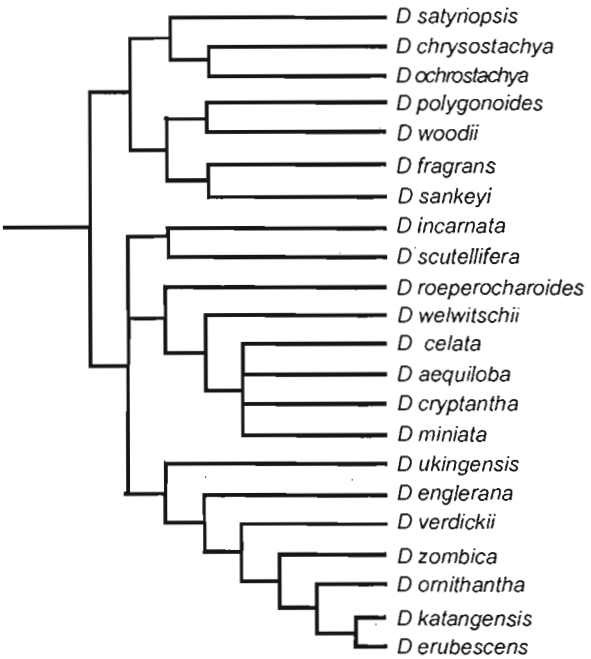


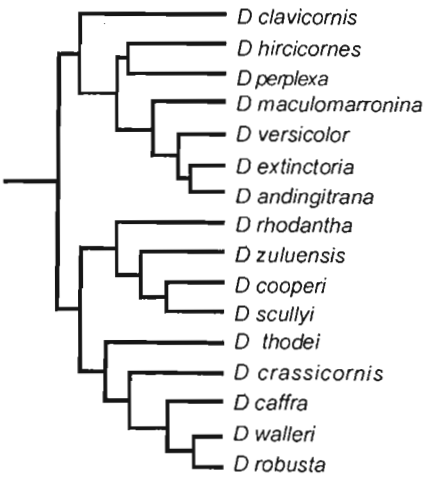
Section *Herschelianthe*



Section *Micranthae*



Section *Hircicornes*



APPENDIX TWO

The strict consensus tree of the 64 000 most-parsimonious trees obtained in the parsimony analysis of the *trnL-F* DNA matrix of 54 orchid taxa and 182 phylogenetically informative sites. Numbers above branches represent decay values followed by bootstrap percentages. Bootstrap values < 50% and decay values of 0 are not included. Numbers below branches are corresponding branch-lengths (BELLSTEDT *ET AL.*, 2001). The sections into which the species have been classified within the genus *Disa* are included (LINDER & KURZWEIL, 1999).

