Horticultural propagation of the threatened species, *Syncarpha recurvata* (L.f.) B.Nord.

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Abstract

Syncarpha recurvata (L.f.) B. Nord. (Asteraceae, Gnaphalieae) is listed as a vulnerable (intermediate priority) species. The propagation methods of achene germination, stem cuttings, air layering and micropropagation were investigated to obtain an efficient propagation method. Smoke water dilutions of 1:100, 1:50 and 1:10 significantly increased germination, with the 1:100 smoke water dilutions showing the highest germination percentage (22.4%). Achenes collected between August and October 2005 showed higher germination and viability than achenes collected between May and July 2006. Fifty percent of the stem cuttings and 32% of the air-layered branches rooted. Embryo culture was the only successful culture type out of the four attempted. Two embryos germinated and four became photosynthetic. Two of the germinated embryos also produced callus cells, a medium supplemented with 5 µM IAA may be used to test for a suitable plant regulator for organogenesis or embryogenesis. *Syncarpha recurvata* plants were also transplanted into pots and 60% survived. A growth rate of 1.466 mm y^{-1} was obtained from total shoot length measurements. In this study, it was found that air-layering branches of transplanted *Syncarpha recurvata* plants is the most efficient propagation method.

Chapter 1: General introduction

Syncarpha recurvata (L.f.) B. Nord. is listed as a vulnerable (intermediate priority) species nationally and in the Eastern Cape (Hilton-Taylor 1996). This species is endemic to the Eastern Cape and limited to calcareous sediment deposited on ridges (Watson 2002). This sediment essentially consists of shallow sandy soils overlaid on a substantial calcrete layer (Campbell 1995). *Syncarpha recurvata* is vulnerable principally due to the disturbance of populations by mining activities and urban expansion (Golding 2002). It is exploited as a cut flower and makes an attractive everlasting (Golding 2002).

Syncarpha recurvata occurs in Bontveld (Watson 2002) in an area of about 500 km^2 within the Eastern Cape (Todkill 1996). Bontveld consists of a mosaic of bushclumps and grassveld. The bushclumps have thicket affinities while the grassland consists principally of dwarf shrubs and grasses (Watson 2002). The populations of *S. recurvata* are small, with mature individuals in a continuing state of decline (Golding 2002). Their distribution stretches from around Port Elizabeth up to Alexandria (Todkill 1996). Soils in the Grassridge community of *S. recurvata* typically have a pH of around 8.45 (Weatherall-Thomas 2005). The grassland soil also has higher concentrations of cations (Ca^{2+}, Na$^+$, Mg^{2+}, K$^+$) than bushclump soil (Cowling pers. comm. cited in Watson 2002).

Syncarpha recurvata is a robust, corymbosely branched shrub about 12 to 30 cm in height (Gledhill 1981). Its leaves are linear lanceolate, 12-20 mm long, 4-6 mm wide and infolded (Gledhill 1981). The leaves have a green, glabrous adaxial surface and a densely hairy abaxial surface (Gledhill 1981). Flowers are borne in hemispheral, radiating heads, about 12.5 mm across, which are obtuse at the base (Gledhill 1981). The flowers can be identified by their yellow florets surrounded by bright pink involucral scales (Home and Campbell 1996). These involucral scales become silvery-white with age (Todkill 1996). Similar to *Syncarpha vestita* (L.) B. Nord., propagules are one-seeded fruits (achenes) (Brown 1993).

Syncarpha recurvata belongs to the tribe Gnaphalieae and subtribe Gnaphaliinae within the family Asteraceae (Bayer, et al. 2000). The Gnaphalieae, known locally as everlastings, are a tribe of Asteraceae that show highest diversity in South America, Southern Africa, and Australia (Bayer*, et al.* 2000). *Syncarpha recurvata* was previously known as *Helichrysum recurvatum* (Geldenhuys 1997).

Seven populations of *Syncarpha recurvata* have been recorded within the 500 km^2 area in which the species occurs (Campbell 1995). These populations occur in areas at an elevation range from a few meters above sea level to 200 m above sea level (Todkill 1996). The seven populations were reported to be at Van Stadens, Bethelsdorp, Swartkops, St. George's Strand, Coega, Kaba Valley and Grassridge (Campbell 1995). Of these only four remain. These are the Van Stadens, Coega, Kaba Valley and Grassridge populations (Campbell 1995). Studies have been done on the population ecology of the Coega and Grassridge populations. Geldenhuys (1997) did a study on the population age structure and geographical distribution of the Coega population. Todkill (1996), Eastwood (1998) and Watson (2002) compiled reports on the population structure of the Grassridge population.

The aim of this study was to establish a propagation method for the horticultural development of *Syncarpha recurvata* and to design a protocol for propagation that could ultimately be used for the conservation of *S. recurvata* and the restoration of *S. recurvata* populations in the wild.

To investigate the growth and reproduction of *Syncarpha recurvata*, achenes and plants were taken from the study site and the effectiveness of propagation through germination, stem cuttings, air layering and tissue culture was assessed.

1.1 Transplanting

Home and Campbell (1996) excavated plants and potted them into 540 mm diameter pots. None of these transplanted plants survived (Home and Campbell 1996).

Todkill (1996) transplanted juvenile and adult individuals into shallow containers in a greenhouse (Todkill 1996). Juveniles were defined as shorter than 15 cm and unbranched or slightly branched and adults defined as plants taller than 15 cm and repeatedly branched. These shallow containers were filled with calcareous soil obtained from Grassridge (Todkill 1996). After 35 days 50% of the adults and 57% of the juveniles remained alive (Todkill 1996). After about 6 months two thirds of the surviving plants died, with no individuals surviving after one year (Todkill pers comm.). These mortalities were however largely due to neglect (Todkill pers comm.) and do not reflect the survival of transplanted individuals as they were not propagated under suitable conditions.

1.2 Propagation by Achenes

Achene germination was the only successful propagation method in all previous studies in which the propagation of *Syncarpha recurvata* was attempted (Home and Campbell 1996, Todkill 1996, Weatherall-Thomas 2005).

In all studies involving propagation from achenes, achenes were harvested from mature inflorescences collected from the Grassridge population. Home and Campbell (1996) obtained the best germination results using achenes soaked in distilled water for 24 hours and subsequently placing the achenes on three layers of paper towel and treating them with a 500 mg l^{-1} gibberellic acid solution. The paper towel and achenes were placed in petri dishes (Home and Campbell 1996). These were covered and placed in a dark controlled environment at 22°C. A percentage germination of 20% was obtained (Home and Campbell 1996).

Todkill (1996) had three achene germination treatments . In the most successful one achenes were soaked in distilled water for 24 hours, placed in soil obtained from Grassridge and covered with a single layer of filter paper. Todkill (1996) also treated the soil with a 500mgl $^{-1}$ gibberellic acid solution and 'Kaptan', a fungicide. After germination the seedlings were transplanted to a Petri dish containing soil from Grassridge and watered with distilled water (Todkill 1996). These seedlings were exposed to 12 hours of light and 12 hours of darkness at 23°C (Todkill 1996). The percentage germination was not reported.

After reaching a height where they were too tall for the Petri dish they were transplanted into a seed tray containing soil obtained from Grassridge and kept under the same light and temperature regime (Todkill 1996). All of these seedlings died within 24 hours of transplantation. Better results were obtained when the seed tray the seedlings were transplanted to was covered with a glass sheet (91% survival after 50 days) (Todkill 1996).

Weatherall-Thomas (2005) germinated *Syncarpha recurvata* achenes on two layers of double-ply paper towel placed in a Petri dish and covered with a layer of cling film. He found that a 100 mM $CaCl_2$ solution and a 1:50 smoke water dilution treatment stimulated germination (Weatherall-Thomas 2005).

Home and Campbell (1996) and Weatherall-Thomas (2005) obtained a low percentage germination for chemical and mechanical scarification treatments compared to gibberellic acid and smoke water treatments. This suggests that it is likely that endogenous dormancy mechanisms control the germination of *Syncarpha recurvata*.

1.3 Propagation by cuttage and air-layering

Home and Campbell (1996) cut stems into 100 mm long portions, removed all or some leaves and treated the cut ends with Seradix[©] no. 3 rooting hormone. None of these cuttings rooted successfully. They also attempted root cuttings, which showed

no success (Home and Campbell 1996). Air-layering has not been attempted in previous studies.

1.4 Micropropagation

Home and Campbell (1996) used agar as a support medium. Agar was made up at a concentration of 13.5 g l^{-1} in distilled water. Apparatus and liquids were sterilised by autoclaving at 121°C for 20 minutes. After boiling, Provasoli nutrient medium was added to the agar solution at a final concentration of 10 ml Provasoli l^{-1} (Home and Campbell 1996).

Stem apices were cut with a sterile scalpel. Cut tissue was subsequently disinfected by dipping it into a 0.5% sodium hypochlorite solution and washed three times in sterile distilled water. Apices with and without leaves were incubated on agar. Glass vials containing the cultures were plugged using cotton wool and incubated in a controlled environment of 12 hours light and 12 hours dark. Day temperature was 22°C and night temperature was 20°C (Home and Campbell 1996).

Although the tissue remained green, no growth was recorded after four weeks. Most of the apices developed mould. According to Home and Campbell (1996) this was probably due to the leaf hairs forming a barrier against the sterilant.

1.5 Implications for restoration, conservation and propagation

Finding an efficient and practical propagation method will aid in the restoration of *Syncarpha recurvata* populations in the wild. Such a propagation method will also aid in conserving this threatened species by enabling a gene bank to be established and allowing the plants to be commercialised.

Chapter 2: Transplanting

2.1 Introduction

The transplantation of plants is important in restoring damaged habitats (Given 1994). The low transplantation success observed by Home and Campbell (1996) as compared to the results of Todkill (1996) could possibly be explained by the low replication used by Home and Campbell (1996).

Growth rate can be used as a measure of the success of transplantation. Due to the branched nature of *Syncarpha recurvata*, total stem length is an appropriate measure of growth. Growth can be expressed as the relative growth rate (RGR) (Raveh, et al. 1993).

Relative growth rate is defined by the equation:

$$RGR = (Lt_2 - Lt_1) / Lt_1$$

Where Lt_1 is the total stem length at the time of transplantation and Lt_2 is the total stem length at time t after transplantation (Raveh, *et al.* 1993).

Transplanting whole plants may be the best option if a population is facing destruction and when there is a complete failure of propagation with propagules (Given 1994). It is sometimes necessary or advantageous to bypass germination in the field and use mature plants to establish populations (Davy 2002). Successful transplantation of *Syncarpha recurvata* plants will be important in rehabilitation success.

Helichrysum obconicum (Gnaphalieae), an endemic plant of Madeira Island (Portugal) showed good adaptation to pots in a study done by Oliveira Dragovic *et al.* (2005).

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Pinching techniques promoted sprouting, originating 2-6 new sprouts in the stem of *Helichrysum obconicum*. Such pinching techniques involve removing the top apical meristem so that apical dominance is suppressed (Oliveira Dragovic*, et al.* 2005).

The aims of this experiment were to investigate transplantation success and to assess the suitability of translocating whole *Syncarpha recurvata* for rehabilitation.

The individuals transplanted by Todkill (1996) had a 57% survival for individuals shorter than 15 cm and a 50% success for individuals taller than 15 cm. The tallest plant used in this study was 15.8 cm tall, while the average main stem length was 7.2 cm. It was expected that the survival obtained in this study would be close to that of Todkill (1996) and that whole plants will be suitable for translocation as with *Helichrysum obconicum*.

2.2 Materials and methods

2.2.1 Plant collection

A total of 150 individuals less than 158 mm in height were collected from the Grassridge population on 11 May 2006. Grassridge is situated along the R335 road, 40 km north of Port Elizabeth and 20 km south of Addo (Watson 2002). It consists of a number of farm units owned by PPC Cement (Pty) Ltd. with a total area of 8000 ha (Watson 2002). The plants were potted in the Nelson Mandela Metropolitan University Botany department greenhouse. The soil used was a 1:2 mixture of peat and calcareous soil collected from Grassridge. The plants were placed under shade cloth adjacent to the greenhouse.

2.2.2 Survival

Survival was recorded on a monthly basis for four months. Survival was defined as plants whose foliage was not totally discoloured.

2.2.3 Growth rate

The plants were numbered from 1 to 150 and their total stem length was measured using a pair of callipers.

2.2.4 Statistics

All data was positively skewed and leptokurtic and hence non-parametric statistics were used. To compare the position of data sets Mann-Whitney U tests were done. A Spearman rank correlation was done to test for correlations between variables. All statistical tests were done using Statistica$^{©}$ 7.0, copyright StatSoft, Inc., at 95% confidence limits.

2.3 Results

2.3.1 Survival

Out of the 150 plants collected from the study site for transplantation, 90 survived (60%). To examine which plant sizes would be the most suitable for transplantation the total stem lengths were grouped into 10 length classes (or size classes), each of consisting of 15 individuals (Figure 1). Eight of these size classes had fewer individuals after 5 months, while two size classes increased in size.

The $261 < x <= 357$ and the $524 < x <= 906$ size classes appear to have the highest percentage survival with 120% and 140% survival respectively. These new recruits to are likely to have come from the preceding size classes, the $196 < x <= 261$ size class and the $357 < x <= 524$ size class. These two smaller size classes may also be suitable for transplantation. These four size classes can be grouped into a single size class of $196 < x <= 906$. The average plant height of the plants in this size class was 93 mm ± 3 S.E. when measured directly after transplantation.

Figure 2 shows the number of surviving plants recorded on a monthly basis after transplantation. In the first month, 91% of the plants still appeared healthy. The second month showed the highest number of mortalities (26) and left 75% of the plants. Numbers declined steadily after this with 65%, 62% and 60% of the plants surviving after three, four and five months respectively.
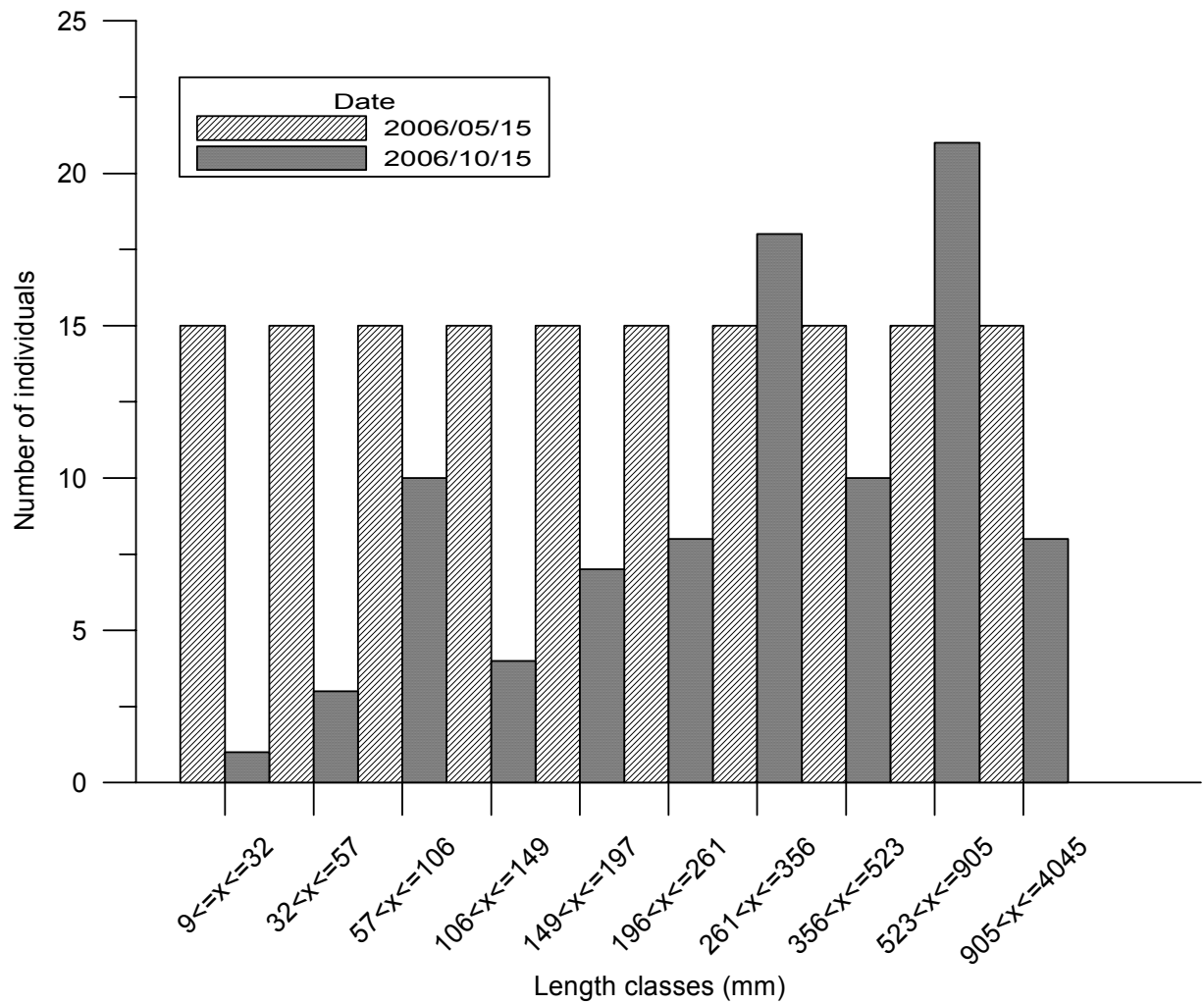
Figure 1: Number of transplanted individuals of *Syncarpha recurvata* in each stem length class as well as the number in each stem length class that survived after a 5 month period.

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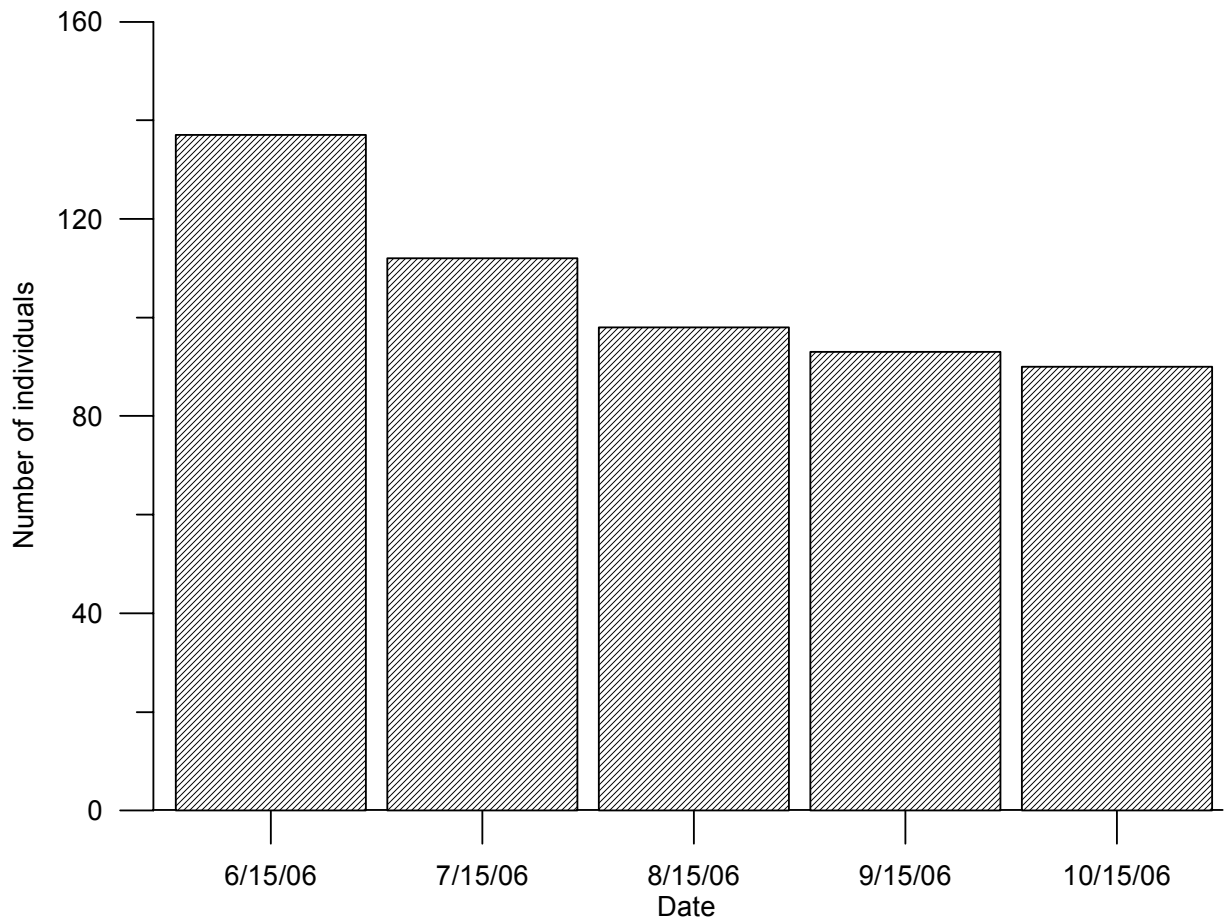
Figure 2: Number of surviving transplanted *Syncarpha recurvata* plants over a 5 month period.

2.3.2 Growth rate

To determine relative growth rate the total stem lengths of the surviving transplanted plants were compared to the total stem lengths measured directly after transplantation using the equation presented in Section 2.1. Growth rate was calculated to be 1.466 mm y^{-1}. Total stem length increased significantly after five months, from 381± 45 S.E. to 460 ± 43 S.E. (U = 2983, p = 0.002, N = 90). There was no significant difference between the number of stems after a 5 month period (U = 3485, p = 0.105, N = 90). Relative growth rate was significantly negatively correlated with total stem length (r = -0.209, p < 0.05, N = 90; Figure 3).

Figure 3: The negative correlation between total stem length and relative growth rate in transplanted *Syncarpha recurvata* plants, the red line represents a linear fit.

2.4 Discussion

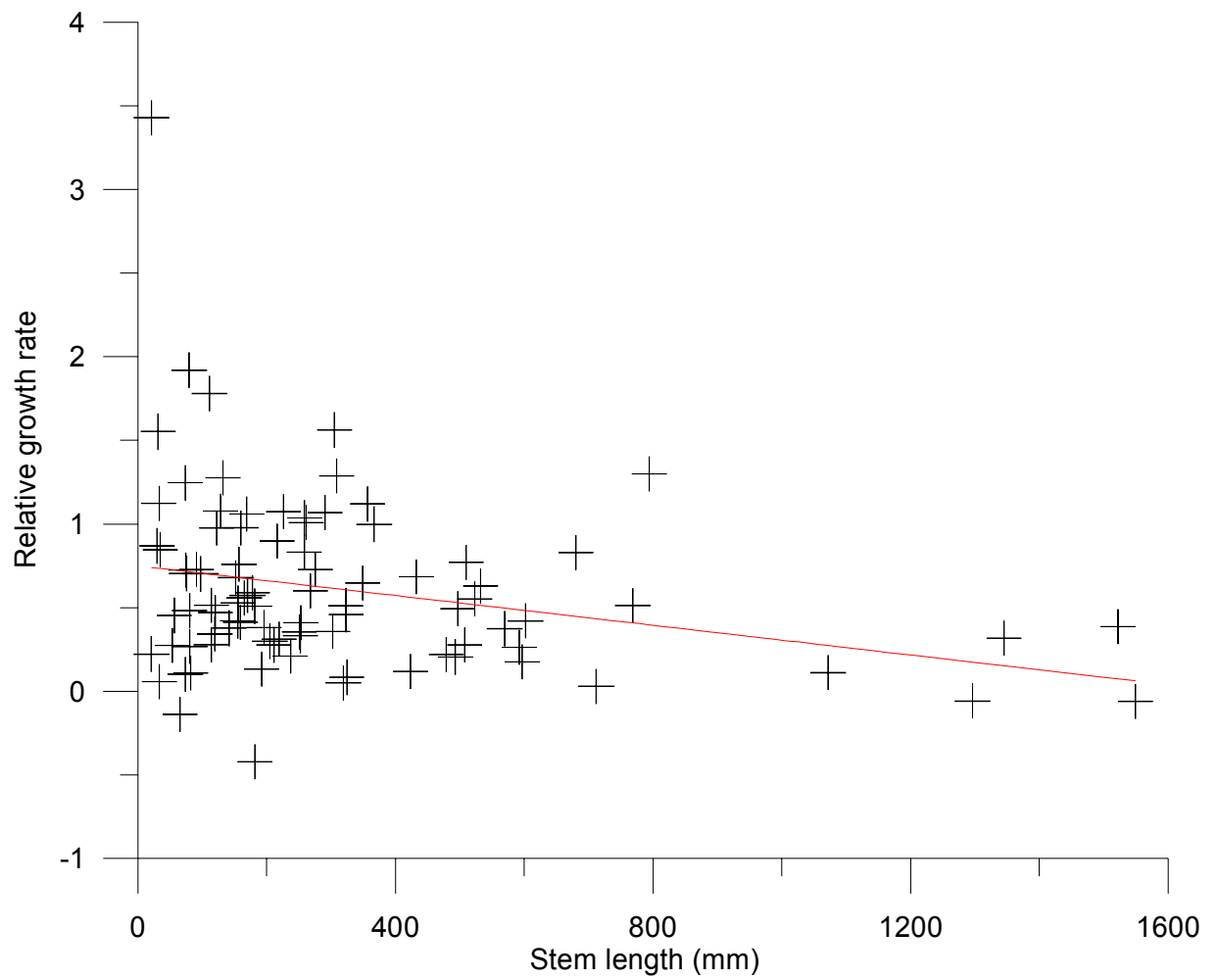A large number of the smaller individuals (9<=x<=196) did not survive the stress of transplantation (Figure 1). Despite their higher growth rate (Figure 3) they are not suitable for transplantation because of the high mortality observed.

The 261<x<=357 and the 524<x<=906 size classes appear to have the highest percentage survival (120% and 140% respectively). The 196<x<=261 and the 357<x<=524 size class should also be regarded as suitable for transplantation as they were the source of the new recruits in these size classes. A single measurement of plant height (93 mm ± 3 S.E.) can be used to select plants suitable for transplantation for rehabilitation.

The percentage survival obtained in this study after one month was 34% higher than that obtained after a month by Todkill (1996) for individuals under 150 mm and 41% higher than the percentage survival of the individuals taller than 150 mm after one month. The higher percentage survival is probably due to the addition of peat to the soil mixture. The porous nature of peat allows unrestricted root growth and supplies oxygen to the roots.

The significant increase in stem length shows that the transplanted plants were able to obtain enough resources during propagation. After 5 months, roots emerged from the holes underneath the pots showing that the plants had re-established their root systems in the new substrate.

The results obtained in this study and those of Todkill (1996) show that whole *Syncarpha recurvata* plants are suitable for translocation, however a large number of them will not survive transplantation. It is also not known from this study how successful in the long term the transplantation process will be.

The information reported above about the success of transplanting *Syncarpha recurvata* will aid in the design of a successful restoration protocol. It is of concern that plant mortality was as high as it was. Survival of plants translocated in the field may be better than the survival of plants placed in pots, which potentially limits root growth. Most mortality occurred after two months. After this time period the rehabilitation site should be checked for mortalities for the first time. Dead plants should be replaced with another plant in the 93 mm ± 3 S.E. size class. Many of the plants flowered and fruited during the transplantation experiment. The achenes produced by these flowers could germinate at the rehabilitation site. In this way an individual that flowers and fruits may contribute to the population at the restoration site although it only survives long enough to allow achene distribution to take place.

Chapter 3: Propagation by achenes

3.1 Introduction

3.1.1 Seed structure

An angiosperm seed is typically comprised of the embryo, which is the result of fertilization of the egg cell in the embryo sac by a male pollen tube nucleus; the endosperm, which arises from the fusion of two nuclei in the embryo sac with the other pollen tube nucleus; the perisperm, which is developed from the nucellus; and the protective testa or seed coat, which is formed from one or both the integuments around the ovule (Bewley and Black 1994, Hartmann and Kester 1965).

The embryo is the diploid result of fertilization and is a minute autotrophic plant (Edmond, et al. 1964). The embryo principally consists of an embryonic axis and at least one cotyledon (Bewley and Black 1994). The axis further consists of the embryonic root (radicle), the hypocotyl with attached cotyledons and the shoot apex with the first true leaves (plumules) attached (Bewley and Black 1994).

The nourishing tissue is generally either the endosperm or cotyledons (Edmond, *et al.* 1964). In the case of endospermic seeds, the endosperm, which is present in the mature seed, serves as food storage organ (Hartmann and Kester 1965). Here the testa and endosperm are the two layers covering the embryo (Bewley and Black 1994). In non-endospermic seeds the cotyledons serve as sole food storage organs (Bewley and Black 1994). During development, the cotyledons absorb the food reserves from the endosperm. Here the embryo is enclosed by the testa and the endosperm is all but completely degraded in the mature seed (Bewley and Black 1994).

In some cases the testa exists in a rudimentary form only and the prominent and outermost structure is the pericarp or fruit coat derived from the ovary wall (Hartmann and Kester 1965). In such cases the embryo is also encased in a fruit (Bewley and

Black 1994). In achenes these fruit and seed layers are continuous (Hartmann and Kester 1965). The seed coverings provide mechanical protection to the embryo and make transportation and storage of seeds possible (Hartmann and Kester 1965).

Hairs or wings, which aid in seed dispersal, sometimes develop as a modification of the enclosing fruit coat (Bewley and Black 1994). These are attached via the hilum (Bewley and Black 1994). The hilum is a funicular scar on the seed or fruit coat that marks the point at which the seed was attached via the funiculus to the ovary tissue (Lawrence 2000). In many cases a small hole, called the micropyle, can be seen at one end of the hilum (Bewley and Black 1994).

Seeds store various substances that are important for germination and early seedling growth. These primarily include carbohydrates, fats and oils, and proteins (Bewley and Black 1994, Mayer 1977). Other important substances that are only stored in small amounts include alkaloids, lectins, proteinase inhibitors, phytin, and raffinose oligosaccharides (Bewley and Black 1994, Raghavan 1976). Most seeds store their major food reserves within the embryo; usually the cotyledons (Bewley and Black 1994). Some plants also have their seed storage reserves within extra-embryonic tissues. These extra-embryonic tissues used for storage include the endosperm (Gymnosperms) or the perisperm (*Coffea arabica*). Both embryonic and extra-embryonic tissues can also be used for storage; such as in maize (Bewley and Black 1994).

3.1.2 Seed germination

Germination starts with the uptake of water by a seed and ends with the onset of elongation of the embryonic axis, usually the radicle (Bewley and Black 1994). It also includes the steps of protein hydration, subcellular changes, respiration, macromolecular syntheses and cell elongation (Bewley and Black 1994, Raghavan 1976). The combined effect of these steps is to transform a dehydrated, dormant embryo into an embryo which grows actively and accumulates water (Bewley and Black 1994, Mayer 1977). A seed in which none of these processes have taken place is said to be quiescent. They characteristically have low moisture content (5-15%)

and an extremely slow metabolic rate (Bewley and Black 1994). Seeds are able to survive in this state for a number of years.

Quiescent seeds require an environment of suitable temperature, hydration and the presence of oxygen in order to germinate (Bewley and Black 1994). In dormant seeds however, some of these processes may occur without subsequent cell elongation and germination (Bewley and Black 1994). In these seeds dormancy must be broken by certain priming treatments (Heydecker 1977).

The major cellular processes involved in the initiating and facilitating of radicle emergence include respiration, RNA and protein synthesis and enzyme and organelle activity (Bewley and Black 1994, Raghavan 1976).

During imbibition various structural and physical changes occur (Bewley and Black 1994). The completion of imbibition requires a small amount of water (not more than three times the seed's dry weight). For successful subsequent root and shoot growth a larger and more constant supply of water is essential (Bewley and Black 1994).

The uptake of water by the seed can be seen as triphasic (Figure 4) (Bewley and Black 1994). Phase 1 involves imbibition, where a strong osmotic gradient results in the uptake of water from the soil (Bewley and Black 1994). Different areas of the testa and different organs within the seed often absorb variable amounts of water. Phase 2 is seen as a lag phase. Here the matric forces of the seed cells that caused the strong osmotic gradient in phase 1 are no longer active (Bewley and Black 1994). During this phase major metabolic events take place in preparation for radicle emergence (Bewley and Black 1994). Only germinating seeds, and not dormant seeds, enter phase 3. This stage is associated with changes in the cells of the radicle and radicle elongation (Bewley and Black 1994, Jann and Amen 1977). These changes are facilitated by the uptake of water (Jann and Amen 1977). This uptake of water is a result of the production of low-molecular-weight osmotically active substances (Bewley and Black 1994). These substances are produced as a result of

hydrolysis of stored reserves (Bewley and Black 1994). The kinetics of water uptake is however more complex than this, as many seeds distribute water to different parts at different rates.
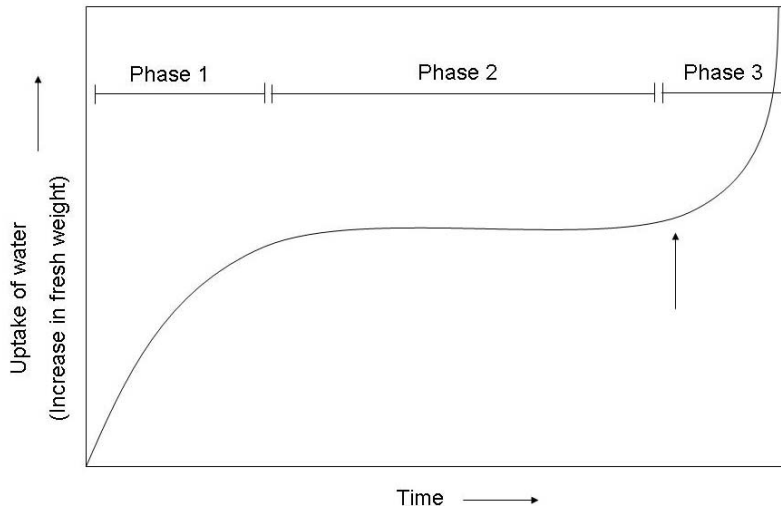
Figure 4: The triphasic pattern of water uptake by germinating seeds, with arrow showing the time of radicle protrusion (Bewley and Black 1994).

For germination to be completed, the radicle must expand and penetrate the surrounding structures (Bewley and Black 1994). This does not require cell division. Instead, the radicle cells elongate as the radicle penetrates through the surrounding tissues and cell division starts some time after this penetration.

There are a number of possible requirements for radicle elongation (Bewley and Black 1994). One such requirement is the lowering of the osmotic potential as a result of the accumulation of solutes within the radicle; this increases water uptake and raises the turgor pressure, which facilitates cell elongation.

The initial growth of the seedling follows one of two distinct patterns (Hartmann and Kester 1965). The seedling either follows the pattern of epigeous germination, where the hypocotyl elongates and raises the cotyledons above the ground, or hypogeous germination, where the lengthening of the hypocotyl does not cause the cotyledons to rise above the ground and only the epicotyl emerges (Hartmann and Kester 1965).

3.1.3 Measuring germination

It is incorrect to equate germination to seedling emergence from soil, as germination ends sometime before this (Bewley and Black 1994). Emergence of the axis can however be used as a precise measurement of termination of germination (Bewley and Black 1994).

The progress of germination is expressed as a percentage of the total number of seeds tested at time intervals throughout the germination period (Bewley and Black 1994). When this relationship is expressed graphically it ordinarily yields a sigmoid curve.

Some valuable conclusions can be drawn from variations in the shape of such a curve. If the curve flattens off when only a low percentage of the seeds have germinated it indicates that the seeds have a low germinating capacity (Bewley and Black 1994). The shape of the curve also describes the uniformity of germination (Bewley and Black 1994).

3.1.4 Promotion and inhibition of germination

<u>3.1.4.1 Gibberellin and abscisic acid</u>

Gibberellins (GA) promote the induction of cell wall hydrolases and thereby promote endosperm weakening and endosperm rupture (Marion-Poll 1997). Abscisic acid (ABA) inhibits the induction of cell wall hydrolases and thereby inhibits this weakening and rupture (Marion-Poll 1997).

GA promotes and ABA inhibits the embryo growth potential. ABA, however, also plays an important role in seed development and germination, acquisition of desiccation tolerance, accumulation of proteins and lipid reserves, and induction and maintenance of seed dormancy (Marion-Poll 1997).

The expression of genes encoding enzymes that mobilise food reserves is induced by several known GA signalling factors. These food reserves include the starches, proteins and lipids that are stored in the endosperm (Peng and Harberd 2002). During protein rehydration GA biosynthesis is induced by a phytochrome-mediated light signal and the newly synthesised GA down-regulates the expression of protein repressors of germination. These are also activated by rehydration, by protein degradation, suppression of transcription or mRNA degradation (Marion-Poll 1997). This newly synthesized GA also initiates signals to induce the expression of hydrolytic enzymes that modify the cell wall and weaken the endosperm cap, thus facilitating germination (Peng and Harberd 2002).

It has also been proposed that GA could promote the formation of low molecular weight mono- and disaccharides, which assist the intracellular generation of negative water potentials, thus aiding radicle emergence (Tian, et al. 2003).

3.1.4.2 Ethylene

Increased ethylene evolution accompanies seed germination of many species (Baskin and Baskin 1998). Ethylene promotes its own biosynthesis during pea seed germination by positive feedback regulation of 1-aminocyclopropane-1-carboxylic acid oxidase.

3.1.4.3 Smoke

When germinating the fynbos fire ephemeral, *Syncarpha vestita,* with smoke water, Brown and van Staden (1997) obtained a mean percentage germination of 100% germination, opposed to a germination percentage of 30% for the control. Although it is known that there is a general lack of fire in Bontveld (Cowling pers. comm. cited in Watson 2002) a positive response of *Syncarpha recurvata* to smoke water was shown by Weatherall-Thomas (2005). Many plant species have shown increased percentage germination and vigour after exposure of seeds or seedlings to smoke water (Minorsky 2002). These include plants naturally adapted to areas of high fire frequency as well as some species that are not specifically adapted to these conditions. These include agriculturally useful species, such as maize (Sparg, et al.

2005) and lettuce (Brown and van Staden 1997), as well as many aesthetically useful species, such as those naturally occurring in fynbos (Brown and van Staden 1997). Species originating from natural fire-prone habitats include many species of South African fynbos such as the fire-climax grass, *Themeda triandra* (Poaceae) and members of the Mesembryanthemaceae (Brown and van Staden 1997). Other examples of such smoke water stimulated plants include species of the California chaparral and many other fire-prone communities (Blank and Young 1998). It has been suggested that the promotive effect of smoke is independent of seed size and shape, plant life form and fire sensitivity (Brown and van Staden 1997). Smoke can also be utilised as an effective seed pre-sowing treatment, as the stimulatory effect of smoke is irreversible and can not be leached (Light, et al. 2002). Seeds treated with smoke are known to retain this stimulatory effect even after a year of storage (Minorsky 2002). The inhibitory effects of high smoke concentrations appear to be reversible and seeds grow with increased vigour after the smoke has been leached to a tolerable level (Light*, et al.* 2002). These inhibitory compounds are however not the same compounds that are involved in the promotion of germination (Light*, et al.* 2002). This effect would be a favourable adaptation to a post-fire environment as the inhibitory compounds will only leach with sufficient rainfall (Light*, et al.* 2002). Smoke thus enables seeds to germinate at the right time, grow faster and have a more robust root system and so have a major competitive advantage in their natural environment (Blank and Young 1998).

In early experiments by de Lange and Boucher (1990), smoke was generated by burning a mixture of fresh and dry plant material in a metal drum. This smoke was then fed into a polythene tent and allowed to settle on the soil where the seeds were stimulated to germinate. One disadvantage is that the germination cue of smoke is easily confused with the effect of temperature on germination. This is because temperatures slightly higher than ambient temperature significantly increase seedling emergence in many species (Baskin and Baskin 1998). Due to the complications of separating smoke from high temperatures, direct exposure to smoke is not recommended.

Aqueous smoke extracts were pioneered by De Lange and Boucher in 1990 and since then many authors have demonstrated that the active component of airborne smoke is soluble in water (Brown and van Staden 1997, Sparg, *et al.* 2005, Taylor and van Staden 1998). The method for preparing such a solution usually involves forcing smoke that has been generated in a drum to bubble through water (Brown and van Staden 1997). Combustion usually proceeds slowly and the burning material is made to smoulder, thus releasing relatively large quantities of smoke (Brown and van Staden 1997).

Blank and Young (1998) reported that smoke increases the permeability to solutes of a subdermal seed membrane for some species of California chaparral. They also stated that these specific mechanisms of fire cue stimulation may be species dependent. This response involves triggering via elevated nutrient content or via the presence of stimulating gases in the smoke or triggering chemicals that permeate the embryo and induce enzymatic changes that trigger germination. In an experiment done by Brown and van Staden (1997) the dormancy of celery seeds was broken by a combination of plant-derived smoke, benzyladenine and gibberellins in the dark at temperatures between 18°C and 26°C. From these results it could be argued that smoke extracts act in a similar way to cytokinins in the celery seed as it enhances gibberellin activity.

In a study conducted at the ultrastructure level by Egerton-Warburton (1998a) the causal factor(s) associated with seed dormancy and the stimulation of germination were investigated for *Emmenanthe penduliflora* (Hydrophyllaceae) seeds. It was found that a short exposure to smoke resulted in two major morphological changes. These changes are closely associated with the stimulation and acceleration of germination. The first major and most visible smoke induced morphological change observed was an intense chemical scarification of the external cuticle (Egerton-Warburton 1998a). This has a direct and destabilising effect on the external cuticle and is manifested as the formation of oil-like spheres or micelles. These micelles

increase the surface area of the seed for the exchange of water and solutes, as well as altering the hydrophobicity of the seed surface (Egerton-Warburton 1998a)

The second, more important smoke induced morphological change occurred at the internal cuticle. Here the exposure to smoke stimulus caused a significant increase in the number and diameter of permeate channels in the cuticle of *Emmenanthe penduliflora* seeds (Egerton-Warburton 1998a). The creation of such channels within the cuticle increases the permeability of this layer. It was shown through the use the fluorescent apoplastic tracer dye, lucifer yellow (carbohydrazide) that these channels permit the rapid exchange of water and solutes between the external environment and the seed.

Even though these major morphological changes have a large influence on the function of the cell, they do not significantly alter the general shape or dimensions of the cells (Egerton-Warburton 1998a). Egerton-Warburton (1998a) speculated the mechanism by which such morphological changes benefit the plant may be a synergy between the observed increased cuticular permeability and a simultaneous leaching of endogenous inhibitors of germination during imbibition.

They also further described the mechanism of formation of permeate channels. Because the permeate channels occurs in the cuticle, the formation of channels has to be the result of a preceding reaction between certain constituents of smoke and the semi-crystalline structure of waxes (Egerton-Warburton 1998a). This is a rather promising argument as the pyrolysis of cellulose alone produces a collection of compounds, such as aromatic hydrocarbons, ketones and a number of organic acids, which all have the potential to dissolve or modify waxes.

Smoke also contains a number of compounds that may act as surfactants (e.g. alcohols) (Egerton-Warburton 1998a). These surfactants modify cuticular layers by plasticizing the molecular structure of waxes (Egerton-Warburton 1998a). The sorption of such surfactants to the cuticle also creates hydrophilic channels. This

increases the area of channels within the cuticle that leads to accelerated transcuticular penetration of solutes in several species.

Using liquid chromatography, Blank and Young (1998) discerned over 30 organic anions in aqueous extracts of soil heated between 250°C to 450°C. Blank and Young (1998) speculated that these unknown compounds or combinations of compounds could be cueing agents. Flematti *et al.* (2004) recently identified the UV absorbance maximum and molecular weight of a germination-enhancing compound in smoke. They also assigned a molecular formula of $C_8H_6O_3$ to the compound. Recently they also identified the compound as the butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one and were able to synthesize it. They compared the activity of this synthesized butenolide with that of smoke water dilutions and found a high similarity in the germination delivered by these two compounds.

In an experiment conducted by Jager *et al.* (1996) it was found that aqueous smoke extracts prepared from a range of plants, as well as extracts prepared by heating agar and cellulose contained compounds that stimulated the germination of Grand Rapids lettuce seed. They also demonstrated that the same active compound is produced by burning *Themeda triandra* leaves, agar and cellulose by providing evidence obtained by thin-layer chromatography and high-performance liquid chromatography. This was also demonstrated with the same methods by Brown and van Staden (1997).

3.1.5 Seed dormancy

The imposition of dormancy is normally controlled endogenously and germination is initiated in response to a certain combination of environmental variables. Fluctuating environmental variables include cold, availability of minerals and light (Koller 1972). With the termination of dormancy, the metabolic processes of synthesis and growth are resumed (Koller 1972).

There are, generally speaking, two types of organic seed dormancy; endogenous and exogenous (Baskin and Baskin 1998). In endogenous dormancy there is some

characteristic of the embryo that prevents germination, while in exogenous dormancy it is some characteristic of the structures that cover the embryo that prevents germination. These structures include the endosperm, perisperm, testa and fruit walls (Baskin and Baskin 1998).

Seeds may, for example, be unable to germinate because of seed or fruit coats that are impermeable to water (Baskin and Baskin 1998). Before water uptake and subsequent germination can take place these blocks to germination must be removed.

There are a number of endogenous and exogenous dormancy types (Table 1).

Table 1: Organic seed endogenous and exogenous dormancy types (Modified from Baskin and Baskin (1998).

	Type	Cause	Broken by
Endogenous	Physiological	Physiological inhibiting mechanism (PIM) of germination	Warm/cold stratification
	Morphological	Underdeveloped embryo	Appropriate conditions for embryo germination/growth
	Morphophysiological	PIM of germination and underdeveloped embryo	Warm/cold stratification
Exogenous	Physical	Seed/fruit coats impermeable to water	Opening of specialized structures
	Chemical	Germination inhibitors	Leaching
	Mechanical	Woody structures restrict growth	Warm/cold stratification

Endogenous physiological dormancy is generally caused by a physiologically inhibiting mechanism of the embryo that prevents radicle emergence. The structures that cover the embryo, such as the endosperm and testa, may however also play a substantial role (Baskin and Baskin 1998). Physiological dormancy can be differentiated into non-deep, intermediate and deep physiological dormancy (Baskin and Baskin 1998). Embryos of seeds in non-deep and intermediate dormancy tend to germinate when isolated from the surrounding tissues while those of seeds in deep physiological dormancy do not (Baskin and Baskin 1998).

In the case of morphological dormancy, germination is prevented at the time of maturity due to the morphological characteristics of the embryo. The embryo is underdeveloped or even undifferentiated at the time of dispersal and a period of growth is required before the seed can successfully germinate (Baskin and Baskin 1998). Morphological dormancy occurs in seeds with rudimentary and linear embryos. Most of the interior of the these seeds is occupied by endosperm and the embryo may only be 1% of the seed volume or less (Baskin and Baskin 1998).

Morphophysiological dormancy is essentially a combination of the two dormancy types. In this case the embryo must grow to a species-specific critical size and the physiological dormancy of the seed must occur before germination can take place (Baskin and Baskin 1998).

The primary reason for dormancy of seeds with physical dormancy is the impermeability of their seed or fruit coats to water (Baskin and Baskin 1998). Seeds with physical dormancy frequently have a palisade layer of lignified cells in the testa or pericarp (Baskin and Baskin 1998). Physical dormancy is often found in combination with chemical dormancy.

Chemically dormant seeds do not germinate due to the presence of inhibitors in the pericarp. These inhibitors are usually removed by leaching (Baskin and Baskin 1998). These inhibitors are usually chemicals such as ABA (Abscisic acid).

Mechanical dormancy is usually the result of a hard, woody fruit wall. This woody structure is usually the endocarp or the mesocarp (Baskin and Baskin 1998). Dormancy can often be broken with a period of cold stratification. Upon germination the endocarp often splits into two halves (Baskin and Baskin 1998).

3.1.6 Seed viability and longevity

Some seeds are viable after several years, decades or even after a few hundred years (Bewley and Black 1982). There are several reports of herbarium specimens remaining viable for 80-120 years (Bewley and Black 1982).

Longevity is largely dependent on storage conditions (Bewley and Black 1982). Factors that influence the longevity of seeds in storage include temperature, moisture and oxygen pressure (Bewley and Black 1982). A low temperature and moisture content usually equates to a longer period of viability (Bewley and Black 1982). Higher oxygen pressure results in a shorter period of sustained viability (Bewley and Black 1982).

Unorthodox or recalcitrant seeds cannot withstand drying (Bewley and Black 1982). These seeds must retain a relatively high moisture content to remain viable (Bewley and Black 1982). Even in relatively moist storage conditions they are rarely viable for more than a few months (Bewley and Black 1982).

The majority of seed plants are however orthodox and can remain viable for a prolonged period under suitable storage conditions (Bewley and Black 1982). Various mathematical equations have been derived to relate the viability of seeds with their storage environment (Bewley and Black 1982).

The germination percentage is the relative number of normal seedlings produced by seed germinated under natural conditions and serves as a comparative measure of seed viability (Hartmann and Kester 1965). To yield good results at least 400 seeds should be randomly selected and germinated under optimum conditions (Hartmann and Kester 1965). Such a germination test usually runs from 10 days to 4 weeks (Hartmann and Kester 1965). Dormancy can however severely interfere with the reliability of the test (Hartmann and Kester 1965). Dormant seeds can usually be distinguished from non-viable seeds in that the former are firm, swollen and free from moulds (Hartmann and Kester 1965).

Excised-embryo tests are used to test seeds in cases where embryos require long periods of after-ripening before germination will take place (Hartmann and Kester 1965). The embryo is essentially excised from the seed and germinated. Before excision is attempted, seeds must be soaked for a few days, changing the water once or twice daily. A viable embryo shows some indication of germination, whereas a non-viable embryo becomes discoloured and deteriorates. Signs of germination include the spreading of cotyledons, the development of chlorophyll and the growth of the radicle and plumules. The time required for this test ranges from 3 days to 3 weeks.