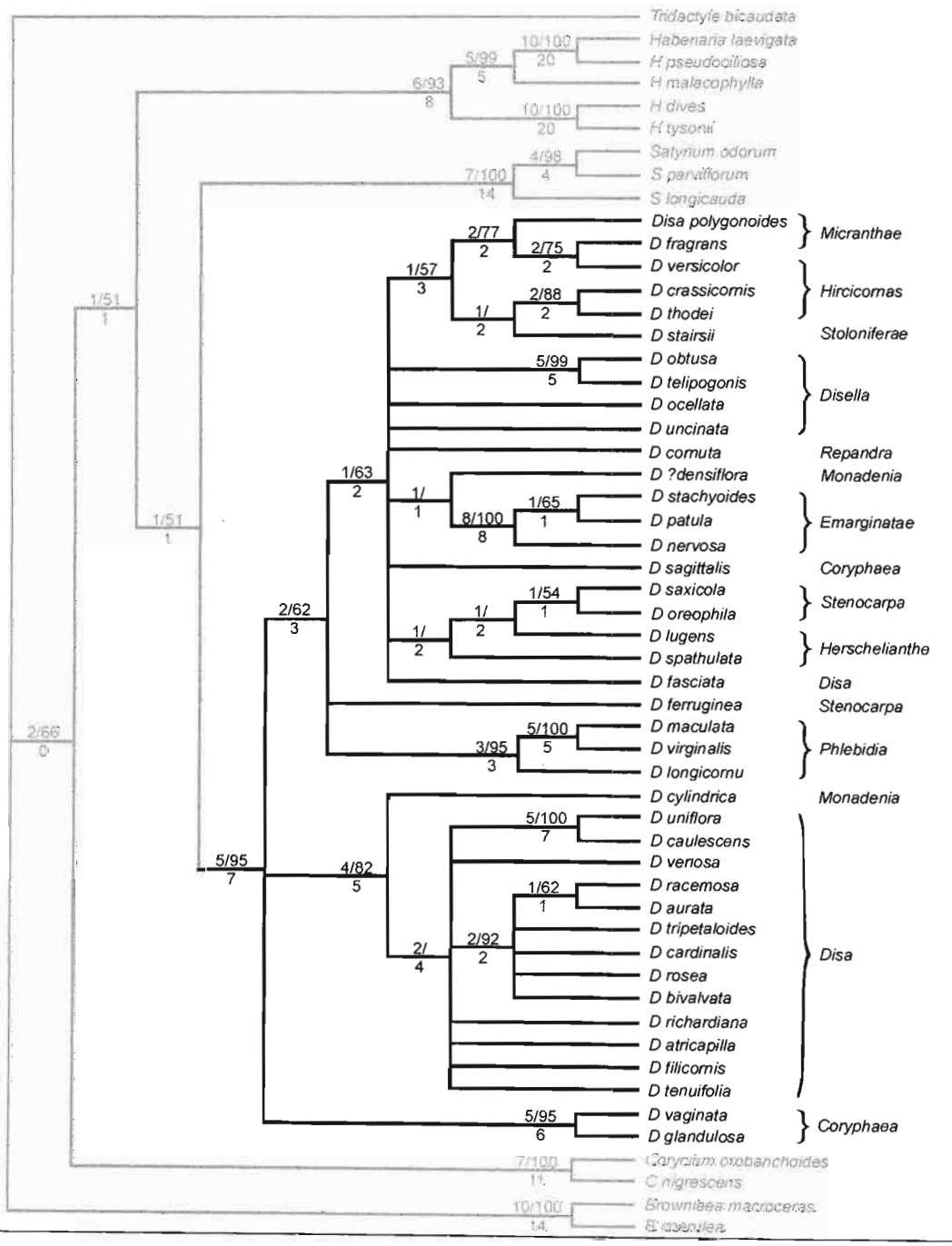


Plate 2.1. **Polymorphic seed morphology in the South African *Disa*. *Disa uniflora* – (A, B) and *Satyrium* – type (C – F) seed are included. The former were comparatively large and pyriform or subglobular in shape, the latter small and variously fusiform.**
Disa uniflora (A); ***D. tripetaloides*** (B); ***D. pulchra*** (C); ***D. versicolor*** (D); ***D. saxicola*** (E) and ***D. cornuta*** (F).

Plate 2.2. Polymorphic colouration in mature, *Satyrium* – type *Disa* seed. Seeds were variously brown in colour, with mid-brown representing the primitive condition in the genus. Alternative colour morphs evolved independently on multiple occasions. Comparative seed colouration (A) for *D. pulchra* (1), *D. nervosa* (2), *D. patula* (3) and *D. cooperi* (4); tan seed of *D. pulchra* (B); golden seed of *D. crassicornis* (C); mid-brown seed of *D. cooperi* (D); red-brown seed of *D. patula* (E); black-brown seed of *D. versicolor* (F) and red-brown seed of *D. nervosa* (G). Intraspecific colour polymorphisms occurred in *D. nervosa* and *D. versicolor*.

Plate 2.3. Testa anatomy and cell arrangement in the South African *Disa*. Filiform, colourless seeds (a) occurred in both *Satyrium* – (A) and *D. uniflora* – type (F) seeds. The testa cells of aborted seeds displayed a reticular-foveate arrangement, indicating similar ontogenetic pathways in the separate seed types. The testae of *Satyrium*- type seeds (B) were uninterrupted and consisted of collapsed cells with the outer periclinal walls (pw) adpressed to the inner ones, and to the anticlinal walls (aw). In contrast, the periclinal walls of *Disa uniflora* – type seeds (C – E) were convex, raised above the anticlinal walls. Circular perforations (cp) and cracks (c) disrupted the integrity of the testae in these species.
Disa versicolor (A); *D. pulchra* (B); *D. tripetaloides* (C and E, F); *D. uniflora* (D).