The tetrazolium test for seed viability is used by soaking seeds in a solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Hartmann and Kester 1965). This chemical is absorbed by living tissue and changed into an insoluble red compound, formazan, by NADPH dehydrogenases (Band and Hendry 1993, Hartmann and Kester 1965, Leadem 1984). Non-living tissue remains uncoloured (Hartmann and Kester 1965). The reaction takes place equally well in dormant and non-dormant seeds and results are obtained in less than 24 hours. The test is used as a rapid assessment of viability or as a viability test of dormant seeds that do not respond to other methods (Hartmann and Kester 1965). A 1% solution is commonly used, although a 0.05% solution may sometimes be satisfactory (Band and Hendry 1993, Hartmann and Kester 1965). It should be used at a pH of 6 or 7 (Band and Hendry 1993, Hartmann and Kester 1965). General procedures to be followed before staining include the removal of any hard covering, imbibing of the seeds and sectioning of the seeds so that the embryo may be exposed to the TTC solution (Hartmann and Kester 1965). The embryo is then incubated in 1% tetrazolium for 2 hours in the dark after which the excess tetrazolium is washed off with water (Band and Hendry 1993). The amount of staining is then observed.

The location and intensity of the formazan stain is important to accurately define the viability of the embryo (Leadem 1984). A number of broad classes, defining the germinability of the embryo are given in Table 2.

Table 2: Topographic stain evaluation classes for the tetrazolium test (Leadem 1984).

Class	Description	Viability
1	Embryo completely stained	Germinable
2	Very pale staining	Possibly germinable
3	Cotyledons unstained	Non-germinable or possibly germinable
4	Radicle unstained	Non-germinable or probably not germinable
5	No staining	Non-germinable

The meristems should be well stained in order to insure healthy germination and growth of the embryo (Leadem 1984). A typical viable embryo should be at least 75% stained and its tissue should be firm with a smooth surface (Leadem 1984).

The tetrazolium stain test leaves a number of uncertainties. A sample may, for example, not be stained because the stain never penetrates the tissue and the non-enzymatic reduction of tetrazolium is also possible in dead and living tissue (Band and Hendry 1993). A second stain test should be used to confirm the tetrazolium result (Band and Hendry 1993).

Blue indigo carmine is oxidised to a colourless compound in living tissue (Band and Hendry 1993, Scott and Gross 2004). The samples are prepared as with the tetrazolium test. The samples are then incubated in a 0.05% indigo carmine solution made with water at 30ºC for 2 hours in the dark (Band and Hendry 1993). After the samples are rinsed with water the amount of staining is noted. Viable embryos would be completely unstained or may possess a small blue spot at the radicle end (Kamra 1972). Embryo's can be classed as completely white and germinable when stained from the radicle end to less than a quarter of their total size and when only parts of the cotyledons are stained (Kamra 1972).

3.1.6 Aims and hypotheses

The aims of this study were to provide information on the viability of *Syncarpha recurvata* achenes, to investigate the effect of various treatments on germination and to investigate the suitability of achenes as method for re-establishing *S. recurvata*.

Based on the results obtained by previous authors (Home and Campbell 1996, Todkill 1996, Weatherall-Thomas 2005) all the treatments were expected to result in a higher percentage germination compared to the control. The seeds collected between August and October 2005 would have had a longer ripening period and will be more viable than seeds collected between May and July 2006.

3.2 Methods

Seeds were harvested from whitened capitulae of the Grassridge population. This was done between August and October 2005 and again in May to July 2006. Whitened heads with retracted involucral bracts were picked from the plants. The achenes were extracted in the laboratory and sorted using a stereomicroscope. The achenes were first separated from the wings attached to the hilum of the achenes. Achenes that were underdeveloped, desiccated, had severe indentations or had fungal infection were discarded, while only the black achenes which were large and had a swollen appearance were used in the experiments. Achene viability tests were done to investigate the effect of storage on their viability.

3.2.1 Germination treatments

Achenes were germinated (ten different treatments) in petri dishes on pieces of moist sterilized paper towel on 19 August 2006. The petri dishes with achenes were placed inside a culture cabinet at 21.5°C under a 16:8 h light:dark regime. A fungicide, 'Virikop', was applied to each treatment. Achenes were watered every third day using distilled water.

The treatments consisted of smoke water dilutions of 1:10, 1:50, 1:100 and 1:500, $CaCl_2$ concentrations of 10, 100, 500 and 1000 mM, pre-treatment with a 500 mg l^{-1} gibberellic acid solution and a control, which was watered with distilled water. Each treatment consisted of four petri dishes, each containing 25 achenes. A total of 2000 achenes were used, 1000 collected in 2005 and 1000 collected in 2006.

Smoke water was made in accordance with the methods described by Brown (1993) in a previous study (Swart 2005). All dilutions were made with distilled water. The sterilized paper towel inside the petri dishes was moistened with diluted smoke water before placing achenes in the Petri dish. After this the achenes were watered with distilled water every third day, as were the other treatments.

For the $CaCl_2$ treatments the paper towel inside the petri dishes was moistened with 10, 100, 500 and 1000 mM solutions of $CaCl_2$. After this the achenes were watered with distilled water every third day, as with other treatments.

For the gibberellic acid treatment the achenes were soaked overnight in a 500 mg l^{-1} solution (Day 2000). The achenes were then placed on moist paper towel in a Petri dish and watered every third day with distilled water.

The control treatment was set up by placing achenes on a moist paper towel in a Petri dish which was moistened every third day with water.

Germination data was recorded every third day. Germination was taken to have occurred when the radical was at least 2 mm long. After germination the seedlings were either used for *in vitro* root and shoot culture or placed in polyethylene tents.

3.2.2 Achene viability tests

3.2.2.1 Germination test

The germination data was also used as a comparative viability test.

<u>3.2.2.2 Tetrazolium and Indigo-carmine test</u>

Tetrazolium and indigo-carmine tests were used to indicate the amount of viable tissue present in achenes. For each stain, 40 achenes were used, 20 collected in 2005 and 20 collected in 2006. Before the achenes were tested they were imbibed for 6 hours with distilled water.

Achenes were soaked in a 1% solution of 2,3,5-triphenyltetrazolium chloride or a 0.05% indigo-carmine solution for 2 hours. The achenes were examined using a stereomicroscope and photographs taken with a JVC GC-X3E digital camera for image analysis.

<u>3.2.2.3 Embryo excision test</u>

In vitro embryo culture was done in conjunction with the germination treatments and also serves as a viability test. A total of 396 embryos of both the 2005 and 2006 achene collection were excised. The achenes were placed on agar under various treatments explained in Chapter 6. There were 66 treatments each with six replicates. The lengths of the embryos were measured using Image-Pro$^{©}$ Express 4.5 Image analysis software, Image Processing Solutions, Inc. to determine whether embryos grew and whether there was a difference in embryo length for embryos collected in 2005 and 2006. Separation of the cotyledons was also noted.

3.2.3 Statistics

All the statistical analyses were done with Statistica$^{©}$ 7.0, copyright StatSoft, Inc., using non-parametric statistics as the data was found to be positively skewed and leptokurtic. The correlation between various data sets was investigated using Spearman's rank correlation at 95% confidence limits.

3.3 Results

3.3.1 Germination

The achenes followed the pattern of epigeous germination. No germination was recorded for the 1:1000 smoke water treatment or for any of the $CaCl_2$ treatments (Figure 5). There was no significant difference (Mann-Whitney U test) between the germination recorded for the 1:10 smoke water treatment and any of the other treatments

Table 3. There was no significant difference between the 1:50 and 1:100 smoke water treatments either (Table 3). The germination of the 1:50 smoke treatment was significantly higher than the germination recorded for either the 1:500 smoke water, gibberellic acid or the control treatment. The 1:100 smoke water solution treatment also showed a significantly higher germination success than the latter treatments (Table 3). There were no significant differences between the 1:500 smoke water treatment and the gibberellic acid and control treatments and also no significant difference between the gibberellic acid treatment and the control.
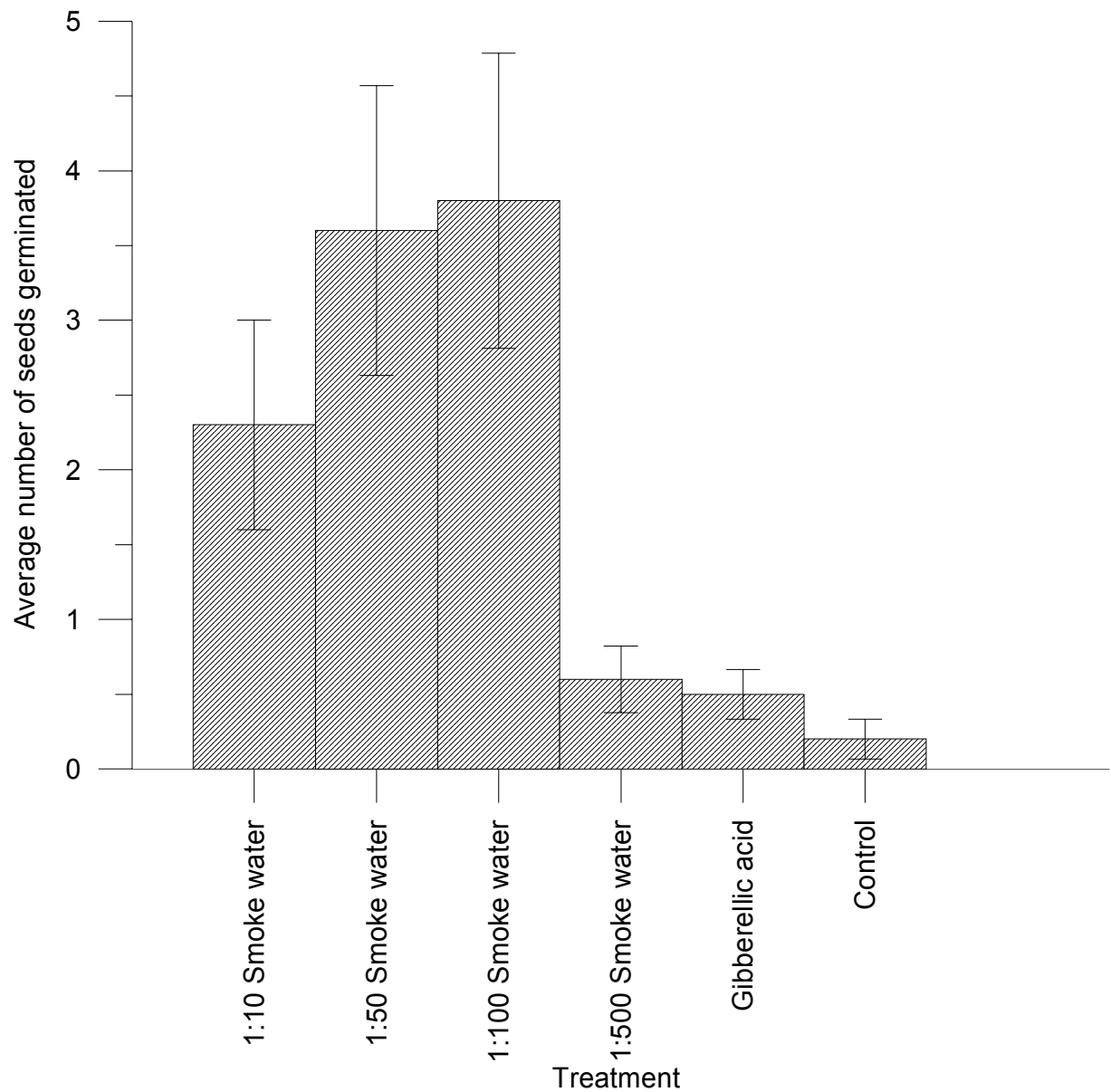
Figure 5: The average number of achenes that germinated for each treatment which consisted of 100 achenes. Vertical bars indicate ±1 standard error of the mean at 95% confidence limits.

Table 3: Mann-Whitney U significant statistical comparisons of achene germination treatments. Significant differences are indicated by an asterisk.

Comparison	N	U	p
1:10 smoke water solution vs. 1:50 smoke water solution	10	37	0.326
1:10 smoke water solution vs. 1:100 smoke water solution	10	35.5	0.273
1:10 smoke water solution vs. 1:500 smoke water solution	10	29	0.112
1:10 smoke water solution vs. Gibberellic acid	10	27.5	0.089
1:10 smoke water solution vs. control	10	20	0.012
1:50 smoke water solution vs. smoke water solution 1:100	10	47.5	0.85
1:50 smoke water solution vs. 1:500 smoke water solution*	10	16.5	0.011
1:50 smoke water solution vs. Gibberellic acid*	10	15	0.008
1:50 smoke water solution vs. Control*	10	12	0.004
1:100 smoke water solution vs. 1:500*	10	16	0.01
1:100 smoke water solution vs. Gibberellic acid*	10	15	0.008
1:100 smoke water solution vs. Control*	10	12	0.004
1:500 smoke water solution vs. Gibberellic acid	10	47.5	0.85
1:500 smoke water solution vs. Control	10	34	0.226
Gibberellic acid vs. Control	10	35	0.257

3.3.2 Viability

3.3.2.1 Germination

The cumulative percentage germination over time for the two achene collections showed significant differences between the cumulative percentage germinations of them (Figure 6; Wilcoxon matched pairs test: $Z = 3.2$; $n = 55$; $T = 12$; $p = 0.001$).

There were no significant differences between the cumulative percentage germination of the two sets of seeds for the first 22 days after the experiment was initiated (Table 5). After that, there were significant differences between the cumulative germination percentages on each date that germination was recorded (

Table 4).

Table 4: Wilcoxon matched pairs statistics recorded for the specified days since the initiation of the experiment. Significant differences indicated with an asterisk.

Time (days since start of experiment)	N	Z	T	p
10	30	0.535	2	0.592
13	30	0	10.5	1
16	30	0.63	13.5	0.529
19	30	1.47	7.5	0.141
22	30	1.886	9	0.059
25*	30	2.547	1	0.011
28*	30	2.863	2.5	0.004
31*	30	3.15	7	0.002
34*	30	3.243	8	0.001
37*	30	3.419	7	0.001
40*	30	3.419	7	0.006
44*	30	3.34	15	0.001
47*	30	3.201	12	0.001
50*	30	3.201	12	0.001
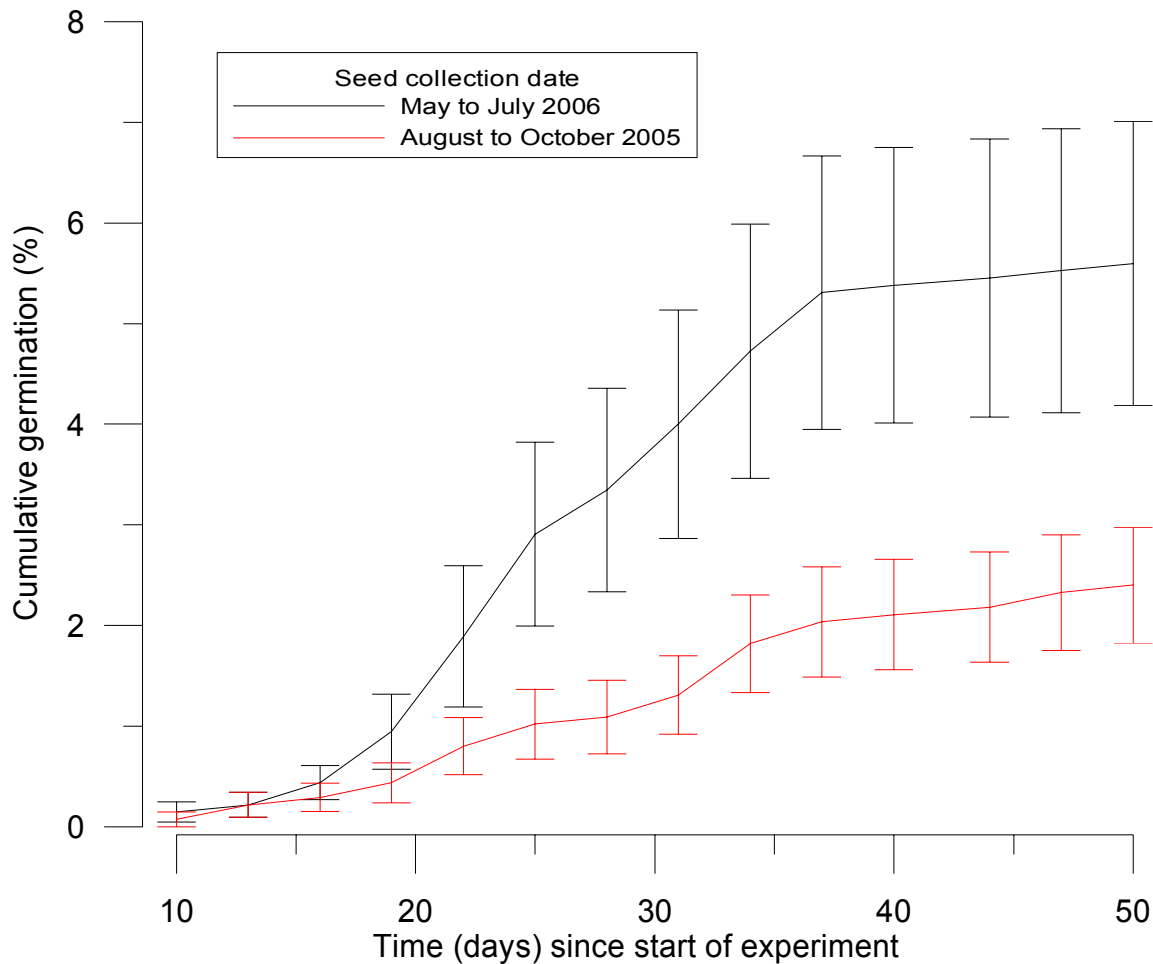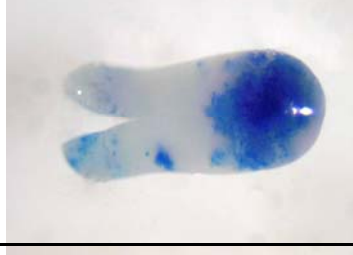
Figure 6: Cumulative percentage germination of achenes of *Syncarpha recurvata*. Vertical bars indicate + 1 standard error of the mean at 95% confidence limits.

<u>3.3.2.2 Tetrazolium and Indigo-carmine test</u>

None of the 40 embryo's treated with tetrazolium stained red. Indigo carmine stained the embryo's differentially. The stains were interpreted according to the inverse of table 2, as indigo carmine stains non-living tissue blue (Table 5) and tetrazolium stains living tissue dark orange. According to these results only 1 of the 20 achenes of the 2006 c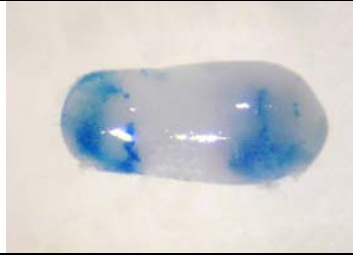ollection had a possibility of germinating. Out of the 20 achenes tested from the 2005 collection 1 was germinable, 5 possibly germinable, 6 had lower probabilities of germinating and 8 were non-germinable.

Table 5: Assumptions made on the viability of the embryos from two achene collections according to the results of the indigo carmine test

Viability (relative to the inverse of table 2)	Number of embryo's from achenes collected in:		Representative photograph
	2005	2006	
Germinable	1	0	
Possibly germinable	5	1	
Non-germinable or possibly germinable	5	0	
Non-germinable or probably not germinable	1	0	
Non-germinable	8	19	

3.3.2.3 Embryo excision test

Table 6: Treatments that showed a notable embryo response

Treatment	Embryo response	Number of embryos from achenes collected in:		Time (weeks) after excision
		2005	**2006**	
Control	Germinated	1 out of 10		1
5 µM of IAA and 0.5 µM of Zeatin	Germinated		1 out of 6	1
1µM IAA	Germinated		1 out of 6	2
1µM 2,4-D and 0.5 µM kinetin	Turned green	1 out of 6		1
0.5 µM 2,4-D and 0.5 µM kinetin	Turned green	1 out of 6		1
0.5 µM 2,4-D and 10 µM Kinetin	Turned green	1 out of 6		2
5 µM IAA and 1 µM Zeatin	Turned green	1 out of 6		2
1 µM IAA and 1 µM Zeatin	Turned green		1 out of 6	2

Only 1 out of 10 achenes from the 2005 collection germinated on the control agar
medium (Table 6). Germination occurred in less than a week. Germination of an
embryo excised from an achene from the 2006 collection occurred in less than 1
week on a medium supplemented with 5 µM of IAA and 0.5 µM of Zeatin. One out of
six embryos from the 2005 collection turned green on a medium supplemented with 1
µM 2,4-D and 0.5 µM kinetin D1*K0.5 and a medium supplemented with 0.5 µM 2,4-D
and 0.5 µM kinetin within one week. A single achene from the 2005 collection
germinated out of 6 replicates on a medium supplemented with 0.5 µM 2,4-D and 10
µM Kinetin and on a medium supplemented with 5 µM IAA and 1 µM Zeatin within 2
weeks. On a medium supplemented with 1 µM IAA and 1 µM Zeatin 1 achene from
the 2006 collection turned green out of 6 replicates within 2 weeks.

The embryos excised from achenes collected in 2005 had a significantly greater length than those excised from the 2006 collection (U = 63429.5; m = 389, n = 375, p = 0.002).

The cotyledons of a total of 109 of the embryos of the 2006 achene collection (n = 396) separated, whereas only 75 of the cotyledons of the 2005 achene collection (n = 396) separated.

3.4 Discussion

3.4.1 Germination treatments

Diluting the smoke water used in this study to 1:50 and 1:100 dilutions were most successful in inducing germination (Figure 6 and Table 3). Neither low concentrations of smoke water (e.g. 1:500; Figure 6, Table 3), nor a gibberellic acid treatment had a significant effect on germination. The optimal smoke water dilution (when prepared with smoke water used in this study) for this species was 1:100. This can not be used as a standard dilution for all smoke water solutions, as the amount of smoke that the water is saturated with can not be quantified. A standardised solution or primer is therefore necessary.

Brown and van Staden (1997) obtained a mean percentage germination of 100% when germinating *Syncarpha vestita,* a fynbos fire ephemeral, with a 1:100 smoke water dilution, apposed to a germination percentage of 30% for the control. This does not show that a suitable smoke water dilution of 1:100 is genus specific as a standardised smoke water solution was used. Such comparisons between studies and species can only be possible if a standardised solution is used.

Weatherall-Thomas (2005) found that a 1:50 smoke water dilution treatment and a 100 mM $CaCl_2$ treatment stimulated germination. The efficacy of his 1:50 smoke water treatment is supported by this study. The efficacy of the 100 mM $CaCl_2$ treatment is however not supported by this study. This is not due to differences in replication or chemical treatment as the same number of replicates were used, the

methods of the treatment was standardized after personal communication with Weatherall-Thomas and the same smoke water solution was used. The physical environment of propagation was different, as Weatherall-Thomas (2005) germinated his achenes on a laboratory bench with fluorescent lights and no temperature control, opposed to the culture cabinet used in this study.

GA is known to promote embryo growth potential (Marion-Poll 1997). Home and Campbell (1996) and Todkill (1996) showed that a gibberellic acid treatment increased germination of *Syncarpha recurvata*. The lack of a significant difference between the gibberellic acid and the control in this study could be because of a lack of replication. It may also be explained by the physical conditions as these were different in the study done by Home and Campbell (1996) and Todkill (1996). Home and Campbell (1996) germinated the achenes in the dark and Todkill (1996) germinated the achenes in a 12:12 h light:dark regime. In this study the achenes were germinated in a 16:8 h light:dark regime. Home and Campbell (1996) and Todkill (1996) also germinated the achenes at 22°C and 23°C respectively, whereas the achenes were germinated at 21.5°C in this study.

Weatherall-Thomas (2005) concluded that *Syncarpha recurvata* had endogenous dormancy, based on his findings and that of Home and Campbell (1996) who showed that mechanical and chemical scarification does not have a significant effect on germination. He also suggested that the successful gibberellic acid and smoke water solution treatments of Home and Campbell (1996) and his study support an endogenous dormancy. The results obtained in this study and studies done by previous authors point toward both endogenous and exogenous dormancy.

Smoke water induces morphological change of the external and internal cuticle of *Emmenanthe penduliflora* (Hydrophyllaceae) achenes (Egerton-Warburton 1998b). Egerton-Warburton (1998b) speculated that such morphological changes benefit the plant by increased cuticular permeability and a simultaneous leaching of endogenous inhibitors of germination during imbibition.

The shortening of the germination period by removal of the fruit coat of *Syncarpha recurvata* supports an exogenous dormancy. Both of the embryos which fully germinated in the embryo excision test germinated within one week of culture initiation, whereas the achenes with their fruit coat intact only germinated ten to fifty days after the experiment was prepared.

Physical exogenous dormancy is caused by impermeability of the achene coat to water and is broken by the opening of specialised structures (Baskin and Baskin 1998). These specialised structures include a chalazal plug, a strophiole, a plug-like structure near the micropyle, a lid like operculum and other modifications to the seed or fruit coat, with some to still be described (Baskin and Baskin 1998). Smoke water is known to alter the hydrophobicity and increase the surface area of the external cuticle and increase the number and diameter of permeate channels in the internal cuticle (Egerton-Warburton 1998b). It could be said that smoke water stimulates the opening of specialized structures and, as a result, the chemical interaction of the embryo with the external environment.

Embryos with morphophysiological dormancy can usually be induced to germinate by a gibberellic acid treatment. In this study gibberellic acid had no significant effect on germination. The positive results obtained by Home and Campbell (1996) and Todkill (1996) for gibberellic acid suggests that morphophysiological dormancy might also be involved.

Egerton-Warburton (1998a) speculated that the enhanced germination observed when subjecting *Emmenanthe penduliflora* (Hydrophyllaceae) achenes to smoke water treatments may also partially be the result of leaching of endogenous inhibitors of germination through an altered, more permeable, achene coat. This could indicate chemical exogenous dormancy as well as non-deep physiological dormancy, as the chemical inhibitor can be present in either the fruit coat or the embryo (Baskin and Baskin 1998).

The aims for this section were achieved as germination treatments that significantly increased germination were used. The highest percentage germination obtained in this study was 22.4% (1:100 smoke water dilution treatment of seeds collected in 2006). This is too low to reliably use achenes as a propagation method. Many of the germination treatments did not show a significantly higher germination percentage compared to the control. The reasons for this are discussed above.

3.4.2 Viability

Figure 6 shows cumulative percentage germination over time. This figure and the statistics (

Table 4) show that the cumulative percentage germination significantly differed between achenes collected in 2005 and 2006 in the last 24 days of germination. The achenes collected in 2005 have a greater germination rate. This will result in more achenes germinating per area in a given time.

The indigo carmine test also shows that the achenes collected in August to October 2005 were more viable. The staining showed that a total of 55% of the achenes collected in 2005 had a possibility of germinating, while only 5% of the achenes collected in 2006 had a possibility of germinating. This higher possibility of germinating definitely points toward a higher viability of the seeds collected from August to October 2005.

The possibility of germination occurring is higher than germination observed. This indicates that the potential germination of the embryos is higher than that observed with the fruit coat intact and points toward exogenous or endogenous dormancy.

According to the number of excised embryos germinated the seeds collected in 2006 are more viable. The embryos germinated from the 2006 collection were rather abnormal compared to the embryos germinated from the 2005 collection.

These abnormalities may indicate better suitability of 2005 collection. The abnormalities observed are potentially connected to the composition of the medium, this possibility discussed further in Chapter 6. The excision test does not clearly show that the 2005 collection is more or less suitable for germination.

The abundance of embryos obtained from the 2005 collection that greened show that the 2005 collection is more viable as the development of chlorophyll can be taken as a sign of germination (Hartmann and Kester 1965).

The higher germinability of the embryos excised from achenes collected in 2005 is also supported by their significantly greater lengths. The achenes of *Syncarpha*

recurvata appears to be non-endospermic, as no endosperm was observed (Bewley and Black 1994). The size of the achene as a whole is largely dependant on the size of the embryo. Achenes with large reserves have an increased chance of survival under unfavourable conditions whereas small achenes are associated with high fecundity and allows for effective dispersal (Hodgson and Thompson 1993).

The fact that the cotyledons of the achenes collected in 2006 seem to separate more readily than those of the 2005 collection indicate that the seeds collected in 2006 were more viable.

These results, with the exception of the germination observed for the embryo excision test and the cotyledon separation results, indicate that the seeds collected in August to October 2005 are more viable than those collected in 2006. This higher viability can be the result of either endogenous or exogenous dormancy. An appropriate after-ripening time increases germination percentage, this points towards morphological or morphophysiological dormancy.

The achenes were not collected in the same season. The higher viability observed for the achenes collected in August to October 2005 might be a result of seasonal variation rather than after-ripening. When the achenes were collected in July 2006 it was observed that the number of fruiting flowers was low and that the number of flowers in the budding stage was substantial. This might indicate a larger spring fruit production period, bearing more germinable seeds. The day length, light quality, mineral nutrition and soil moisture to which the mother plant is subjected often has a significant effect on the germination and viability of its achenes (Baskin and Baskin 1998). In many species achenes produced early in the growing season are heavier than those produced late in the growing season (Baskin and Baskin 1998). This corresponds with the greater length observed for seeds collected in August to October 2005.

Although it can be concluded that the achenes collected in August to October 2005 are more viable than those collected in May to July 2006, this does not tell us enough about the viability and dormancy of *Syncarpha recurvata*. Collection of achenes in August to October or May to July should be used in future studies to identify seasonal variations, if any, and to verify the positive effect of after ripening. Collecting the achenes at the right time may increase the potential of *Syncarpha recurvata* achenes to be used as a propagation method.

From this study it can be concluded that achenes is not a suitable propagation method as the percentage germination is too low. Using achenes for propagation will lead to loss of genetic material that may have been incorporated in to the population under natural conditions. On the other hand, the germination percentages of *Syncarpha recurvata* achenes in a natural population are not known.

Other propagation methods, using different plant material, need to be investigated. Such propagation methods are investigated in the following two chapters. Other plant material should also be used for a gene bank for conservation, as an achene collection is not suitable with such low percentage germination. Other combinations of treatments should be attempted and different physical conditions and chemical stimuli should be investigated in future studies to improve germination. A biochemical and morphological study of the fruit coat is needed to further investigate the dormancy of *Syncarpha recurvata* achenes. The biochemical and morphological effect of smoke stimulus on the fruit coat of *S. recurvata* should also be investigated.

Chapter 4: Propagation by cuttage and air-layering

<u>4.1 Introduction</u>

4.1.1 Shoot cuttings

According to Hammet (1973) the use of younger plants and stems for cuttings is preferable in species that are difficult to propagate by seed germination. The suitability of a plant part to be propagated varies between parts taken, individuals from which the parts are taken and the species used (Hammet 1973). Lateral shoot cuttings often root easier than terminal ones (Hartmann and Kester 1965).

The production of auxins and carbohydrates is not possible in wilted leaves. Auxins generally stimulate cell elongation and cytokinins cause cell division (Gaba 2004). Together they are involved in the differentiation of phloem and xylem and the formation of adventitious roots (Pierik 1997).

To prevent wilting the number of leaves on a cutting must be reduced and the leaves must be kept turgid by sustaining a high relative humidity around the leaves (Edmond, *et al.* 1964).

The main method of promoting root growth is by retarding shoot growth. This is done by keeping the tops cool and the bottoms warm (Edmond, *et al.* 1964). To sustain such a situation the tops are usually kept cool by fanning and misting, while the bottoms are placed in a medium to which artificial heat is supplied. This low rate of transpiration, caused by the high relative humidity, keeps the guard cells turgid and the stomata open (Edmond, *et al.* 1964). This allows carbon dioxide to diffuse into the tissues and photosynthesis takes place, allowing the manufacture of carbohydrates and hormones (Edmond, *et al.* 1964). The higher temperature of the cut surface promotes rapid oxidation of fatty acids and the formation of suberin and speeds up mitotic division of cambium cells and the development of the root system (Edmond, *et al.* 1964). For an enhanced rate of photosynthesis and carbohydrate production the

application of mist to the tops is advantageous because of its cooling effect on the leaves (Welch 1973). This decreases respiration and increases net production (Welch 1973). It is said that daytime air temperatures of 21 to 27°C and night temperatures of ~15°C is sufficient for rooting (Hartmann and Kester 1965).

High relative humidity promotes low rates of transpiration while allowing the plant to absorb sufficient light energy for carbohydrate production (Edmond, *et al.* 1964). Such high relative humidity can be sustained by a mist system (Edmond, *et al.* 1964). A high relative humidity can also build up inside a small cover or container by a "greenhouse effect". This method does not allow results that are as fast and efficient as a mist system though. This is because the mist also has a cooling effect and allows a larger leaf area, and thus a higher photosynthetic rate, to be sustained (Hammet 1973). This in turn results in faster carbohydrate production and faster root development and growth, leaving less time for infection by internal and external contaminants (Hammet 1973).

High light intensities will result in wilting if not sustained by high relative humidity (Welch 1973).

Oxygen and sufficient water are needed for the formation of suberin and the mitotic division of cambium cells (Edmond, *et al.* 1964). Rooting media commonly used include coarse river sand, mixtures of sand and peat moss, vermiculite and mixtures of sand and perlite (Welch 1973). Excessive moisture can result in excessive formation of callus covering the cut shoot, so that root primordia are unable to penetrate through it (Welch 1973). A high alkalinity is said to cause hard and corny growth of the callus, making it impregnable to the developing root primordium and retarding root growth (Welch 1973). The growth medium should be kept at a pH just above neutral.

Natural root growth substances are synthesised in apical buds and young leaves (Bleasdale 1973). These include auxins, cytokinins and gibberellins. Of these, auxins are said to have the largest influence on root formation.

Auxins were the first plant hormones to be discovered (Wareing and Phillips 1978). Their existence was investigated by naturalists such as Charles Darwin, Boysen-Jensen and Paàl and the chemical was successfully isolated for the first time by F.W. Went in 1926.

The name auxin is derived from the Greek *auxem*, which means 'to grow'. This was the name originally given to the hormone produced from the tip of the coleoptile (Wareing and Phillips 1978). It was later found that this same chemical is found in all higher plants. Auxins are mainly synthesized in the meristematic tissues of the stem and root apex, young developing leaves, flowers and fruits (Wareing and Phillips 1978). It was chemically identified in 1934 as indole-3-acetic acid (IAA) and various synthetic auxins have since been synthesised (Wareing and Phillips 1978).

The process of the development of adventitious roots from stem cuttings can be divided into three important stages (Hartmann and Kester 1965). The first stage involves the development of the root initials, which are essentially groups of meristematic cells. Secondly these groups of cells differentiate into distinct root primordia. In the third stage the roots develop to a stage where they rupture other stem tissues and emerge as new roots, complete with vascular connections to the connective tissue of the cutting (Hartmann and Kester 1965).

Oliveira Dragovic *et al.* (2000) obtained 62.2% rooting success in an experiment with cuttings of *Helichrysum obconicum*. Branches of 13 cm were removed from the plants, after which ~1cm of the epidermal layer was removed from the base of the cutting followed by a 5 seconds dip in a 4 ppm ethanolic solution of indole-3-butyric acid (IBA) (Oliveira Dragovic*, et al.* 2000)

Several species of the genus *Anaphalioides* (Gnaphalieae) root from layered branches in the wild and under controlled conditions in the laboratory (Glenny 1997).

The use of cuttings for conservation places limits on size of the samples as a limited number of cuttings can be obtained from each plant (Given 1994). A method such as micropropagation allow a larger sample size (Davy 2002).

Cuttings are ideal for sampling genetic traits such as flower colour variants (Given 1994). Numerous flower colour variants were observed in both the Grassridge and Coega populations in this study. The success of propagating species using cuttings will enable the preservation of these flower colour variants.

4.1.2 Root cuttings

Home and Campbell (1996) also attempted propagating *Syncarpha recurvata* from root cuttings. Excavated roots were cut into 50 mm lengths, treated with hormone powder, placed horizontally and covered with 15 mm of substrate. Different substrates were used including fine soil, potting soil, and a 1:1 ratio of potting soil and peat. None of the root cuttings sprouted stems (Home and Campbell 1996).

In root cuttings new shoots develop from adventitious buds (Edmond, *et al.* 1964). In young roots these buds often arise from the pericycle near the vascular cambium, whereas buds may arise exogenously in a callus-like growth from the phyllogen in old roots (Hartmann and Kester 1965). Buds also arise this way from cut ends or injured surfaces (Hartmann and Kester 1965). Roots normally develop after this, from the old root or the base of the new individual (Edmond, *et al.* 1964). Root cuttings may be planted either horizontally or vertically (Hammet 1973)

Bud initiation in root pieces are normally stimulated by cytokinins such as kinetin (Hartmann and Kester 1965). Home and Campbell (1996) treated the root cuttings with auxins. This may have been the incorrect way to initiate the *in vivo* multiplication using root cuttings.

For plants propagated in this way shoots usually arise from the root system under natural conditions (Hartmann and Kester 1965). As this was not observed for *Syncarpha recurvata*, this propagation method was not investigated.

4.1.3 Air-layering

Air-layering involves the production of new individuals before they are severed from the parent plant (Hartmann and Kester 1965). There are two advantages to using this method when compared to propagation with cuttings; the parent plant supplies the new plantlet with water, minerals, carbohydrates, proteins and plant growth regulators, and the environment of propagation does not have to be as strictly controlled as with cuttings (Edmond, *et al.* 1964). The amount of equipment and skill needed for layering is also less compared to cuttings (Hartmann and Kester 1965). This method of propagation is however limited to plants that form growth points readily (Edmond, *et al.* 1964). A smaller number of plantlets are produced per plant when compared with propagation from cuttings (Edmond, *et al.* 1964).

Air layering consists of surrounding stems of the previous season's growth with peat moss held in place by a split pot or sheets of plastic film (Edmond, *et al.* 1964). The stem is usually girdled to facilitate the production of roots just above the girdle (Edmond, *et al.* 1964). This is done to such an extent that the phloem and cambium is completely removed (Hartmann and Kester 1965). A root promoting substance such as 'Seradix©' is applied to this girdling cut (Hartmann and Kester 1965). The stem is severed from the parent plant as soon as sufficient roots have been produced (Edmond, *et al.* 1964). Root formation can be viewed through the transparent film (Hartmann and Kester 1965). Severed plantlets may require a period of misting followed by a period of hardening (Hartmann and Kester 1965).

Air layering has been in use for more than a thousand years (Hartmann and Kester 1965). It is known in various parts of the world as Chinese layerage, pot layerage, circumposition, marcottage and gootee (Hartmann and Kester 1965). Factors that influence the production of roots and shoots on layered stems are similar to those

factors that influence the production of roots and shoots on cuttings (Hartmann and Kester 1965).

A common problem in air layering is keeping the rooting medium moist (Hartmann and Kester 1965). In ancient Chinese gootee or marcottage methods peat is plastered to the stem using clay (Hartmann and Kester 1965). A water receptacle is placed above the gootee (Hartmann and Kester 1965). From this receptacle a string extends which leads to the gootee, continuously keeping it moist (Hartmann and Kester 1965). Most methods of air layering require a humid atmosphere to prevent desiccation of the rooting medium (Hartmann and Kester 1965).

Various enclosures have been used to surround the rooting medium (Hartmann and Kester 1965). These include metal or wooden boxes, split flower pots, paper cones and rubber sheeting. If the layering is done in an area of high humidity or if daily syringing with water is applied, covering the girdle with peat moss may be sufficient.

Many of these devices have been replaced by polyethylene film with properties of high gas permeability and low water permeability (Hartmann and Kester 1965). This enables the exchange of carbon dioxide and oxygen and prevents the escape of water vapour. Many plants that do not root easily as cuttings can be propagated by layering (Hartmann and Kester 1965). Most methods of layering can be carried out in the field or in a greenhouse.

4.1.4 Aims and hypotheses

As seen with the studies done by Glenny (1997) and Oliveira Dragovic *et al.* (2000) success has been shown with other species in the tribe Gnaphalieae. The study done by Home and Campbell (1996) showed no success with rooting shoot cuttings.

The main aim of the chapter was to test whether *Syncarpha recurvata* is capable of asexual reproduction *in vivo* using methods different than those used by Home and Campbell (1996).

It was hypothesised that the results would be similar to the results obtained by Home and Campbell (1996) so that no rooting will be observed for either the cuttings or the air layered branches. This will potentially be a limiting factor in the use of whole plants as translocation method. If rooting success is observed air layering would probably deliver the highest percentage rooting, as the new plant has a chemical environment more favourable when attached to the mother plant, whereas a cutting only has its own resources available (Edmond, *et al.* 1964).

4.2 Methods

4.2.1 Stem cuttings

A total of 20 100 mm long stem cuttings were placed in a polyethylene tent. Leafy cuttings were taken from healthy, acclimated plants. All the leaves were removed from the lower 20 mm of the cutting after which the base of the cutting was treated with 'Seradix$^©$ no 1' rooting powder, Bayer Pty. Ltd. The cuttings were placed in a 1:1 mixture of peat and calcrete rich soil collected from Grassridge. After four weeks the cuttings were removed from the rooting medium and any indication of rooting was recorded.

4.2.1.1 Construction of the polyethylene tents

Because a high humidity environment is favourable for the rooting of stem cuttings and air-layered plants and the hardening of micropropagated plants (Ahloowalia, et al. 2002, Edmond, *et al.* 1964, Hartmann and Kester 1965) two polyethylene tents were constructed.
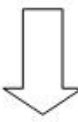
The tents were constructed along the lines of the Quonset type greenhouses suggested by Ahloowalia and Prakash (2002). Some modifications were however made to provide some cooling, as cooling allows a larger leaf area, and thus a higher photosynthetic rate, to be sustained (Hammet 1973): a fan controlled by a 'Kovcostat' thermostat was installed and misters inside the tunnels were supplied with water using a pump placed in a 50l plastic drum. The position of the fans is indicated by the

hashed square and the position of the pump is indicated by the dotted sphere in figure 7. An analogue timer was connected to the pump to control the misting intervals. This analogue timer was placed in an electrical box underneath. This electrical box and all other electrical connections are indicated by the dashed line in figure 7.

The thermostat probe was mounted inside the polyethylene tent and submersed in motor oil. The thermostat was adjusted to 25°C. Because the tents were placed outside, the air inlet of the fan was covered with voile (a lightweight translucent fabric made from cotton, synthetic fibres, or wool) to prevent small pieces of dust and debris from being deposited on the plants.

Air entered the propagation tunnels through the voile (indicated by box arrows) and exited through the spaces left between the Velcro at the end of the tunnel (indicated by black arrows) (Figure 7).

The misters served to irrigate the plants and to cool and humidify the air inside the tunnel. The cross inside the crossed spheres indicates the position of the misters (Figure 7).

Legend	
⊗	Position of misters
▨	Fan
◉	Water reservoir
○	Pump
⬡	Thermostat with probe
▬	Irrigation piping
....	Electrical connections
▯	Location of electrical box
⬇	Direction of airflow into tunnels
↓	Direction of airflow out of tunnels
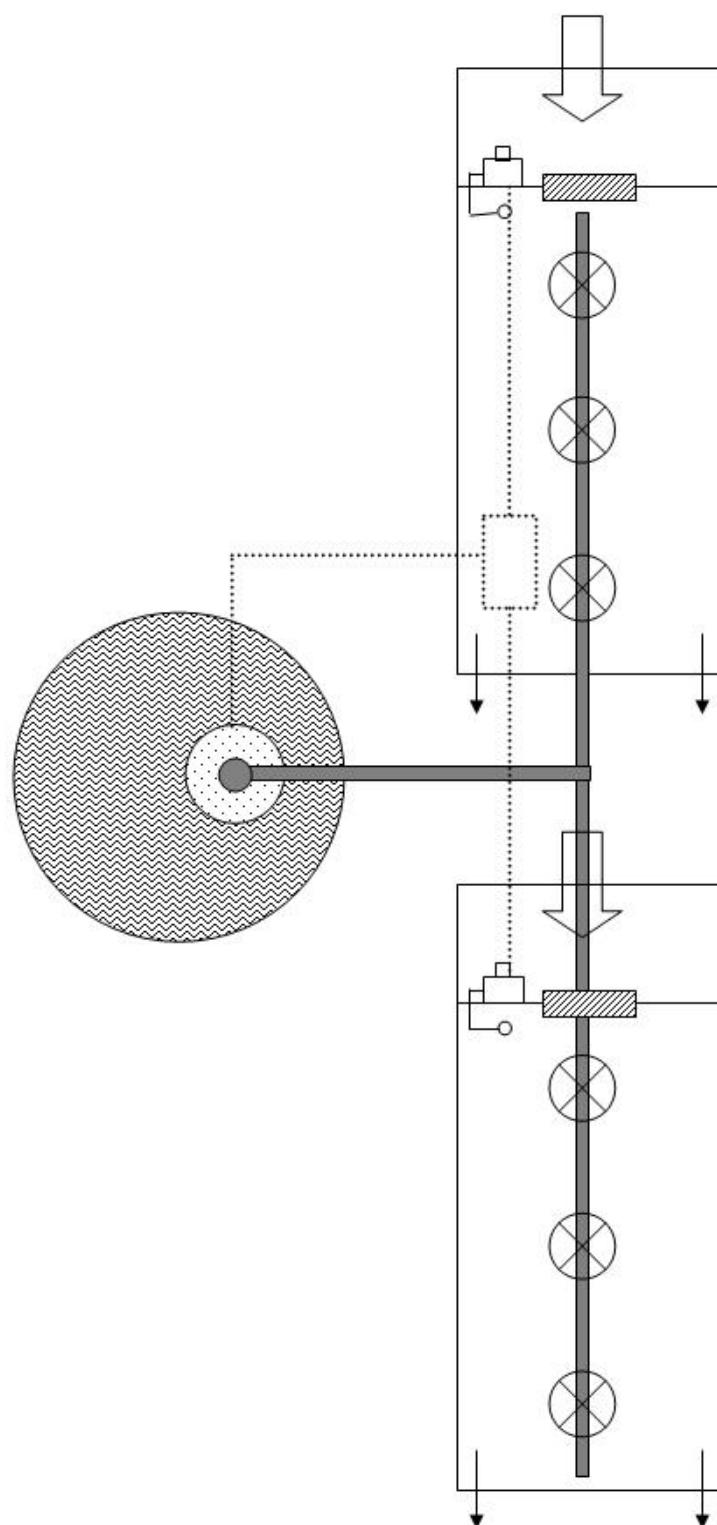
Figure 7: Diagram of propagation tunnels showing design and function

The tunnels were placed inside the NNMU Botany department research enclosure under the canopy of a *Brachylaena discolor* tree. This resulted in the tunnels being shaded until well after midday, reducing high temperatures during the day. Because the amount of shade that covered, the tunnels gradually increased from the direction of the plastic drum throughout the day, the side closest to the drum received a longer period of shade during the day.

Because *Syncarpha recurvata* does not appear to grow well in excessively moist soil, as low survival (15%) was observed in a preliminary transplantation experiment under such conditions.

The timer was set so that the plants were misted two times a day, for 5 minutes at 10:00 and for 5 minutes at 15:00. On rainy or cloudy days the second misting interval was cancelled manually.

4.2.2 Air layering

Fifteen transplanted individuals were air-layered. A 5 mm section of bark was removed from a few (2 to 5) healthy stems of each individual (Edmond*, et al.* 1964). The section of the stem was then treated with 'Seradix[©] no.1', Bayer Pty. Ltd. (Hartmann and Kester 1965). The wound was covered with 50 ml of the soil mixture used for cuttings. The soil was secured to the stem with aluminium foil. After 4 weeks the foil and soil mixture was removed and any indication of rooting was recorded.

4.2.3 Statistics

All data sets were non-parametric. A Mann-Whitney test was used to compare the root lengths obtained through cuttage with that obtained from the air-layered plants.

4.3 Results

4.3.1 Stem cuttings

A total of 10 out of the 20 cuttings rooted after a period of 1 month (50%). The rooted cuttings had an average of 16 roots ± 2 S.E. with an average root length of 17.41 mm ± 1.01 S.E. The average total stem length of the successful cuttings was 130.15 mm ± 12.97 S.E. All the successful cuttings were found to be on the side of the tunnel that received the most amount of shade during the day.

There was no significant difference between the shoot lengths of the 10 rooted cuttings and that of the 10 non-rooted cuttings (U = 45, p = 0.705, N = 10)

4.3.2 Air layering

Six of the 15 air layered plants showed signs of rooting after a month (40%). Out of a total of 21 air layering treatments placed on branches of these six plants 17 rooted (81%). The air layering treatments therefore had a success rate of 32%. The plantlets had an average of 7 roots ± 1.4 S.E. with an average root length of 20.57 mm ± 1.42 S.E. There was no visible correlation between the positions of the plants in the tunnel and their rooting success.

There was no significant difference between the root lengths obtained through making cuttings and the root lengths obtained through air-layering the branches (U = 10263.5, p = 0.432, n = 163, m = 133).

4.4 Discussion

The number of cuttings and air layered branches that rooted was not particularly high. The observation that the cuttings on the more regularly shaded side of the tunnel rooted more readily indicates that the tunnels should be subjected to more shade in future studies. This indicates that more shade or less morning sun (perhaps only until ~11h00) is more suitable.

The difference in light in the tunnel could not have been so great and the one side of the tunnel may not have been shaded for a ling enough time to cause a significant difference in photosynthetic rate. The difference in rooting success may have been caused by other environmental gradients as the tunnel had various design and construction flaws. One side of the tunnel could have air-flow because of the fan being mounted slightly skew or the side could have become more ventilated because of it being torn loose by the wind periodically. The effect of temperature and levels of irradiance on the rooting of *Syncarpha recurvata* branches should be tested in future studies.