Plate 2.4. **Testa anatomy in the South African *Disa*. Testa cells for all *D. uniflora* – type seeds were without ornamentation, as were the majority of *Satyrium* – type seeds. Linear concavities** (lc) **occurred on the testae of *D. cooperi*** (A) **and *D. saxicola*** (B) **seed, two unrelated species. *Satyrium* – type seeds comprised three and four cells in their long and short axes respectively. *Disa woodii*** (C) **revealed the extraordinary length attained by the cells** (el) **of select species. In *Satyrium* – type seeds the anticlinal walls** (aw) **of the testa cells were variously fused and the periclinal walls** (pw) **variously adpressed (*D. chrysostachya*;** D**). Incomplete wall fusion in *D. patula*** (E) **was revealed by triangular concavities** (tc) **and medial grooves** (g) **between adjacent cells. The determination of cell margins in *D. uniflora* – type seeds was difficult due to the convex nature of the periclinal walls (*D. tripetaloides*;** F**).**

Plate 2.5. Embryo morphology in the South African *Disa*. *Satyrium* – type seeds were typified by globose (*D. cornuta*; A) or sub-globular (*D. woodii*; B) embryos. Embryonic cellular detail was obscured by the carapace (ec), a derivative of the inner integument. Separation of testa cells revealed the thickness (t) of the testa. A blind-ended opening (o) dominated the suspensor (*D. pulchra*; C).

Figure 3.1. Germination and primary seedling development for small-seeded, summer-rainfall *Disa* species. Mature (A) and immature seed culture (B) protocols are included, together with beneficial media manipulations. Immature seed cultures were initiated using transverse capsule sections (c). Media with increased water potential (decreased inorganic salt concentrations and decreased media viscosity) enhanced the success of both protocols. The combination of immature seed and media manipulation resulted in > 50 % germination, the highest percentage recorded for a summer-rainfall *Disa* under conditions of single-phase media. Primary seedling development was independent of seed maturity, with protocorms emerging 12 – 20 weeks after initiation (C). Protocorms displayed rhizoids (r) and single apical meristems (m) from protrusion. Four to six weeks after germination (D), protocorms developed leaf primordia (lp), especially in cultures with increased water potential. True leaves (l) and roots were observed 8 – 10 weeks after germination (E) and define primary seedlings, which are expected to reach maturity. The attainment of primary status was enhanced by increased media viscosity and the provision of charcoal.

Plate 4.1. Ultra-structure and mature seed nutrient reserves in small-seeded *Disa* species. These species contained abundant lipid (li) and protein (p) reserves, but never starch. Light microscopy of sectioned *D. stachyoides* seed (A) revealed lipids as pink and nuclei (n) as purple (haemotoxylin-eosin *Y* staining). Several cells contained large areas free from reserve material (*). The seed testa (t) and embryo carapace (ec) are indicated. Scanning electron microscopy of *D. pulchra* (B), D. *crassicornis* (C) and *D. versicolor* (D) embryos revealed transparent lipids and comparatively dense protein reserves. Typically smaller lipid bodies formed a layer (indicated) adjacent to the cell wall (cw). By two weeks after germination reserves were depleted and *D. pulchra* (E) protocorm cells were highly vacuolate (v), with organelles being pressed against the nucleus (n).

Plate 4.2. Ultra-structure and mature seed nutrient reserves in large-seeded *Disa* species. Storage in these species was dominated by starch (s), which is atypical for members of the Orchidaceae, but is permitted by their increased size. Starch reserves are characteristic of immature and post-imbibition orchid seed and may account for the rapid germination observed in the large-seeded species. Scanning electron microscopy of *D. uniflora* (A) and *D. tripetaloides* (B, C) embryos revealed that lipid reserves were negligible and protein absent. The cell wall (cw) is also indicated. By two weeks after germination reserves were depleted and *D. uniflora* (D) protocorm cells were highly vacuolate (v), with organelles being pressed against the nucleus (n). Numerous mitochondria (m) were present.