The fact that the shoots of *Syncarpha recurvata* plants were rooted in this study is inconsistent with the results of Home and Campbell (1996), who obtained no rooting for stem cuttings of *Syncarpha recurvata* and observations made of *S. recurvata* in the field. The positive results obtained in this study might be explained by a number of differences between the methods used in this study and that of Home and Campbell (1996). They used a different rooting hormone; 'Seradix[©] no. 3' apposed to the 'Seradix[©] no 1' used in this study. The substrate used by Home and Campbell (1996) can also be considered to be acidic relative to the calcareous habitat of *S. recurvata* and the 2:1 mixture of calcareous soil from the study site and peat used in this study.

The vegetative material was also kept under partially controlled physical conditions in this study, whereas Home and Campbell (1996) placed the cuttings in a greenhouse with no temperature control. The greenhouse probably became too hot and at one point and the cuttings became necrotic. In this study such heat build up was avoided by placing the tunnels under partial shade and by attaching fans to a thermostat, so that air circulation prevented further build up of heat when temperatures reached above 25°C.

Their use of sprinklers every 40 minutes might also have been excessive, so that the roots were subjected to too much moisture and became necrotic. In this study there

were only two irrigation intervals every day, allowing water to drain from the roots and the roots to come into contact with oxygen, a prerequisite for root growth.

The substantial rooting observed in this study means that *Syncarpha recurvata* plants can be asexually multiplied *in vivo* and the degree of rooting can presumably be increased by optimizing the method. Successful rooting and asexual propagation is a prerequisite for restoration by using vegetative parts (Davy 2002). Plants are able to asexually reproduce under physically and chemically controlled conditions through cuttage or air-layering.

A higher percentage rooting success was obtained for cuttings than for air-layered branches. This was not expected. The air-layered plants were expected to show a higher percentage rooting success and a higher root growth rate as the connection with the parent plant supplies the new plantlet with water, minerals, carbohydrates, proteins and plant growth regulators (Edmond *et al.* 1964). There was also no difference in the root lengths obtained. If this technique is applied to rooted, intact plants in the field the success rate could be higher.

Air-layering must not be disregarded because of its lower percentage rooting success. None of the stems that did not produce roots were damaged in air-layering whereas the stems used for cuttage were damaged, as they were removed from the plants. A branch that did not root in air-layering could be used for cuttage or could remain on the plant to attempt air-layering later or to aid in additional growth of the mother plant. Air-layering is therefore less destructive.

4.5 Conclusion

Syncarpha recurvata readily produces roots from its shoots as a means of asexual reproduction under physically and chemically controlled conditions. Cuttage and air-layering are suitable propagation methods for *S. recurvata*. The potential exists for air-layering on whole plants and cuttings to be used in propagation of this species. Plants can be planted in nurseries and seeds and air-layered plantlets can be

harvested in a sustainable fashion. This will enable multiple clones to be made from single individuals so that cloned material can be produced for conservation and horticultural purposes.

Similar studies should be done for all the other and rare endangered species of the tribe Gnaphalieae as it is evident that numerous species in this tribe can be efficiently cloned through rooting of cuttings.

Chapter 5: Micropropagation

5.1 Introduction to micropropagation

When plants are multiplied vegetatively, through cuttings or by tissue culture, all the offspring can be classified as clones. The term "tissue culture" is actually a misnomer inherited from the field of animal tissue culture. Plant micropropagation involves the culture of a whole individual from isolated tissues, while animal tissue culture involves the culture of isolated tissues (Kyte and Kleyn 1996).

According to Ahloowalia *et al.* (2002), the process of micropropagation can be divided into five stages: the pre-propagation step (stage 0); the initiation of explants (stage I); the subculture of explants for multiplication or proliferation (stage II); shooting and rooting of the explants (stage III); and hardening off the cultured individuals (stage IV). The pre-propagation stage involves preparing the explant for aseptic culture (Ahloowalia*, et al.* 2002).

The explant and its response *in vitr*o is significantly influenced by the phytosanitary and physiological condition of the donor plant (Kane 2004). Plant material used in clonal propagation should be taken from mother plants that have undergone the appropriate pre-treatment with fungicides and pesticides to minimize contamination in the *in vitro* cultures (Ahloowalia*, et al.* 2002). Such a piece of plant material is called an explant (Smith 2000). A single explant can theoretically produce an infinite number of plants (Kyte and Kleyn 1996). Explants can be obtained from meristems, shoot tips, macerated stem pieces, nodes, buds, flowers, peduncle pieces, anthers, petals, pieces of leaf or petiole, seeds, nucellus tissue, embryos, seedlings, hypocotyls, bulblets, bulb scales, cormels, radicles, stolons, rhizome tips, root pieces or protoplasts (Kyte and Kleyn 1996). The explants should be surface sterilised with antibiotic sprays before they are introduced into culture (Ahloowalia*, et al.* 2002, Kane 2004).

In Stage I an aseptic culture is initiated by inoculating the explant onto a sterile medium (Ahloowalia, *et al.* 2002). Once such an explant is established it can be multiplied a number of times (Ahloowalia, *et al.* 2002). The explants are then transferred to a contaminant free *in vitro* environment (Ahloowalia, *et al.* 2002). During this process minute pieces of plant tissue are surface sterilised using chemicals such as sodium hypochloride or ethyl alcohol. These minute plant pieces are also washed with sterile distilled water before and after treatment with chemicals (Ahloowalia, *et al.* 2002). Explants that show contamination after the first 3 to 5 days are discarded (Ahloowalia, *et al.* 2002). The surviving uncontaminated explants are maintained and used in further subculture (Ahloowalia, *et al.* 2002).

In stage II or the propagation phase explants are cultured onto a medium that promotes the multiplication of shoots (Ahloowalia, *et al.* 2002). Propagation must be achieved without excessive mutation (Ahloowalia, *et al.* 2002). The culture of various organs in stage I leads to the multiplication of propagules in large numbers. These propagules can be cultured further and used for multiplication (Ahloowalia, *et al.* 2002). These cultured shoots are often placed onto different media for elongation (Ahloowalia, *et al.* 2002).

The result of stage III is the production of complete plants, as the shoots derived from stage II are rooted (Ahloowalia, *et al.* 2002). If shoot clumps are present they should be separated after rooting and never before rooting. Many plants can be rooted on half strength Murashige and Skoog without any growth regulators (Ahloowalia, *et al.* 2002). Successful and sufficient rooting is essential for survival of the plant during hardening and transfer to soil (Ahloowalia, *et al.* 2002).

The complete plants are weaned and hardened during stage IV (Ahloowalia, *et al.* 2002). The plants should at this stage be autotrophic. Hardening consists of gradually altering the humidity, light and nutrition available to the plant. The plant is moved gradually from a high to a low humidity, from a low light intensity to a high light intensity and the agar is removed by gently washing it away with water. After

sufficient hardening, plants can be transplanted to a suitable substrate and hardened further (Ahloowalia*, et al.* 2002).

5.1.1 Explant selection

Physiologically younger tissues are generally much more responsive to tissue culture (Smith 2000). In many cases, older tissues will not form callus that is capable of regeneration. Younger tissue is usually the newest formed and therefore easier to surface disinfect (Kyte and Kleyn 1996, Smith 2000). Plant material at the base of a plant may, however be more suitable than explants that grew higher up (Kyte and Kleyn 1996).

The season of the year can have an effect on contamination and the response of the explant in culture (Smith 2000). Contamination tends to increase as summer progresses.

The smaller the explant, the harder it is too culture (Smith 2000). Larger explants have more nutrient and plant growth regulator reserves to sustain the culture. A large explant is often more difficult to decontaminate (Kyte and Kleyn 1996, Smith 2000).

Explants should be obtained from plants that are healthy as apposed to plants under nutritional or water stress or plants exhibiting disease symptoms (Smith 2000). Plant material in state of active growth is cleaner, and more suitable for aseptic culture, compared to dormant tissue. To control contamination, donor plants should be pre-screened for diseases (Ahloowalia*, et al.* 2002).

Plant material obtained from the field is often more contaminated than material obtained from greenhouse or growth chamber grown plants (Kyte and Kleyn 1996, Smith 2000). Mother plants should ideally be maintained under dust, insect and disease free conditions (Ahloowalia*, et al.* 2002). These plants should also not be stressed and they should preferably be grown under controlled conditions that promotes active growth (Kane 2004). Such conditions should preferably include

conditions of low relative humidity. Drip irrigation should preferably be used as the misting will facilitate contamination by wetting the foliage (Kane 2004).

Placing the plant material in a less humid and dry environment a few weeks prior to taking explant material can reduce contamination of cultures (Smith 2000).

Plant material growing in soil (roots, tubers, bulbs) or near the soil surface (stolons, rhizomes, orchid protocorms, etc.) is often harder to clean and disinfect than aerial plant material (Smith 2000). Explants are much easier to clean if the plant has been growing in an artificial medium, such as washed sand or perlite (Kyte and Kleyn 1996). After cutting the explant from the source plant, it should be placed in a plastic bag containing a moist paper towel and kept refrigerated until culture initiation (Kyte and Kleyn 1996).

5.1.2 Aseptic cultures

The maintenance of aseptic or sterile conditions is essential for successful tissue culture (Gamborg and Phillips 1995a). There are three categories of sterilisation (Gamborg and Phillips 1995a). The first involves the preparation of sterile media and containers. The second involves obtaining explants materials that are sterile and axenic (free from other organisms). The third involves the maintenance of such aseptic conditions during culture.

Cleanliness is one of the most important factors in preparing a facility for tissue culture (Kyte and Kleyn 1996). The selected area should be relatively free from contaminants such as dust, spores, mould, smoke and chemicals. A micropropagation laboratory essentially consists of three distinct areas; a space for preparing the culture media; a chamber used for transferring cultures; and an area in which to grow the cultures. These areas are usually located in three separate rooms.

Each time instruments touch the surface of the explant they are sterilised and the explant is moved to a new portion of the dissection stage (Ahloowalia, *et al.* 2002).

Explants must always be cut with sterilized equipment (Kyte and Kleyn 1996). Instrument packages can be wrapped in aluminium foil or placed in a stainless steel instrument tray and autoclaved at 121°C for 15-20 minutes or placed in a dry oven at 140-160°C for 4 hours (Smith 2000). The instruments are placed in a test tube with 95% alcohol when not in use so that two thirds of the instrument is submerged in alcohol (Smith 2000).

The test tube should be placed in a sturdy flask or beaker with water (Smith 2000). The instrument is flamed and allowed to cool before use. The alcohol and Bunsen burner should be placed on opposite sides of a laminar flow hood.

It has been reported that *Bacillus macerans*, a bacterial contaminant, could be viable on forceps stored in 95% ethanol for several weeks (Smith 2000). These bacteria even remain viable after flaming and can only eliminated by autoclaving at 121°C for 20 minutes or by heating for 6-8 seconds over a Bunsen burner. *Clavibacter* sp. may survive alcohol flaming and so instruments should be washed to remove excess plant material and autoclaved (Smith 2000).

To kill *Bacillus macerans* the pH of the sodium hypochlorite solution used for sterilisation can be adjusted to 7 (Smith 2000). This solution is however also toxic to cut plant surfaces and delicate plant material. A dilution of 1000 ppm is used for 30 seconds on instruments to kill *Bacillus macerans*. After instruments are placed in this solution they are dipped in ethanol and flamed.

Using bead sterilizers is an effective and safe option for instrument sterilization (Smith 2000). A bead sterilizer consists of heated housing filled with glass beads. The instrument is placed in the sterilizer for approximately 10 seconds at 240°C. Blades however dull faster in a bead sterilizer compared to sterilisation using alcohol.

Bacticinerators are also used in instrument sterilisation (Smith 2000). This however reduces instrument life, due to heat, which causes warping.

Culture tubes containing spent medium should be autoclaved for at least 30 minutes and the contents promptly discarded, after which the glassware should be washed (Smith 2000). Glassware should be scrubbed in warm, soapy water, rinsed three times with tap water, rinsed three times in distilled water and place in a clean area to dry (Smith 2000).

5.1.2.1 Surface sterilization technique

Explants require surface-disinfection before they can be placed in culture on the nutrient agar for *in vitro* culture (Smith 2000). Explants are washed in sterile water and rinsed in ethanol and the surface is sterilised using chemicals with a chlorine base (Ahloowalia*, et al.* 2002). There are a number of products used for surface infections, the most commonly used is commercial chlorine bleach (Smith 2000).

For soft, herbaceous material a calcium or sodium hypochlorite based solution is often used at a concentration of 1-3% (Ahloowalia*, et al.* 2002). Before surface-disinfection any remaining soil or dead parts should be removed from the explant (Pierik 1997). An inexpensive and ready-made alternative is a 5-7% solution of Domestos® (a toilet disinfectant by Lever Bros. Ltd., UK), which contains 10.5% sodium hypochlorite, 0.3% sodium carbonate, 10.0% sodium chloride and 0.5% sodium hydroxide and a patented thickener (Ahloowalia*, et al.* 2002). Explants are washed in sterile water before and after sterilization (Ahloowalia*, et al.* 2002).

A general procedure for preparing the explant involves washing the explant in warm, soapy water after which it is rinsed in tap water (Pierik 1997, Smith 2000). The explant is then rinsed in a freshly made chlorine bleach solution (Pierik 1997). One to 2 drops of wetting agent should be added to every 100 ml of bleach solution. The explant is then rinsed in sterile water three to five times.

Pierik (1997) stated that a dealing a brief alcohol rinse or swab is needed with hairy or wax coated surfaces. Epidermal hairs may trap air bubbles, in such cases these have to be evacuated under vacuum.

Sterilized forceps and scalpels must be used for the transfer of explants to fresh solutions (Ahloowalia, *et al.* 2002). Sterile containers must be used throughout the protocol of surface sterilization (Ahloowalia, *et al.* 2002). If explants become brown or pale the strength of sterilizing agent should be reduced (Gamborg and Phillips 1995b, Pierik 1997).

A cut explant such as a stem or leaf that is surface sterilised often shows tissue damage from surface sterilisation (Pierik 1997). The damaged tissue should be removed before culture (Smith 2000).

Gamborg and Phillips (1995) suggest that a procedure for seed sterilization should include washing the seeds in detergent, after which they rinsed with tap water and subsequently alcohol, a bleach solution and autoclaved demineralised water respectively.

Seeds can be germinated on filter paper in petri dishes or on an agar medium (Gamborg and Phillips 1995a). A single seed should ideally be placed in each container so that a single contaminated seed does not contaminate other seeds.

Contamination resulting from improperly sterilised tissue will generally arise from the explant and be located in the medium adjacent to the explant (Smith 2000). Contamination that is due to poor technique will generally appear over the entire agar surface (Smith 2000). Examples of poor technique include contaminated transfer hood filters and culture cabinets and improperly sterilised media.

Contamination of cultures by fungi appear as fuzzy growth whereas bacterial contamination appears as smooth pink, white or yellow colonies and contamination from insects appears as tracks across the medium which are visible due to surrounding fungal or bacterial growth (Smith 2000).

Explant material may harbour internal micro-organisms (Smith 2000). In such a case it is very difficult to establish clean cultures. Explants that are least likely to harbour internal contaminants include explants taken from growing shoot tips, ovules of immature fruit, immature and mature flower parts and runner tips (Smith 2000). Explants that are more likely to harbour internal contaminants include explants taken from bulbs, slow-growing shoots or dormant buds, roots, corms and underground rhizomes (Pierik 1997, Smith 2000). In such cases seeds are often aseptically germinated to provide clean explants from the root, hypocotyl, cotyledon and shoot (Pierik 1997). In such cases using of antibiotics or fungicides in the medium is generally not useful (Smith 2000). Although these agents can repress the growth of some microorganisms, they can also suppress the growth of the plant tissue or even kill it (Smith 2000).

5.1.3 Prevention of Contamination

It is important to wash hands, fingernails and arms with warm soapy water and a finger-nail brush. If sterile latex gloves are not used, hands and arms must be treated with 70% alcohol. Hair nets, masks, and a clean laboratory coat are also beneficial. Talking inside or next to the laminar flow cabinet should be minimised (Smith 2000).

Dust helps spread contaminants (Smith 2000). Contamination from foot traffic and air vents can be a problem (Smith 2000). The tissue culture areas should be kept clean at all times to ensure clean cultures and reproducible results (Smith 2000). Potted plants and other plant material can be a source of mites and other contaminating organisms (Smith 2000). If work is done in the field or greenhouse it is preferable to shower and change clothes before entering the culturing facility (Smith 2000). It is more convenient to prepare culture media on a laboratory chemical bench with a pH meter, balances and a sink in close proximity.

Most nutrient media are sterilised using an autoclave (Pierik 1997). Flasks containing between 20 and 50 ml of nutrient media are autoclaved for 20 minutes at 121°C,

flasks containing between 50 and 500 ml of nutrient media are autoclaved for 25 minutes at 121°C and flasks containing between 500 and 5000 ml of nutrient media is autoclaved for 35 minutes at 121°C (Pierik 1997). Empty tubes, flasks and filter paper is autoclaved for 30 minutes at 130°C (Pierik 1997). When autoclaving bottles and flasks with tops they should not be tightly packed and their tops should be loose (Pierik 1997). Other items such as test tubes, petri dishes, paper and instruments can be dry sterilised for 2 to 3 hours at 160°C (Pierik 1997). Autoclaving breaks down, amongst others, sucrose, zeatin, gibberellic acid (90% loss in reactivity) and Vitamin B_1 (Gamborg and Phillips 1995b, Pierik 1997). Most auxins and cytokinins however remain active after autoclaving (Gamborg and Phillips 1995b). The pH of autoclaved media is generally lowered by 0.3 to 0.5 units (Pierik 1997). Autoclaving media at a high temperature caramelizes sugars, leading to toxic effects (Pierik 1997) and may also precipitate salts and depolymerise the agar (Pierik 1997).

Most culture rooms consist of a room with shelves equipped with lights with photoperiod control timers (Beyl 2005). High humidity in the culture room should be avoided as it increases contamination (Smith 2000). Adequate airflow is therefore essential (Beyl 2005). The culture room temperature should generally be kept between 24 and 29°C, with 8 hours of darkness and 16 hours of light (Kyte and Kleyn 1996). Most cultures are incubated in a temperature range of 25°C to 27°C (Smith 2000). It is usually preferable to set the timer so that the 8 hours of darkness occur during the day and the 16 hours of light occurs during the night (Kyte and Kleyn 1996). In this way the heat given off by the lights at night aids in keeping the room warm when it is most needed and the absence of light during the day allows the room to cool (Kyte and Kleyn 1996).

5.1.4 Culture media

A number of standard formulas for tissue culture media have been developed to provide optimum nutrients and growth regulators for specific plants (Kyte and Kleyn 1996). The selection or development of a suitable culture medium is vital to the success of the culture (Smith 2000). If literature on a particular species is not

available, the development of a suitable medium is based on trail and error (Smith 2000). The approach to the development of a suitable medium will depend on the purpose of the culture (Smith 2000). The medium generally contains water, inorganic salts, plant growth regulators, vitamins, a carbohydrate and a gelling agent (Smith 2000). High quality water should be used as an ingredient of the plant culture media (Beyl 2005, Pierik 1997). Ordinary tap water contains cations, anions, particulates, micro-organisms and gases that may influence the reaction of the tissue culture media with the tissue (Beyl 2005). The most commonly used method of water purification involves a deionization treatment followed by one or two glass distillations (Beyl 2005, Pierik 1997).

<u>5.1.4.1 Salts</u>

The distinguishing feature of Murashige and Skoog inorganic salts is their high content of nitrate, potassium and ammonium in comparison to other salt formulations (Smith 2000). Table 7 shows the composition of the Murashige and Skoog formula.

Table 7: The standard Murashige and Skoog formula (From Kyte & Kleyn (1996).

			mg/l
MAJOR SALTS	Ammonium nitrate	NH_4NO_3	1650
	Calcium chloride	$CaCl_2 \cdot 2H_2O$	440
	Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	370
	Potassium nitrate	KNO_3	1900
	Potassium phosphate	KH_2PO_4	170
	Subtotal		4530
MINOR SALTS	Boric acid	H_3BO_3	6.2
	Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.025
	Cupric sulfate	$CuSO_4 \cdot 5H_2O$	0.025
	Manganese sulfate	$MnSO_4 \cdot H_2O$	16.9
	Potassium iodide	KI	0.83
	Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25
	Zinc sulfate	$ZnSO_4 \cdot 7H_2O$	8.6
	Subtotal		32.83
IRON	Ferrous sulfate	$FeSO_4 \cdot 7H_2O$	27.8
	Na_2EDTA		37.3
	Subtotal		65.1

| **Total mg/l** | **4627.93** |

Murashige and Skoog (MS) (1962) is the most suitable and the most commonly used basic tissue culture medium for plant regeneration from tissues and callus (Beyl 2005). Salt stocks are best stored in the refrigerator and are stable for several months (Smith 2000).

Plant growth and developmental processes are controlled by plant growth regulators (Gaba 2004). The study of plant growth regulator function is complex because several plant growth regulators usually work in concert with each other and their concentration within plant tissues changes with time, season and developmental stage (Gaba 2004). The effect of plant growth regulators on plant growth and development depend on the chemical structure of the plant growth regulators used, the plant tissue used and the genotype of the plant (Gaba 2004). The type and concentration of the plant growth regulators used will vary according to the culture purpose (Pierik 1997).

5.1.4.2 Auxins

Auxin (for example: IAA, NAA, 2,4-D, or IBA) is required by most plants for cell division and root initiation (Pierik 1997, Smith 2000). Auxin, or indole-3-acetic acid (IAA), was the first plant growth regulator to be isolated (Gaba 2004). IAA is rapidly degraded in growth media and inside the plant (Gaba 2004). For this reason chemical analogues of IAA with similar biological activity are often substituted (Gaba 2004). These more stable synthetic auxins include 2,4-D, IBA and NAA (Gaba 2004). IAA is added at a concentration of 0.01 to 10 mg l^{-1}, while synthetic auxins, such as IBA, NAA and 2,4-D, are used at concentrations of 0.001 to 10 mg l^{-1} (Pierik 1997). IAA can be considered to be a weak auxin (Pierik 1997). Cultures in which a large quantity of IAA has been added are often less successful than cultures to which low concentration of a stronger auxin, such as NAA have been added (Pierik 1997).

At high concentrations auxin can suppress morphogenesis (Smith 2000). Auxins have numerous effects on plant growth and differentiation, depending on their chemical

structure, their concentration and the affected plant tissue (Gaba 2004). Auxins generally stimulate cell elongation, cell division in cambium tissue and, together with cytokinins, the differentiation of phloem and xylem and the formation of adventitious roots (Pierik 1997). High concentrations of auxins can induce somatic embryogenesis (Gaba 2004).

The essential function of auxins and cytokinins is to reprogram somatic cells that were in a state of differentiation (Gaba 2004). Such reprogramming causes dedifferentiation and then redifferentiation into a new developmental pathway (Gaba 2004).The mechanism of dedifferentiation is not understood (Gaba 2004).

The use of 2,4-D should be avoided, as it may induce mutations (Pierik 1997). This growth regulator is however important in callus initiation for many species.

2,4-D is widely used for callus induction, while IAA (0.6-60 µM), IBA (2.5-15 µM) and NAA (0.25-6 µM) are mainly used in root initiation (Gaba 2004, Smith 2000). Higher than optimum levels of auxins causes callus production and a reduction in root growth and root quality (Gaba 2004). Combinations of auxins at low concentrations can sometimes produce better results than using individual auxins (Gaba 2004). High concentrations of auxins are sometimes necessary to induce rooting (Gaba 2004). This can however have undesirable side effects such as growth inhibition of induced roots (Gaba 2004). In such cases the elevated auxin levels should be administered as a pulse treatment (Gaba 2004). To do this the shoot is incubated with auxin for several days before it is transferred to a medium with no plant growth regulators to allow root growth and development (Gaba 2004).

Somatic embryogenesis is typically induced by auxins, sometimes in combination with cytokinins (Gaba 2004). 2,4-D is commonly used at this stage, although other auxins can be used (Gaba 2004). Auxins induce the cells to become embryogenic and promote subsequent repetitive cell division of embryogenic cell (Gaba 2004). High concentrations of auxins prevent cell differentiation and embryo growth (Pierik 1997).

5.1.4.3 Cytokinins

As the name suggests, cytokinins cause cell division (Gaba 2004). Such cell division can lead to shoot regeneration *in vitro*, by stimulating the formation of shoot apical meristems and shoot buds (Pierik 1997). This cell division caused by cytokinin can also cause the production of undifferentiated callus (Gaba 2004). A high concentration of cytokinins can cause the release of shoot apical dominance and will block root development (Pierik 1997). Cytokinins include Kinetin, Zeatin, 2-iP, BA, BAP and Thidiazuron (Pierik 1997). A high concentration of cytokinins can cause many small shoots to initiate but fail to develop (Gaba 2004).

Shoots are induced into forming roots by placing them in a regeneration medium, containing a high level of cytokinin, and then in a medium with no plant growth regulators (Gaba 2004). Cytokinins inhibit rooting and can be effectively removed from the plant material by placing shoots in a medium without plant growth regulators (Gaba 2004). Such a treatment can also be used to reduce endogenous cytokinin levels (Gaba 2004).

5.1.4.4 Gibberellin

Gibberellins are, in most cases, non-essential for plant development and *in vitro* culture (Pierik 1997). In tissue culture Gibberellic Acid (GA) is used to stimulate either shoot elongation or the conversion of buds into shoots (Pierik 1997). Gibberellins reduce root formation and embryogenesis *in vitro* (Pierik 1997). Gibberellins are primarily used to stimulate cell elongation and to produce elongated shoots in plant tissue culture (Gaba 2004). Unwanted side effects caused by gibberellins include reduction in the number of buds produced, the elongation of leaf structures such as petioles and lamina, the excessive elongation of shoots and reduced root production (Gaba 2004).

Yasmin *et al.* (2003) found that GA dissolved in 0.2% ethanol inhibited adventitious rooting of mungbean cuttings but when dissolved in water, GA promoted adventitious

rooting at 10^7 M and 10^8 M concentrations (Yasmin, *et al.* 2003). Ethanol thus suppresses the promoting effects of GA (Yasmin, *et al.* 2003).

<u>5.1.4.5 Abscisic acid</u>

Abscisic acid is rarely used in tissue culture protocols and has a negative effect on growth in most cases (Pierik 1997). It is mainly used in plant tissue culture to facilitate somatic embryo maturation (Gaba 2004) but may also be used in some regeneration processes and rarely used to produce somatic embryos (Gaba 2004). ABA induces the formation of essential LEA (late embryogenesis abundant) proteins found at late stages of embryogenesis in somatic and sexual embryos (Gaba 2004). LEA proteins are associated with tolerance to water stress resulting from desiccation and cold shock (Goyal, et al. 2005).

<u>5.1.4.6 Ethylene</u>

Ethylene or physiological reactions similar to that caused by ethylene is produced by certain plastic containers, plant tissue and as a result of fire (Pierik 1997). This is the only gaseous natural plant growth regulator and it is naturally produced by all plant tissues in a controlled fashion (Gaba 2004). Endogenously produced ethylene can accumulate in a closed vessel to levels that negatively affect plant growth and development (Pierik 1997). The biological effect of the ethylene depends on how air-tight the vessel is and the sensitivity of the plant material (Gaba 2004).

Ethylene is primarily known for its effects on fruit ripening (Gaba 2004). Exposure to ethylene also results in reduced stem length, restricted leaf growth, premature leaf senescence and may cause increased growth of axillary buds (Gaba 2004). An enhanced ethylene concentration can induce callus formation, while inhibiting bud and shoot regeneration (Gaba 2004). Low concentrations of ethylene stimulate somatic embryogenesis, while high concentrations of ethylene inhibit somatic embryogenesis (Gaba 2004). Explants need a low level of ethylene for correct biological function, but too high an ethylene concentration leads to symptoms of excess (Gaba 2004).

Such symptoms of excess include stunted growth, a reduction in leaf size and leaf drop (Nowak and Pruski 2002, Pierik 1997). These plants when transferred to acclimatise well to the *in vivo* environment and often desiccate shortly after being transferred to soil (Nowak and Pruski 2002).

Endogenous ethylene has an important role in shoot and root growth and differentiation (Pierik 1997).

5.1.4.7 Combinations of plant regulators

A high ratio of auxin to cytokinin induces root formation in shoots of dicotyledonous plants and somatic embryogenesis (Gaba 2004). Intermediate ratios induce callus initiation and adventitious root formation from callus in dicotyledonous plants (Gaba 2004). Such intermediate ratios often involve high levels of both cytokinins and auxins (Gaba 2004). Low ratios of auxin to cytokinin induce adventitious shoot formation and axillary shoot production in shoot cultures (Gaba 2004).

The optimum cytokinin to auxin ratio can be established by using a matrix approach (Table 8) with two axes of increasing plant growth substance concentration (Kyte and Kleyn 1996)

Table 8: Matrix to establish optimal auxin to cytokinin ratios and their concentrations, where the rows represent auxin levels and the columns represent the cytokinin levels (Modified from Kyte and Kleyn (1996).

	0	0.5	1	3	5	10
0						
0.5						
1						
3						
5						
10						

5.1.4.8 Vitamins

The vitamin considered most important for plant cells is thiamine (B_1) (Smith 2000). Other vitamins, such as nicotinic acid (B_3) and pyridoxine (B_6), are also added to culture media, as they may enhance cellular response (Smith 2000).

5.1.4.9 Carbohydrates

Green cells in culture are generally not photosynthetically active and require a carbon source (Smith 2000). Sucrose or glucose at 2-5% (w/v) is commonly used (Smith 2000). Higher levels of sucrose leads to low levels of photosynthesis in the leaves (Roberts, et al. 1990)

Higher levels may be used for embryo culture (Smith 2000). Sugars undergo caramelization when autoclaved too long (Smith 2000). When sugars are heated they degrade and form melanoidins, which are brown, high molecular weight compounds that can inhibit cell growth (Smith 2000).

5.1.4.10 Gelling agent

The type of agar used to gel the medium can affect the response of experiments (Smith 2000).
To minimise problems that arise from agar impurities washed or purified agar should be used (Smith 2000).

5.1.5 Culture preparation and management

Auxin stocks are usually prepared by adding several drops of a 1 N NaOH or KOH solution until the crystals are dissolved, rapidly adding 90 ml of double distilled water and increasing the volume in a volumetric flask (Smith 2000).

IAA stock should be prepared fresh weekly because it is degraded by light within a few days (Smith 2000). IAA is also destroyed by low pH, oxygen, and peroxides (Smith 2000). 2,4-D, a synthetic auxin is more stable than IAA (Smith 2000).

Cytokinin stock is prepared in a similar fashion to auxin except that 1 N HCl and a few drops of water are used to dissolve the crystals (Smith 2000). Gentle heating is also required to completely dissolve the crystals (Smith 2000). Double distilled water is then added quickly to avoid the crystals falling out of solution (Smith 2000). Cytokinin stock may be stored for several months in the refrigerator (Smith 2000). Long term degradation does however take place (Smith 2000).

Zeatin is thermolabile and should be added inside a laminar flow cabinet after the medium has been autoclaved (Stephan-Sarkissian 1990). Additions should be made when the medium is at a temperature 40 to 50°C, so that medium temperature does not damage the Zeatin, but the agar does not set (Stephan-Sarkissian 1990).

Agar should be melted over a hot plate and kept in motion by either a magnetic stir bar or stirring the solution by hand (Smith 2000). When the agar is dissolved it can be dispensed into the culture containers (Smith 2000).

A medium that has grown plant material for a prolonged period of time may develop a change in pH, which can be detrimental to the culture (Kyte and Kleyn 1996). In such cases the culture should be transferred to a fresh medium (Kyte and Kleyn 1996). The old medium should also be checked in order to determine whether a change in pH was the cause of decline in the culture (Kyte and Kleyn 1996). Significant changes in pH could also indicate that the culture should be transferred to fresh media more often (Kyte and Kleyn 1996). The pH of plant tissue culture medium is generally adjusted to between 5.5 and 6.0 (Smith 2000). Below a pH of 5.5, the agar will not gel properly and above a pH of 6.0 the gel may be too firm (Pierik 1997). Media pH generally drops by 0.6 to 1.3 units after autoclaving (Smith 2000). Production of organic acids and nitrogen utilization by plant tissue in culture may cause the pH to drop (Smith 2000). The pH should be adjusted before adding agar with 1.0 or 0.1 N HCl by using a medicine dropper while keeping the medium stirred (Smith 2000). If the pH is too low IAA and gibberellic acid becomes unstable (Pierik 1997). A low pH may also cause the precipitation of phosphate and iron salts (Pierik 1997). Vitamin B_1 becomes less stable in a medium with a low pH (Pierik 1997).

5.1.7 Culture types

<u>5.1.7.1 Callus culture</u>

Explants, when cultured in the appropriate medium, usually with both auxin and cytokinin, give rise to a mitotically active, but unorganized mass of cells. It is thought that, under the right conditions, any plant tissue can be used as an explant (Slater, et al. 2003). Callus culture concerns the initiation and continued proliferation of undifferentiated parenchyma cells from explant tissue on clearly defined semi-solid media (Brown 1990).

Callus initiation is the first step in many tissue culture experiments (Brown 1990, Smith 2000). *In vivo*, callus is a wound tissue produced in response to injury or infestation (Brown 1990, Mineo 1990, Smith 2000). Not all the cells in an explant contribute to callus formation (Smith 2000). Only certain callus types, which are competent to regenerate organised structures, display totipotency (Smith 2000).

The level of plant growth regulators is a major factor that controls callus formation in the culture medium (Brown 1990, Smith 2000). The correct concentration of plant growth regulators depends on the species, individual and explant source (Smith 2000). Other culture conditions such as light, temperature and media composition are also important for callus formation and development (Smith 2000).

Explants can be taken from various plant organs, structures and tissues (Mineo 1990). Young tissues of one or a few cell types are most often used as explants (Mineo 1990). The pith cells of a young stem are regarded as a good source of explant material for callus initiation (Mineo 1990).

Callus growth is maintained, provided that the callus is subcultured onto a fresh medium periodically. During callus formation there is a degree of de-differentiation in both morphology (usually unspecialised parenchyma cells) and metabolism. As a consequence most plant cultures lose their ability to photosynthesise (Slater*, et al.*

2003). This means that the metabolic profile does not match that of the donor plant and the addition of compounds such as vitamins and a carbon source is necessary (Slater, *et al.* 2003).

Callus culture is often performed in the dark as light can result in differentiation of the callus (Slater, *et al.* 2003). The culture often loses its requirement for auxin and/or cytokinin during long-term culture (Slater, *et al.* 2003). This process is known as habituation and is common in callus cultures from some species such as sugar beet (Slater, *et al.* 2003).

By manipulating the auxin to cytokinin ratio whole plants can subsequently be produced from callus cultures (Phillips, et al. 1995). Callus culture can also be used to initiate cell-suspension cultures (Phillips, *et al.* 1995).

Endogenous levels of plant growth regulators and polar growth regulator transport can drastically influence callus induction, which refers the initiation of callus formation (Pierik 1997, Smith 2000). Explant orientation of different sectioning methods affect callus induction (Smith 2000).

Callus cultures subcultured regularly on agar media exhibit a sigmoidal growth curve (Phillips, *et al.* 1995). Phillips *et al.* (1995) describe five phases of callus growth. Phase I is a lag phase, where cells prepare to divide. Phase II is an exponential phase, where the rate of cell division is the highest. Phase III is a linear phase, where cell division slows, but the rate of cell expansion increases. Phase IV is a deceleration phase, where the rates of both cell division and expansion decrease and phase V is a stationary phase, where the number and size of cells remain constant.

Callus growth can be monitored in a non-destructive manner using fresh weight measurements (Phillips, *et al.* 1995, Stephan-Sarkissian 1990). Dry weight measurements are more accurate, but involve the destruction of the sample (Phillips, *et al.* 1995). Mitotic index measurements of cell division rates are not easy to perform

as they require numerous measurements to be made at various time intervals with very small amounts of tissue (Phillips, *et al.* 1995). Fresh weight measurements are performed by culturing a known weight of callus for a given time (typically 4 weeks) and weighing the callus after this time using aseptic techniques (Stephan-Sarkissian 1990).

<u>5.1.7.2 Root cultures</u>

Root cultures were one of the first achievements of modern plant tissue culture (Slater, *et al.* 2003). Root cultures can be established *in vitro* from explants of the root tip of either primary or lateral roots and can be cultured on fairly simple media (Pierik 1997, Slater, *et al.* 2003). The growth of roots *in vitro* is theoretically unlimited as roots are indeterminate organs (Slater, *et al.* 2003).

<u>5.1.7.3 Shoot section and meristem culture</u>

The tips of shoots can be cultured *in vitro*, producing clumps of shoots, from either axillary or adventitious buds. This method is often used for clonal propagation (Slater, *et al.* 2003).

Shoot meristem cells retain the embryonic capacity for unlimited division (Kane 2004). Micropropagation is defined as the true-to-type propagation of selected genotypes using *in vitro* culture techniques (Kane 2004).

In vitro propagation can be achieved via enhanced axillary shoot proliferation, node culture, *de novo* formation of adventitious shoot organogenesis and nonzygotic embryogenesis (Kane 2004). The most frequently used micropropagation method for commercial production utilizes enhanced axillary shoot proliferation from cultured meristems (Kane 2004). Advantages of this method include provision of genetic stability and shoot explants that can easily be obtained from many plant species (Kane 2004). Such cultures usually include plant growth regulators such as cytokinin. The axillary shoots that are formed by micropropagation are either subdivided into shoot tips and nodes that serve as secondary explants for further propagation or rooted to produce plantlets (Kane 2004). The shape and size of the shoot apices of

various species differ significantly. Taking larger shoot tip explants results in enhanced survival rate and a more rapid growth response. Such large explants are however difficult to surface sterilize (Kane 2004).

New shoots provide relatively cleaner explants with increased juvenility (Kyte and Kleyn 1996). Juvenility is an important factor in selecting explants for tissue culture, as it increases the likelihood of success (Kyte and Kleyn 1996). There are numerous ways to enhance or stimulate the production of juvenile growth (Kane 2004). These include altering the physiological status of the stock plant through trimming, thus stimulating lateral shoot growth, pre-treatment sprays containing cytokinins or gibberellic acid or using forcing solutions of 2% sucrose and 200 mgl^{-1} 8-hydroxyquinoline citrate for the induction of bud break and delivery of growth regulators to target explant tissues (Kane 2004).

Numerous factors can affect successful stage I stem culture establishment (Kane 2004). These include the time it takes to prepare the explants for culture, the position of the explant on the stem, explant size, and polyphenol oxidation. Cytokinins or auxins are often added to stage I media to enhance explant survival and growth. Types and levels of growth regulators used are dependant on species, genotype and explant size. Smaller explants are more dependant on media supplemented with cytokinin and auxin (Kane 2004). Most media at least contain a cytokinin such as BA, 2-iP and thidiazuron (Kane 2004). Auxins used include IAA, NAA and IBA (Pierik 1997). For many species, especially herbaceous and woody perennials, consistency in growth rate is only achieved after multiple subcultures on stage I media (Kane 2004). Physiological stabilization may take 3 to 24 months and 4 to 6 subcultures. Failure to allow stabilization before the transferral to Stage II media with higher cytokinin levels may result in lower shoot multiplication rates or production of callus and adventitious shoots (Kane 2004).

Stage II of shoot culture is usually characterized by repeatedly enhanced formation of axillary shoot tips or lateral buds (Kane 2004). This is normally done on a medium

supplemented with a slightly higher cytokinin concentration compared to stage I. The higher cytokinin levels disrupt the apical dominance of the shoot tip. A subculture interval of 4 weeks with a three- to eightfold increase in shoot numbers is common in stage II of shoot culture of many species. This means that a total of 4.3×10^2 shoots could be produced yearly from a single starting explant (Kane 2004).

In stage II, cultures are often subdivided into smaller clusters, individual shoot tips or nodal segments for further propagation (Kane 2004). The main factors that affect stage II of axillary shoot proliferation is the source and orientation of the explant on the culture medium. In many species subcultures inoculated with explants that had been shoot apices in the previous subculture often exhibit multiplication rates up to three times higher than lateral bud explant multiplication rates (Kane 2004).

The amount of time an explant can be maintained in stage II with monthly subculture depends on the species and its genetic stability (Kane 2004). A suitable type and concentration of cytokinin should be selected according to resulting shoot multiplication rate, shoot length and frequency of genetic variation. Shoot proliferation is enhanced by a high cytokinin concentration, this may however cause the shoots to be smaller and to exhibit symptoms of hyperhydricity (Kane 2004). Hyperhydricity refers to a condition in which the explant is excessively hydrated or water soaked (Prakash, et al. 2002). In some species auxins mitigate this inhibitory effect of cytokinin and increases the number of shoots suitable for rooting. The addition of auxin results in a greater chance for callus formation. Shoot elongation may be achieved by the addition of gibberellic acid (Kane 2004).

Stage III, the pre-transplant or rooting stage, may involve the elongation of shoots prior to rooting, the rooting of individual shoots or shoot clumps and the pre-hardening of cultures to increase survival (Kane 2004). This stage is sometimes bypassed by rooting the microcuttings in soil. Costs for Stage III range from 35 to 75% of production costs. *In vitro* formed roots are largely non-functional and often die after transplantation (Pierik 1997). Direct transplantation of the Stage II microcutting into

soil is however, for various reasons, not always suitable (Kane 2004, Pierik 1997). Kane (2004) proposed that Stage III should solely be used for elongation of Stage II shoots.

Stage III rooting of herbaceous plants is usually achieved on a medium without auxins (Kane 2004). Roots should not be excessively elongated *in vitro* to prevent injury during transplantation.

5.1.7.4 Embryo culture

An embryo culture is prepared by excising the embryo from the seed or fruit at some stage during its development and germinating it on an aseptic medium (Hartmann and Kester 1965). Embryo culture is a well established branch of tissue culture and is known as one of the oldest and most successful culture procedures (Hu and Zanettini 1995, Reed 2005).

This technique has three principle uses in propagation (Hartmann and Kester 1965). It is used to grow embryos that are excised from the seed at an early stage of development. In this way an embryo that would otherwise not have survived can be "rescued". It can also be used to circumvent dormancy and to produce immediate germination of seeds that otherwise would have required a long or ineffective pre-germination treatment (Hartmann and Kester 1965). Embryos can be used as explants to generate callus cultures or somatic embryos (Slater*, et al.* 2003). This can be done using either immature or mature explants. Immature, embryo-derived callus is the most popular method of monocotyledonous plant regeneration.

Seed dormancy of many species is due to chemical or mechanical inhibition caused by the structures covering the embryo, rather than the dormancy of the embryonic tissue (Hu and Zanettini 1995). In embryo rescue, the artificial medium substitutes for the endosperm (Reed 2005). Murashige and Skoog, and Gamborg's B-5 media are the most frequently used basal media for embryo culture.

Embryo development occurs in two phases, a heterotrophic and an autotrophic phase (Reed 2005). In the heterotrophic phase, the young embryo, or "proembryo", requires a complex medium. *In vivo* grown embryos at this stage are dependent on the endosperm. Amino acids such as glutamine and asparagine are often added to the culture medium.

Young embryos require a medium of high osmotic potential (Pierik 1997). A high osmotic potential prevents precocious development and promotes normal embryogenic development (Reed 2005). Sucrose is usually added to serve both as an osmoticum and carbon source (Pierik 1997). A medium with 232 mM to 352 mM (8-12%) sucrose is used for the culture of heterotrophic embryos (Reed 2005).

The autotrophic phase is usually initiated in the late heart-shaped embryo stage (Reed 2005). Embryos that are excised during this development stage are completely autotrophic (Hu and Zanettini 1995). Such embryos germinate and grow on a simple inorganic medium with a supplemental energy source (Hu and Zanettini 1995). An inorganic medium supplemented with 58 mM to 88 mM (2-3%) sucrose is used as a standard medium for the germination of autotrophic embryos (Reed 2005).

Growth regulators often have inconsistent effects on embryo culture (Reed 2005). They have however been extensively used in embryo rescue protocols, especially protocols involving heterotrophic embryos (Reed 2005). Low concentrations of auxins promote normal growth whereas gibberellins cause embryo enlargement and cytokinins inhibit growth (Reed 2005).

5.1.7.4.1 Seed dissection

Hard-coated seeds are first soaked in water for a few hours up to a few days before dissection (Hu and Zanettini 1995). Seeds are surface sterilised before and after soaking (Hu and Zanettini 1995).

The most suitable point of incision into the ovule differs amongst species (Reed 2005).

The embryos of some species can be extracted by cutting off the micropillar end of the ovule and applying gentle pressure at the opposite end of the ovule, so that the embryo is pushed through the opening (Reed 2005). Small seeds are dissected by making a longitudinal section using sterile microdissection needles (Hu and Zanettini 1995)

After excision, large embryos should immediately be transferred into culture vessels, using a pair of forceps (Hu and Zanettini 1995, Reed 2005). Small embryos can be handled using the moistened tip of a dissection needle (Hu and Zanettini 1995).

5.1.8 Plant regeneration

5.1.8.1 Somatic embryogenesis

In plants, morphologically and functionally correct nonzygotic embryos can arise from an array of cell and tissue types at a number of different points within both the gametophytic and sporophytic phases of the plant life cycle (Gray 2005). Somatic (or asexual) embryogenesis involves the formation of embryo-like structures, which have the potential to develop into whole plants in a way analogous to zygote embryos from somatic tissues (Slater, *et al.* 2003). Plant regeneration by somatic embryogenesis was first observed in carrot in 1958 (Phillips, *et al.* 1995). These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or a small group of cells without the production of an intervening callus (Finer 1995). Direct somatic embryogenesis is however rare and only common in reproductive tissues (Slater, *et al.* 2003). In indirect somatic embryogenesis, a callus is first produced from the explant before embryos are produced.

Synthetic auxins, especially 2,4-D, are most often used in protocols involving somatic embryogenesis (Gray 2005). Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage (embryo initiation), a high concentration of 2,4-D is used (Finer 1995). In the second stage (or the embryo production stage) embryos are produced in a medium with no or little 2,4-D (Slater, *et al.* 2003).

As with zygotic embryos, nonzygotic embryos arise from a single cell (Gray 2005). Nonzygotic embryos can mostly only be initiated from juvenile or meristematic tissues (Gray 2005). The structure of the dicotyledonous embryo changes from globular through heart, torpedo, cotyledonary to mature stages (Gray 2005, Phillips, *et al.* 1995).

Auxins activate pathways that induce the formation of embryogenic cells (Gray 2005). Auxins promote division, while suppressing differentiation and growth of embryogenic cells (Gray 2005). When using embryogenic cells as an explant, auxins are often not required as there is no need for an induction step (Gray 2005). In many dicotyledonous species cytokinins are also required to induce embryogenesis (Gray 2005). BA is the cytokinin most often used in embryogenesis (Gray 2005). Other cytokinins that have been used include TDZ, Kinetin and Zeatin (Gray 2005).

5.1.8.2 Organogenesis

Organogenesis involves the *de novo* production of organs directly from an explant or through initial callus culture (Schwartz, et al. 2004). An example of organogenesis from non-meristematic tissues is the process of adventitious rooting of stem cuttings. This process was used by the ancient Chinese to root woody cuttings and predates the time of Aristotle (382-322 BC) and Theophrastus (371-287 BC) (Schwartz, *et al.* 2004).

Organogenesis is regulated by altering the components of the culture medium (Brown and Charlwood 1990). Most important of these components is the auxin to cytokinin ratio, which determines the developmental pathway the regenerating tissue will take (Brown and Charlwood 1990). Shoots are usually induced to form first by increasing the cytokinin to auxin ratio of the culture medium (Brown and Charlwood 1990). These shoots can then be easily rooted (Slater, *et al.* 2003).

De novo organ formation via indirect organogenesis, which includes an intermediate callus formation and differentiation phase, may increase the possibility for somaclonal

variation (Schwartz, *et al.* 2004). Any stage in the process of organogenesis that involves callus growth should be minimized.
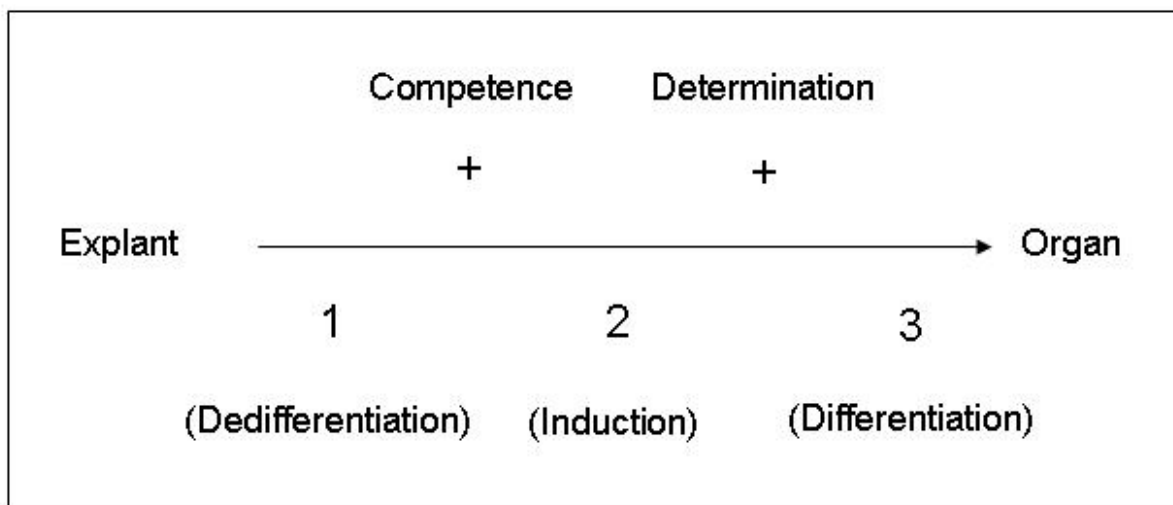
Competence   Determination

+   +

Explant ——————————————————————→ Organ

1   2   3

(Dedifferentiation)   (Induction)   (Differentiation)

Figure 8: Diagram to explain organogenesis as a developmental process (modified from Schwartz *et al* (2004).

After dedifferentiation (Figure 8) the explant acquires a state of competence, defined as its ability to respond to organogenic stimuli (Schwartz, *et al.* 2004) (Figure 8). The attainment of competence can not always be achieved with a single step.

The induction phase occurs between the time of competence and determination (Schwartz, *et al.* 2004). During induction, processes resulting from the expression of genes guides developmental processes and precede morphological differentiation. It has been suggested that such a genetically determined developmental process can be interrupted by certain physical and chemical stimuli (Pierik 1997). At the end of the induction phase the cells are fully committed to the production of shoots or roots.

At this point the tissue can be removed from the root or shoot producing medium and placed on a basal medium without plant growth regulators (PGR's), containing mineral salts, vitamins and a carbon source (Schwartz, *et al.* 2004). The desired organ is then produced on this medium. Successful determination is partially

dependent on the chemical and physical environment to which they have been exposed. The result of failure of explant tissues to express totipotency is a failure of the explant tissues to achieve the state of competence for induction. This makes investigation of the effects of physical and chemical parameters difficult. The use of biochemical or genetic markers that can clearly indicate the developmental disposition of the primary explant tissue have not yet been discovered (Schwartz, *et al.* 2004).

In the next phase the morphological differentiation and development of the nascent organ occurs (Schwartz, *et al.* 2004). Organ initiation involves a rapid shift in polarity followed by a smoothing of this shift into a radially symmetrical organization and the concurrent growth along the new axis to form a characteristic bulge (Schwartz, *et al.* 2004). There is not an absolute certainty as to which tissues are involved and the number of cells involved in meristem initiation (Schwartz, *et al.* 2004).

The initiation of adventitious roots occurs in four stages (Schwartz, *et al.* 2004). A meristematic locus is first formed by the dedifferentiation of a stem or other cells. These cells then multiply to form a spherical cluster. Further cell multiplication occurs with initiation of planar divisions to form a recognizable bilateral root meristem. Lastly, the cells located in the basal part of the developing meristem elongate, resulting in the eventual emergence of the newly formed root (Schwartz, *et al.* 2004). The production of a functional adventitious root system depends on the selection of a microcutting of the appropriate developmental stage and the ability of the *in vitro* environment to initiate the sequence of events described above (Schwartz, *et al.* 2004).

5.1.9 Measuring the *in vitro* response

Reliable and reproducible measurements of growth must be used in order to assess the performance of the explant and the suitability of the culture medium (Stephan-Sarkissian and Grey 1990). The most widely used method of growth determination measurement involves measuring both the fresh and dry mass of explants. Both the

fresh and dry mass should be noted as the wet mass may continue to rise after the dry mass has gone into a state of decline (Stephan-Sarkissian and Grey 1990). This rise in fresh mass after dry mass has declined is caused by the utilization of accumulated starch by the cells.

The viability of the culture gives a measure of its health (Stephan-Sarkissian and Grey 1990). Viability is the capacity of a cell, organ or organism to live (Duncan and Widholm 1990). Viability is most often measured using cytological stains such as fluorescein diacetate or 2,3,5-triphenyltetrazolium chloride (TTC) (Duncan and Widholm 1990, Stephan-Sarkissian and Grey 1990). The use of a single method to measure viability can only be qualitative and often leads to interpretation errors (Duncan and Widholm 1990, Stephan-Sarkissian and Grey 1990). The validity of a viability test should first be established (Duncan and Widholm 1990). Validity can be established by comparing results obtained from actively growing tissue with that of samples that have been frozen and then thawed (Duncan and Widholm 1990). Freezing will provide necrotic tissue to use as a control (Duncan and Widholm 1990).

Culture growth can also be assessed by measuring nutrient uptake (Stephan-Sarkissian and Grey 1990). The analysis of certain key components of the medium will indicate the limitation imposed by a particular nutrient at a given stage of the culture cycle (Stephan-Sarkissian and Grey 1990). Carbohydrate analysis may be used in conjunction with biomass measurements to determine carbon conversion efficiency.

5.1.10 Hardening

Micropropagation of a species is in some cases restricted due to unsuccessful *ex vitro* acclimatization, leading to a low survival rate of cultured plants (Huylenbroeck, et al. 2000). During this period of *ex vitro* acclimatization, plants must acquire the morphological and physiological features required by the *in vivo* environment and develop new patterns of resource allocation (Huylenbroeck, *et al.* 2000). *In vivo*

cultured plants will otherwise not be able to cope with the environmental stresses of the post-propagation environment (Huylenbroeck, *et al.* 2000).

During acclimatization there is a switch to autotrophy and changes in stomatal functioning and cuticular composition (Huylenbroeck, *et al.* 2000). Water is rapidly lost from the *in vitro* cultured plantlet because of the failure of the stomata to respond to stimuli that would normally induce their closure (Roberts, *et al.* 1990). The poorly developed cuticle results in a rapid loss of water (Roberts, *et al.* 1990).

Vitrified plants do not acclimatise well to *in vivo* conditions. Vitrified plants are common where liquid media and low agar concentrations are used (Pierik 1997). In vitrified plantlets there is a reduced deposition of cellulose and lignin, leading to an increase in water uptake by the cells and resulting in glassy swollen leaves and stems (Roberts, *et al.* 1990). Because of this, and the low rates of photosynthesis sustained by the *in vitro* cultured plants, they easily suffer from photoinhibition and water stress; leading to the production of reactive oxygen species (Huylenbroeck, *et al.* 2000). It has been demonstrated that micropropagated plants develop antioxidant mechanisms during acclimatization (Huylenbroeck, et al. 1998).

In vitro grown leaves are the only source of nutrition to cover metabolic demands and to sustain plant adaptation and regrowth during the first days after transplanting micropropagated plants to greenhouse conditions (Huylenbroeck, *et al.* 1998). The good and sustainable health of leaves is therefore essential to the acclimatization and survival of the plant (Huylenbroeck, *et al.* 1998).

Plant hardening is usually carried out under greenhouse conditions to increase the chance of survival (Ahloowalia and Prakash 2002). A commonly used greenhouse is the Quonset type. This consists of movable or fixed benches with hardening tunnels on them (Ahloowalia and Prakash 2002). It is also advantageous to acclimatise plants to lower humidities while they are still under *in vitro* conditions (Ahloowalia, *et al.*

2002). In this way, plants grown in strongly aerated vessels often require little or no hardening (Ahloowalia and Prakash 2002).

There are numerous differences between leaves formed *in vitro* and *ex vitro*: differences in wax composition, pigmentation content, stomatal response and photosynthetic performance (Huylenbroeck*, et al.* 1998). In a study conducted by Huylenbroeck *et al.* (2000) on *Calathea* plants (Marantaceae) it was shown that chlorophyll and carotenoid content in leaves formed *ex vitro* were almost three times higher than *in vitro*.

Stomatal aperture can be measured by applying nail varnish to the abaxial surface of the mature leaf (Roberts*, et al.* 1990). The image of the hardened film can then be analysed (Roberts*, et al.* 1990). This allows us to assess the degree to which the plant has hardened to *in vivo* conditions (Roberts*, et al.* 1990).

5.1.11 Cryopreservation

Cryopreservation refers to the viable storage of cells at the temperature of liquid nitrogen (-196°C) for future use (Withers 1990). Plant cell and tissue cultures change under culture conditions with respect to many desirable features such regeneration capacity (Withers 1990). Such time related factors can be eliminated from the culture process by effectively suspending time itself by immobilizing metabolic activity (Withers 1990). This is done by cryopreservation (Withers 1990).

For genetic conservation of sterile plants and unique clones, shoot cultures are most commonly used as preservation material because of their genetic stability and high regeneration capacity (Withers 1990).

Cryopreservation of seeds is the only possible way of effectively storing recalcitrant seeds that quickly loose viability under normal storage conditions (Withers 1990).

5.1.12 Aims

The aims of this chapter were to investigate the suitability of explants for micropropagation and to obtain, through experimentation, a medium for callus induction.

5.2 Micropropagation of species of the Asteraceae

Within one family there are often similar requirements or difficulties in micropropagation (Kyte and Kleyn 1996). Understanding the culture requirements of species in the same family (Asteraceae) could shed some light on the culture conditions needed for the successful propagation of *Syncarpha recurvata*.

5.2.1 *Chrysanthemum* spp.

Young flower heads are recommended for explants by Kyte & Kleyn (1996). Teixeira da Silva (2003) lists various explant sources for different species within the genus *Chrysanthemum*: stem, shoot tip, capitulum, rescued embryo, tubular floret, root, flower pedicel, leaf, petal, ovary, meristem, mesophyll protoplast, axillary bud or stem node. Regeneration involves the formation of shoots or roots or an intermediate callus phase that are all cultured on a basal Murashige and Skoog medium.

The treatment of young capitula includes washing in water with a few drops of detergent added (Kyte and Kleyn 1996). The heads are then rinsed twice with sterile distilled water, after which they are soaked in a 5% calcium hypochlorite solution. The heads are then cut longitudinally into two halves after which the bracts, disc flowers and ray flowers are removed and discarded. Short shoots develop in three weeks. Once the shoots reach a length of 1 cm they are dipped in 1% IAA in talcum powder and rooted in a potting mix (Kyte and Kleyn 1996).

Media formulas for *Chrysanthemum* species vary considerably.

Table 9 shows a commonly used formula. Kyte & Kleyn (1996) suggests the addition of kinetin instead of BA, an increase in sucrose and the addition of coconut milk for some species.

Table 9: *Chrysanthemum* species medium (Kyte and Kleyn 1996).

Compound	Stage I & II
	mg/l
MS Salts	4.628
Adenine sulphate	80
Inositol	100
Thiamine HCl	0.4
Nicotinic acid	0.5
Pyridoxine HCl	0.5
IAA	0.1
BA	1
Sucrose	30000
Agar	6000
pH 5.8	

5.2.2 *Gerbera jamesonii*

The explants used for *Gerbera jamesonii* are either 2 cm of the emerging shoot tip or whole young flower heads (Kyte and Kleyn 1996). The former is however, the most responsive explant.

The material used must be as clean as possible, as contamination is a big problem (Kyte and Kleyn 1996). Emerging shoot tips with 2 to 3 mm of the base of the crown, the junction between the root and the shoot, should be used. These are washed briefly in running water, after which they are rinsed three times in fresh solutions of sterile water with 1.0% Tween 20 for 30 minutes. These are then rinsed three times, for 15 minutes, in sterile distilled water. Emerging buds of explants can then either be used whole or segments can be excised under a microscope. The explants are then dipped in 1/100 bleach for 1 minute and placed on an agar medium (Kyte and Kleyn 1996).

The formula for the medium suggested for the micropropagation of *Gerbera jamesonii* is given in Table 10. If problems with rooting arise during the rooting stage the

Murashige and Skoog should be reduced to a third of its concentration. Two weeks into stage III before potting should be sufficient (Kyte and Kleyn 1996).

Table 10: Formula for a media for the micropropagation of *Gerbera jamesonii* (Modified from Kyte & Kleyn (1996).

Compound	Stage I & II	Stage III
	mg/l	mg/l
MS Salts	4628	4628
Adenine sulphate	80	-
Thiamine HCl	30	30
Nicotinic acid	10	10
Pyridoxine HCl	1	1
IAA	0.1	2
BA	2	-
Glycine	2	-
Sucrose	30000	20000
Agar	8000	8000
pH 5.7		

5.2.3 *Helichrysum italicum* and *H. stoechas*

Helichrysum italicum and *Helichrysum stoechas* are native to the Mediterranean (Giovannini, et al. 2001). *Helichrysum italicum* and *Helichrysum stoechas* have been used in folk medicine because of their antibacterial, antitoxic, diuretic and anti-allergic properties (Giovannini*, et al.* 2001). The flower heads of these plants contain essential oils, flavonoids and tannins. The flowers of both species are also often dried and used as "everlasting" flowers.

Explants were obtained from *in vivo* germinated seedlings (Giovannini*, et al.* 2001). Stem explants were surface sterilised with 70% ethanol for 30 seconds, followed by sterilisation for 20 minutes in a solution of 1% sodium hypochlorite and two drops of Tween 20. After this, the explants were washed three times in sterile distilled water. The explants were then placed on a medium consisting of basal salts and vitamins of

Murashige and Skoog (Murashige and Skoog 1962), 30% sucrose, 0.8% agar and various growth regulators (Giovannini, *et al.* 2001). The pH of the medium was adjusted to 5.7. All cultures were kept at 23 to 25°C under a 16:8 h light:dark regime.

Shoots of *Helichrysum italicum* were regenerated on a medium supplemented with 10 µM Zeatin and 0.5 and 1 µM IAA, while Thidiazuron at a concentration of 4.54 µM promoted callus and shoot differentiation in *H. stoechas* (Giovannini, *et al.* 2001). A system for callus production was also developed on a medium enriched with 10 µM 2,4-D and 1 µM kinetin (Giovannini, *et al.* 2001). Callus growth rate can be improved by modifying the 2,4-D concentration. The explants used for callus culture were cultured in darkness. Rooting of the explants was performed on MS basal medium without growth regulators. Rooted plantlets were acclimated in the greenhouse under mist (Giovannini, *et al.* 2001).

It is expected that explants of *Syncarpha recurvata* will respond to chemical stimuli *in vitro* in a fashion similar to other species of the Asteraceae.

5.3 Methods

All cultures were initiated inside a laminar flow cabinet and placed in a culture cabinet set at 21°C with a 16:8 h light:dark regime.

5.3.1 Establishing an aseptic technique

In order to ensure that the techniques used were aseptic, the culture of an easily cultured species, *Brassica oleraceabotrytis*, was attempted. After this test of the culture conditions was completed, the experiment was initiated.

5.3.2 Preparing and titrating the nutrient agar

Cultures was initiated on microbiological agar (12 g l^{-1}) supplemented with Murashige and Skoog with Gamborg's vitamins inside Nunclon® culture trays. Agar was pipetted into culture tray cells inside a laminar flow cabinet using an autopipette. The composition of this agar was modified according to the various culture media

treatments. A total of 65 different agar solutions of 35 ml were prepared. These were placed in 100 ml culture flasks and autoclaved for 20 minutes at 121°C in 5 l glass beakers covered in foil. The glass beakers with Erlenmeyer flasks were then transported to the laminar flow cabinet. Before placing the glass beakers into the laminar flow cabinet, they were sprayed with 70% alcohol. The Erlenmeyer flasks were then taken out of the beaker inside the laminar flow cabinet where they were placed on a hot-plate, which had been cleaned and sprayed with 70% alcohol before being placed inside the laminar flow cabinet. Before preparing the explants, 1 ml of the appropriate agar was pipetted into each of the culture tray cells.

5.3.3 Preparing the explants

All instruments used were autoclaved for 20 minutes at 121°C. The scalpel, microdissection needle and forceps were placed in a test tube filled with 95% alcohol placed in a beaker of water inside the laminar flow hood after each incision was made (Smith 2000). The explants were prepared on an autoclaved Petri dish in the hood. A total of 390 *Syncarpha recurvata* seedlings were used as explant material for the root tip, stem segment and shoot tip cultures. Explants used for the later three cultures were first rinsed in a 5% solution of Bacterax®, Kemklean® Pty. Ltd., and then in autoclaved distilled water before placing them in the appropriate culture tray cell. Embryos were only rinsed in autoclaved distilled water, because the sensitive embryonic tissue is easily damaged by chlorine-based disinfectants (Kane 2004).

<u>Root tip</u>

Root tips of about 3 mm length were removed with a sterile scalpel and placed horizontally on the growth medium.

<u>Stem segment</u>

Stem segments of about 3 mm length were then removed from the same seedlings and placed horizontally on the growth medium.

<u>Shoot tip</u>

The shoot tips were also used as explants. Shoot tips of about 4 mm in length were placed vertically in the growth medium, so that approximately 2 mm of the stem was submerged in the agar.

<u>Embryo</u>

Before the embryo was excised from the achene, the achenes were soaked for a day in autoclaved distilled water. The embryos were excised from the achene under a stereomicroscope. A longitudinal section was made using a sterile dissection needle, starting at the funicular scar. The embryos of achenes collected in August to October 2005 and May to July 2006 were used as two separate treatments. The embryos were placed on the surface of the agar.

5.3.4 Culture media treatments

For each culture treatment, 6 replicate cultures were prepared. A total of 65 treatments were prepared per explant type, except for the embryos to which a gibberellic acid pre-treatment was also added.

<u>The control</u>

As a control the agar was enriched with 4 628 mg Murashige and Skoog with Gamborg's vitamins and 30% sucrose. The pH of the control medium was 5.8.

<u>Plant growth regulator treatments</u>

The suitability of two sets of auxins and cytokinins was tested, IAA and zeatin, and 2,4-D and kinetin. For each set, there were 24 different treatments. The treatments were conducted in grid pattern (Table 8) so that the morphological effect of the different combinations of plant growth regulators could be easily compared.

Table 11: Bioassay grid to establish optimal auxin to cytokinin ratios and their concentrations, where the rows represent auxin levels (μM) and the columns represent the cytokinin levels (μM) (Modified from Kyte and Kleyn (1996)). The darkened squares indicate the treatments that were conducted.

	$0\,\mu M$	$0.5\,\mu M$	$1\,\mu M$	$5\,\mu M$	$10\,\mu M$
$0\,\mu M$					
$0.5\,\mu M$					
$1\,\mu M$					
$5\,\mu M$					
$10\,\mu M$					

Nutrient media (MS) treatments

The effect of three different nutrient solution concentrations on *in vitro* growth and germination was examined. The three concentrations were a quarter, a half and double the concentration used in the control.

CaCl₂ treatments

To test the effect of various $CaCl_2$ concentrations on *in vitro* growth and germination, three treatments were tested: 10 mM, 100 mM and 500 mM $CaCl_2$.

pH treatments

pH of the agar was measured with an EDT Instruments pH meter RE357 and adjusted with NaOH and HCl. Treatments with a pH of 6.5, 7 and 7.5 were set up.

Smoke water treatments

The effect of smoke water dilutions of 1:10, 1:50, 1:100 and 1:500 on *in vitro* growth and germination was examined. Smoke water was made in accordance with the methods of Brown (1993) and Swart (2005).

Sucrose treatments

Two sucrose treatments were set up: 5% and 30% and a control (0%).

A gibberellic acid treatment was added to the two sets of embryo cultures (from plant material collected in 2005 and 2006).

5.3.5 Documenting and measuring the *in vitro* response

Observations on the condition of the cultures were made on alternate days. The lack or presence of infection was noted for each culture. If an infection was noted, the infection was described and the culture photographed. Photographs of all the cultures were taken a day, a week, two weeks and three weeks after culture initiation so that the morphology of the cultures could be compared using image analysis using Image-Pro© Express 4.5 image analysis software, Image Processing Solutions, Inc. A scale was included with all photographs so that the change in morphology could be quantified.

5.3.6 Statistics

Wilcoxon matched pairs tests were used to determine significant differences in embryo length using Statistica® version 7, copyright StatSoft, Inc.

5.4 Results

5.4.1 Culture conditions

The cultures of *Brassica oleraceabotrytis* that were prepared as test of the culture methodology were regenerated up to stage II. After this stage, all of the cultures were infected and no tissue was left for rooting.

The contamination appeared over the entire agar surface indicating that the culture conditions were not very sterile. Signs of fungal and bacterial infection were observed. Despite this, the culture conditions were considered to be sufficient to test for a culture medium that induces the initiation of a single stage of micropropagation.

5.4.2 Segment and tip cultures

All 96 of the root tip cultures were infected after 3 weeks. All of the 96 shoot segments were infected after 2 weeks and all of the 96 seedling shoot tips were infected after 1 week.

Because the seedling root tip, shoot segment and shoot tip showed such high rates of infection the experiment was terminated after 96 cultures were done. The shoot tip cultures with least infection showed no signs of rooting.

5.4.3 Embryo culture

5.4.3.1 Germinated and photosynthetic embryos

No treatment showed more than one germinated or photosynthetic embryo. A total of three achenes germinated: one each on control medium, a medium supplemented with 1 µM IAA and a medium supplemented with 5 µM IAA and 0.5 µM Zeatin. The germinated control medium achene was collected in 2005 and the other two in 2006.

The embryo obtained from an achene collected in 2005 germinated first, within one week of culture initiation. Callus formed at the crown of the seedling. The seedling appeared normal and was 2.026 mm in length (Photograph 1). At the time the photo was taken for week 2 the plant had already started to show severe signs of necrosis and was reduced in length to 2.022 mm. After 3 weeks, the plantlet's condition had worsened and it was almost completely necrotic with its length reduced to 1.853 mm.

The embryo that germinated on a medium with 1 µM IAA germinated in the third week of culture. This seedling was abnormal as there were no shoots produced, only two large roots, 4.215 mm and 5.727 mm in length (Photograph 2).

The third embryo (on a medium with 5 µM IAA and 0.5 µM Zeatin) germinated within two weeks. The seedling however appeared abnormal (Photograph 3). At the time at which germination was noted the seedling had already begun to develop a second pair of leaves, measuring 1.216 mm and 1.275 mm in length respectively (Photograph

4). In one week, the growth increased substantially and the leaves had grown to 3.032 mm and 3.281 mm, while root length remained unchanged (Photograph 5).

A number of embryos did not germinate, but turned green. These included five embryos from the 2005 collection and three embryos from the 2006 collection (Table 12). Half of the embryos turned green within one week and the other half acquired photosynthetic capacity within two weeks after culture initiation.

Table 12: Summary of the treatments in which a single embryo acquired turned green.

Treatment	Achene collection date	Number of weeks from culture initiation
0.5 μM 2,4-D; 0.5 μM Kinetin	August to October 2005	1
0.5 μM 2,4-D; 10 μM Kinetin	August to October 2005	2
1 μM 2,4-D; 0.5 μM Kinetin	May to July 2006	1
1 μM 2,4-D; 1 μM Kinetin	August to October 2005	1
1 μM IAA; 1 μM Zeatin	May to July 2006	2
1 μM IAA; 10 μM Zeatin	May to July 2006	2
5 μM IAA; 1 μM Zeatin	August to October 2005	2
10 μM IAA	August to October 2005	1

5.4.3.2 Embryo lengths

Seeds can be classed as dwarf if their embryos are from 0.3 to 2.0 mm in length (Nikolaeva 2004). The achenes of *Syncarpha recurvata* can be classed as dwarf, as their embryos have an average length of 1.137 mm.

Embryo lengths were obtained from the photos taken after culture initiation and 1, 2, and 3 weeks after culture initiation. Data were obtained for a total of 524 embryos. After 1 week, the number of uninfected embryos decreased to 333, after two weeks the number of uninfected cultures was 304 and after 3 weeks, all but 264 cultures were infected.

The embryos showed a gradual decline in length over the 3 week culture period (Figure 9). When the length of the 333 embryos that survived the 1st week of culture

were compared with their initial length it was found that there was a significant decrease in length (T = 21253, Z = 3.569, N = 333, p < 0.001). After 2 weeks the surviving embryos were also significantly shorter than they were before culture (T = 14595, Z = 5.596, N = 304, p < 0.001). The embryos were also significantly shorter than they were a week after culture initiation (T = 16307, Z = 4.325, N = 304, p < 0.001). After the 3rd week the embryo lengths were significantly lower than their initial lengths, their lengths at 1 week and 2 weeks after culture initiation (T = 8017, Z = 7.628, N = 264, p < 0.001; T = 9288, Z = 6.604, N = 264, p < 0.001; and T = 12614, Z = 3.926, N = 264, p < 0.001 respectively).
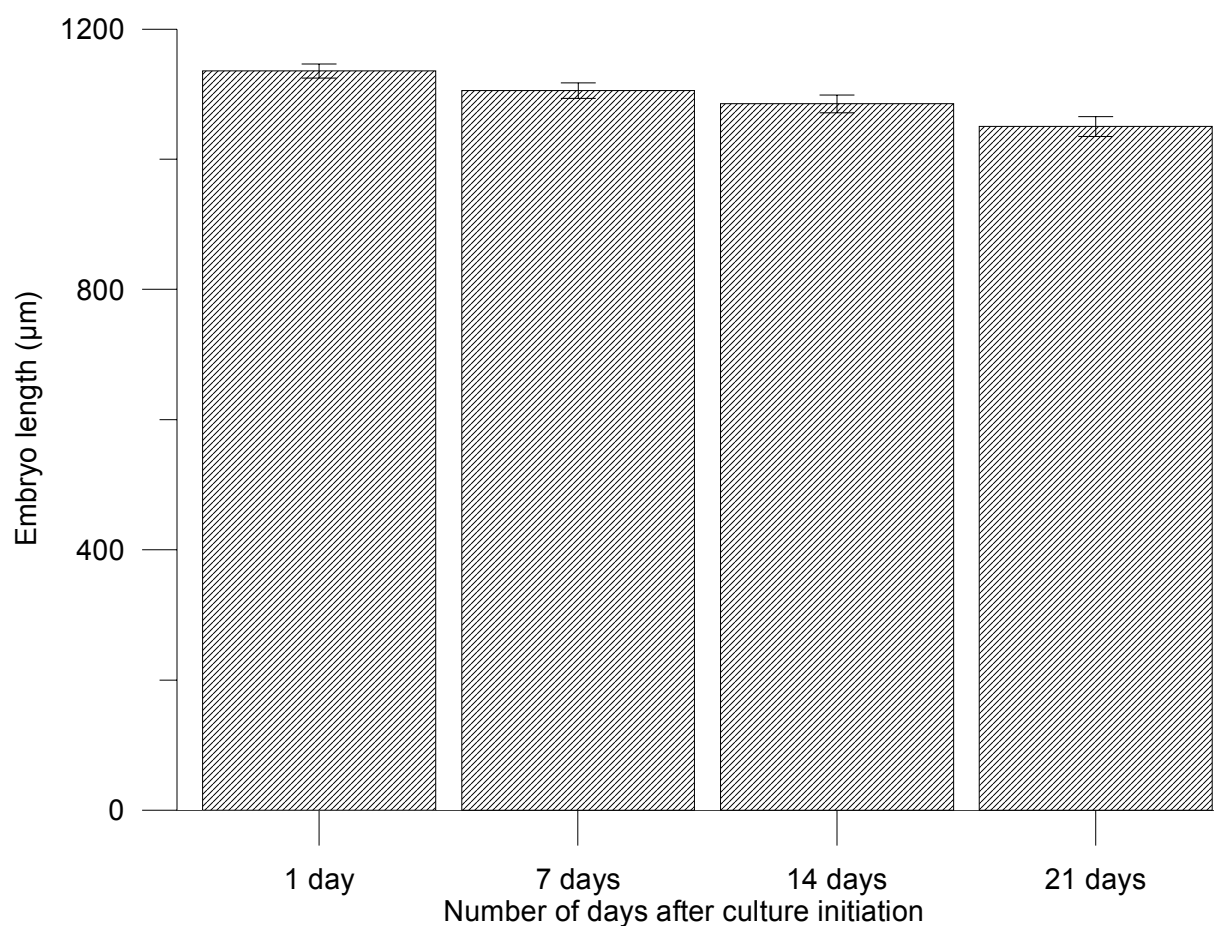

Figure 9: Embryo length (μm) of embryos cultured *in vitro* over 3 weeks

5.4.3.3 Callus formation and growth

There were two instances of callus formation, one from an embryo from the 2006 collection on a medium supplemented with 0.5 μM IAA and 1 μM Zeatin, and the

other from an embryo from the 2005 collection on a medium supplemented with 5 μM IAA. The embryo on the 0.5 μM IAA and 1 μM Zeatin medium increased in area by 11.492%, while the area of the embryo on the 5 μM IAA medium increased by 26.774% (Photograph 9). Callus was also produced on the crown of the embryo germinated on the control medium (Photograph 1).

5.4 Discussion

The absence of any success with the root tip, shoot tip and shoot segment cultures does not indicate that the vascular tissue did not respond *in vitro*, as this was largely a result of contamination. This is consistent with the results of Home and Campbell (1996). They suggested that the leaf hairs might form a barrier to sterilisation. In this study explants were obtained from seedlings germinated in a growth cabinet. The growth cabinet used was, however, not completely aseptic. The achenes and seedlings were exposed to the partially contaminated conditions within the growth cabinet for a prolonged period of time because of the long time for germination required by *Syncarpha recurvata*. The root tip, shoot tip and shoot segments had numerous hairs on them at the time of culture.

The leaves of *Syncarpha recurvata* have large numbers of trichomes, even in stages of high juvenility. Photograph 4 shows that even at a high stage of juvenility leaves are already completely covered in hair. These hairs would increase the chance of contamination because of the increased surface area available for contamination.

The embryo cultures were more successful because of their isolation by the fruit-wall and testa from any potentially contaminating environment before culture initiation. The results obtained for germination of the embryos can only be interpreted descriptively. The three different medium treatments appeared to affect the morphology of the plants. The control treatment resulted in the most normal seedling when compared to an *in vivo* germinated seedling (Photograph 1).

The seedling germinated on the1 µM IAA medium showed rapid and elaborate root growth and the shoot formation seemed to be repressed (Photograph 2). This promotion of root growth is most likely a response to IAA, as IAA is specifically known as an auxin used for root initiation and growth (Gaba 2004, Smith 2000). The absence of cytokinins also promoted rooting as cytokinins inhibit rooting (Gaba 2004). This medium has potential as a root induction medium in stage III of micropropagation of *Syncarpha recurvata*.

The embryo germinated on the medium supplemented with 5 µM IAA and 0.5 µM Zeatin showed stunted root growth and prolific shoot growth (Photograph 5). This morphology was most likely induced by the Zeatin, which is effective at much lower concentrations than IAA. Cytokinins are known to cause the release of shoot apical dominance and effectively blocks root development (Pierik 1997). This medium might have potential as a shoot induction medium in stage II of micropropagation.

The embryos that greened were cultured on a variety of different media, all of which contained plant growth regulators at various concentrations (Table 12). This shows that the embryos were viable to some extent.

The decrease in embryo length over three weeks (Figure 9) is consistent with the general dehydration and necrosis observed, causing tissue to contract and the embryo to shorten. The large number of non-viable embryos is consistent with the low germinability of *Syncarpha recurvata* and points toward an endogenous dormancy of the embryo.

The callus culture observed in embryos formed on a medium with 0.5 µM IAA and 1 µM Zeatin (Photograph 6 and Photograph 7), showed low growth when compared to those formed on a medium with 5 µM IAA (Photograph 8 and Photograph 9). A week after callus initiation, a small amount of greened tissue can be observed in the middle of the callus (Photograph 9).

Callus cultures are usually initiated and maintained by plant growth regulator combinations containing other than IAA, such as 2,4-D (Gaba 2004, Smith 2000). The media also usually contain both an auxin and a cytokinin (Slater, *et al.* 2003). Giovannini and Amoretti (2001) initiated callus on a medium enriched with 10 µM 2,4-D and 1 µM kinetin for *Helichrysum italicum* and *Helichrysum stoechas*. Deka and Kalita (2005) obtained callus from nodal explants of *Spilanthes acmella* (Asteraceae) on a medium supplemented with BAP and IAA, so callus production with IAA has been achieved in the Asteraceae.

According to Pierik (1997) the use of 2,4-D should be avoided, as it may induce mutations. IAA might therefore prove to be more advantageous, as it will increase the chance of normal regeneration from callus.

The contamination observed in this study was high. This is unfortunate, as conditions that are more sterile would have led to more replicates being viable and would have offered more information on the *in vitro* response of *Syncarpha recurvata* embryos to various chemical stimuli. This contamination was probably as result of the dust caused by the excessive foot traffic that surrounded the growth cabinet and laminar growth cabinet throughput the year (Smith 2000). Various types of plant and algal material were also brought into this lab, which could have been a source of contaminating organisms.

The aims of this chapter were met in that embryos were found to be a successful form of explant and callus formation was induced. The effect of other physical and chemical culture conditions on the *in vitro* growth of *Syncarpha recurvata* should also be investigated in future studies. A larger variety of combinations of plant growth regulators at different concentrations that were not used in this study need to be tried. Other culture conditions such as light and temperature, which are also important for callus formation and development, were also not investigated. It would have been desirable to perform the callus cultures in the dark as light can result in differentiation (Slater, *et al.* 2003). The culture medium used for callus induction should be refined

and a large amount of callus used to test for the appropriate conditions for stage III of micropropagation. Once this is done, the callus derived from the various culture treatments must be tested for their degree of totipotency.

Syncarpha recurvata responds to some chemical stimuli *in vitro* in a way similar to other Asteraceous species. Embryos germinated and became photosynthetic on a medium supplemented with the same concentration of Murashige and Skoog and sucrose that is used for basic cultures of *Gerbera jamesonii*, *Chrysanthemum* sp., *Helichrysum italicum* and *Helichrysum stoechas*. An embryo showed excessive root growth and potential suppression of apical dominance on a medium supplemented in IAA, an auxins used for root growth in all the species mentioned above. This information will be useful in future studies that involve the propagation of *Syncarpha recurvata* for conservation purposes.

Chapter 6: Implications for restoration, conservation and propagation

6.1 Introduction

Syncarpha recurvata has a restricted geographical distribution and its survival is threatened by the mining of calcrete and urban expansion. It is necessary to consider the application of this study to rehabilitation after mining so that survival after re-vegetation will be optimised. It is also necessary to consider the implication of this study for the conservation and horticultural propagation of *Syncarpha recurvata*.

6.2 Restoration after mining

6.2.1 Introduction

Mining processes at Grassridge involve the removal of 10 cm of topsoil to a stockpile, the removal of 1 cm of subsoil to a stockpile and the excavation of the calcrete layer (Watson 2002). Endangered species are removed before mining of a target area starts, with 1 to 2 ha being mined at a time (Watson 2002).

The *Syncarpha recurvata* population will have to be restored by translocation of the individuals into nurseries for later repopulation as their habitat will be completely destroyed (Given 1994).

The object of restoration is to create a self-sustainable entity (Guerrant 1996). This is sometimes a long process (Guerrant 1996). With restoration there is no clear predictability, there is excessive complexity, a lack of optimization and no clear endpoint (Pavlik 1996).

Translocation involves the removal of an organism from one site followed by resituating it somewhere else (Given 1994). Translocation strategies need to be carefully planned and involve both *ex situ* and *in situ* techniques (Given 1994). *Ex situ* techniques involve the selection and establishment of correct propagation methods

and treatments, the establishment of a plantlet bank, resistance, survival and transplant tests, acclimatisation and multiplication (Given 1994). *In situ* techniques involve the selection of donor and restoration sites, sampling of plant material from the donor site and the management and execution of repopulation (Given 1994).

According to Urbanska and Chambers (2002) restoration should occur by first assessing the damage to the site and its recovery potential and then applying restoration techniques that include site manipulation, obtaining plant material and post-restoration monitoring.

Pavlik (1996) shows that restoration success should be defined by various goals and objectives. He also adds that success is however never definite as reintroduced and natural populations are subject to continuous, complex and interacting processes. These processes are, in turn, affected by many variables of which only a few can be defined and fewer manipulated (Pavlik 1996).

A reintroduced population should ideally be as capable as its natural equivalent of integrating into ecosystem function of a changing environment (Pavlik 1996). Such integration occurs through evolution and migration (Pavlik 1996). The capability of the population to express its full range of alleles will be advantageous in a changing environment.

A hardening-off period aids transition to the field (Davy 2002). This period involves placing the plants in the open and watering the plants at a frequency similar to field conditions (Davy 2002).

A plant species may only be well established at a certain successional stage and with certain environmental conditions (Primack 1996). It is therefore necessary to implement pre-translocation monitoring of successional development (Sutter 1996).

The aim of this section on rehabilitation after mining is to look at how a successful protocol is designed and to propose a protocol for the translocation of *Syncarpha recurvata* based on the results obtained for propagation in this study and previous studies and guidelines from the literature on how to construct a rehabilitation protocol (Given 1994).

6.2.2 Translocation methods

Planting techniques are variable for different species and cases (Given 1994). Although there are, in theory, a wide variety of horticultural options when considering a reintroduction program there are, in practice, only a few options that might be realistic for a particular case (Guerrant 1996). Every option also has its own embedded options (Guerrant 1996).

The time of execution is also crucial as certain times of the year are more suitable for planting than others (Primack 1996).

Translocation protocols can be divided primarily into those using seeds as a propagation unit and those that use some form of vegetative propagation (Given 1994).

Seeds have been used as founders in many projects for plants with various life histories (Guerrant 1996). Collecting seeds from donor sites has less ecological impact than removing whole individuals (Guerrant 1996).

Using seeds that are propagated off-site for one or more generations can however lead to losses in genetic variability of the population and because of this in some cases transplantation might be a better option (Guerrant 1996).

Transplantation involves the use of any propagules, other than seed, as founders at the restoration site (Guerrant 1996).

Transplantation has many advantages over using seeds (Davy 2002). By establishing a method for cloning, for example, large numbers of plants can be produced relatively rapidly (Davy 2002). Cuttings are also ideal for sampling genetic traits such as flower colour variants (Given 1994). Taking cuttings may however reduce the health of donor populations (Guerrant 1996). The use of cuttings for conservation also places limits on size of the samples (Given 1994).

Plants can also be obtained by raising seedlings obtained from wild collected seed under laboratory or horticultural conditions (Davy 2002). Seeds used for this purpose may be freshly collected or stored in an *ex situ* seed bank (Guerrant 1996). These plants should be raised to a sufficiently large size before transplantation to the field (Davy 2002). This allows the plants to have a sufficiently large above and below ground nutrient reserves at the time of translocation (Davy 2002).

Such matured plants are also closer to reproductive maturity, potentially aiding the acquisition of self-sustainability (Davy 2002). Such plants should ideally be planted in a rooting medium with a texture similar to the substrate of the restoration site (Davy 2002).

In some cases transplantation includes the surrounding soil in which the plants were growing (Guerrant 1996). Such large sections of soil vegetation are called turfs (Davy 2002). With use of suitable machinery turfs of intact vegetation can be removed cutting out the rooting layer of the soil (Davy 2002). These turfs are then transported to the restoration site. This potentially circumvents the majority of the problems associated with establishing the right surface soil conditions (Davy 2002). This method of translocation is especially useful where pedogenesis depends on hundreds or thousands of years of leaching. Using turfs also allow the incorporation of natural seed banks, lichens, bryophytes and microbial and invertebrate soil communities (Davy 2002). The turfs can be placed in their original configuration or spaced (Davy 2002). The latter allows a greater area to be reconstructed by using the turfs as nuclei for colonisation.

Tissue culture can be considered where the number of plants available is small and there is a need to build up numbers rapidly (Given 1994). Plant material can be stored as callus and multiplied and regenerated at a predetermined time, advantageous to translocation success (Given 1994).

Where rapid multiplication is possible multiple replicates of genetically identical populations can be placed into several environments simultaneously to act as "phytometers" at possible reintroduction sites (Guerrant 1996). With micropropagation a large number of plants can be generated from limited material (Pierik 1997). These 'phytometers' tests whether the environment created will be suitable for rehabilitation without risking damage to the population (Guerrant 1996). If these plants fail to establish, other combinations of substrate need to be considered or the hardening procedure needs to be modified (Guerrant 1996).

Some disadvantages of using micropropagation in a translocation protocol is that genetic damage may be caused by somaclonal variation and that such a population will have a narrow gene base, as all the plants are derived mitotically from the same individual (Guerrant 1996).

Where the number of plants available for translocation is small both sexual and vegetative propagation should be used (Given 1994). To preserve endangered species, indicator species associated with rare and endangered species can be used to identify potential safe sites for reintroduction (Primack 1996).

Translocation methods may need to incorporate site manipulations. Site manipulations include landscaping and altering of soil composition (Urbanska and Chambers 2002).

The proportions of species used and the pattern of planting should mimic their natural spatial distribution (Davy 2002). This applies to the micro-environments and spatial

heterogeneity of the population (Davy 2002). It is however often necessary to reduce planting density to minimise competition (Davy 2002). As mimicking the natural spatial distribution and randomising is very expensive, these planting methods should only be used for long-lived perennial species that have a distinctive role in the community (Davy 2002).

The relative biological and financial cost and practicality of each choice should be considered and post-translocation monitoring should be practised (Guerrant 1996).

6.2.3 Sampling the population

The choice of size and sampling strategy is critical to the immediate and long-term success of the founding population (Pavlik 1996).

The rate at which populations lose genetic variability due to random genetic drift is closely correlated with population size (Guerrant 1996). Smaller populations loose genetic diversity at a faster rate than larger populations (Guerrant 1996).

The rate of genetic loss over time should be considered when deciding on the size of the founding population (Guerrant 1996). As much genetic diversity as possible should be captured from the study site to conserve biodiversity (Given 1994). The ceiling should be set by practical limits and conservation of the donor site (Guerrant 1996).

The collection of the most vigorous or attractive plants lead to only a select portion of the total genetic variation of the species being preserved (Given 1994). To avoid this random or representative sampling should be used (Given 1994).

Continuous populations may be composed of a variety of differentially adapted genetic "neighbourhoods", consisting of plants with distinct genetic characteristics (Guerrant 1996). These may be reflected by clustering patterns (Given 1994). Clusters can reflect factors such as patterns of original evolution and migration and

habitat variation (Given 1994). Different clusters may need to be regarded as distinct variants or sampling units (Given 1994). This would also allow variants such as cold-tolerant and altitude tolerant forms to be adequately sampled (Given 1994). Such clusters that appear to be separate may also be similar and may be integrated by gene flow (Guerrant 1996).

Genetic variation increases the chance of species survival through changed circumstances in off-site and on-site conditions (Given 1994).

Genetic variation is expressed by allelic richness, gene diversity or heterozygosity and as genet richness (Given 1994). Allelic richness refers to the diverse array of different alleles needed to meet future environmental requirements, heterozygosity refers to the prevention of inbreeding depression and genet richness refers to the range of distinct genotypes that occur in a particular population (Given 1994).

At least one copy of common alleles should be recovered from each sample unit to prevent a drastic loss in allelic richness (Given 1994). This sample should have a survival probability of 0.90 to 0.95 and a random sample of 50 to 100 individuals is required (Given 1994). Some low frequency alleles governing physiological processes may become important for the survival of the species within the next few decades due to climate change and it is therefore necessary to ensure that the maximum number of alleles is sampled (Given 1994).

An appropriate minimum target for each sample unit is 10 individuals. Such a low sample size can be used if it means that resources can be used to sample other populations (Given 1994).

Genet richness does not take into account the nature and extent of the genetic differences between genotypes or the intensity for testing for genetic differences (Given 1994). This is a useful consideration where there is a predominance of vegetative propagation (Given 1994). When out-breeding non-clonal species the

relationship between genet richness and sample size approaches linearity because each individual is unique (Given 1994).

It is important that the size of the sample should ensure that at least one of those genets is genetically equipped to survive off-site is collected (Given 1994). At least 15 individuals per species should generally be collected (Given 1994). It should be a goal to provide at least 95% probability that all alleles with at least a 5% representation in the population are taken (Given 1994).

Skewed genetic representation may result from selection pressures during cultivation (Guerrant 1996). When seeds are germinated away from the wild a new and sometimes unsuitable set of selection pressures operate (Given 1994). Although this problem can not be avoided it should be recognised (Given 1994, Guerrant 1996).

Selection pressures occur on the genetic information during the sampling, during propagation, as a result of disease or predation and losses of off-site collections and during re-establishment of the plants back to the wild (Given 1994, Guerrant 1996).

It is however sometimes possible to achieve high levels of genetic diversity through off-site breeding programs with a few individuals (Given 1994).

6.2.4 Post-restoration monitoring and maintenance

Monitoring is used to obtain useful information for management decisions (Sutter 1996). The main aim of post-restoration monitoring is to establish if a population is able to carry on its basic life-history processes (Pavlik 1996). These include establishment, reproduction and dispersal (Pavlik 1996). Such initial population stages can be used to evaluate the potential success of reintroduction (Primack 1996).

Reintroductions are judged as successful when a new, expanding population is established (Primack 1996). The number of individuals in the population should be

continually and carefully monitored to check whether numbers are stable, increasing or decreasing (Sutter 1996). Such monitoring takes places over years, decades or even centuries (Primack 1996). The number of new individuals should be noted and compared with the number of dead individuals (Sutter 1996). If there is net population growth it indicates that the population is self-perpetuating through the development of a second generation (Primack 1996). Such self-perpetuation contributes to the viability of the population (Primack 1996).

The site should be monitored for seedlings if seeds were used as the translocation method and seedling persistence should be monitored (Primack 1996). The survival of the transplanted individuals should be monitored over weeks, months and years (Primack 1996).

The reproduction of the plants should also be monitored (Primack 1996, Sutter 1996). The time it takes to reach flowering and the effectiveness of seed production and dispersal should be noted and compared to natural populations (Primack 1996, Sutter 1996).

Successful reintroduction often requires maintenance to keep transplanted individuals alive (Primack 1996). Some sites may need to be altered to suit the requirements of the reintroduced species (Primack 1996).

Plants may need to be watered until the root systems are established, straw or matting may need to be placed around the plants to retain soil moisture and adjacent plant material may have to be removed to decrease potential competition (Primack 1996).

6.2.5 Further studies needed before successful rehabilitation

Numerous further studies need to be conducted on factors concerning rooting, the availability of viable achenes, micropropagation, and population characteristics and dynamics for the success of *Syncarpha recurvata* in rehabilitation studies. The protocol proposed in the following section assumes that these studies will be completed before such a protocol is considered.

6.2.5.1 Rooting

More studies are needed on the requirements of rooting. The fact that all the rooted cuttings were on one side of the propagation tunnel, the side which received the most amount shade during the day, suggests that the percentage rooting can be enhanced by increasing the amount of shading. The amount of shading has a large influence on the rooting of cuttings (Edmond*, et al.* 1964). Further studies on factors such as these should be done.

6.2.5.2 The availability of viable achenes

Home and Campbell (1996) calculated that 800 plants can be produced from each plant found at the Grassridge population by measuring the average number of inflorescences (70), the number of achenes per inflorescence and the average number of achenes per inflorescence (61.7) and combining these measurements with a percentage obtained for germination success (20%). Recalculating this with the percentage germination of the treatment with the highest success (22.4%) in this study (achenes collected by Weatherall-Thomas 2005 (2005) treated with a 1:500 smoke water dilution), approximately 900 individuals could be produced from each plant in the Grassridge population. When we look at this calculation practically for horticultural purposes, it is important to note that the number of fruiting inflorescences and number of dark achenes, which was selected in this study and others (Home and Campbell 1996, Weatherall-Thomas 2005) for propagation, is not incorporated. This can lead to serious miscalculations and overestimation of the potential of seeds for horticultural purposes.

In this study, it was concluded that achenes should be collected in early summer and germinated about 7 months after collection. The exact pattern of achene production and viability is however not known, due to inaccurate documentation of collection dates and the fact that only two time intervals were sampled.

A study is needed to quantify the average number of fruiting inflorescences per plant and the average number of black achenes per inflorescence for at least every 6 months over at least 2 consecutive years. This will allow a more accurate estimation to be made so that consideration can be made regarding the availability of seeds for collection and germination.

To preserve genetic variability it important to create a gene bank, so that the population can be recreated in the case of rehabilitation failure. Achenes are a good source of genes for such a bank. A study is needed to establish achene longevity as this is essential to the functioning of a gene bank in which achenes are used.

The percentage germination of *Syncarpha recurvata* is however low and the use of other plant material in such a gene bank should be investigated.

6.2.5.3 Micropropagation

A full micropropagation protocol must be established to regenerate plants from embryo-derived callus if micropropagation is to be used for experimental and restoration purposes.

Protocols need to be established for stage II, III and IV of micropropagation for successful regeneration (Pierik 1997). The protocol for stage I of micropropagation suggested in this study is limited to the use of embryos as explants. The suitability of other explants should be investigated. An attempt must, for example, be made to obtain callus from shoot cuttings. This could potentially be done by placing the cuttings in agar enriched with 0.5 μm of IAA. By obtaining callus from cuttings, it will be possible to accurately define the phenotype of the plant material before

multiplication. This will potentially enable the effective multiplication of numerous plants with unique characteristics.

<u>6.2.5.4 Population and ecosystem characteristics and dynamics</u>

Possible variants of *Syncarpha recurvata* with regards to flower colour and spatial distribution should be investigated. These variants must be treated as separate sample units in conservation and rehabilitation. A study is therefore needed to quantify the differences between these variants in terms of growth habit, physical appearance and locality and to place these variants in easily identifiable sample units for collection at the time of rehabilitation. Such a study should superimpose the genetic diversity of the *Syncarpha recurvata* at Grassridge over its spatial distribution so that these potential variants or ecotypes can be sampled effectively.

A detailed study on the growth and reproduction of the Grassridge population of *Syncarpha recurvata* is needed before these plants are removed from the study site. The degree to which this data correlates with the data received from post-rehabilitation monitoring will be an informative indicator of rehabilitation success.

The degree of interaction of *Syncarpha recurvata* with other species in Bontveld also needs to be investigated. Studies similar to this one should be conducted for other important species in Bontveld so that they can be planted together with *S. recurvata* in a way that mimics the original Grassridge population.

6.2.6 A protocol for the rehabilitation of a proposed area at Grassridge to be mined by PPC

<u>6.2.6.1 Pre-mining</u>

Before plants are taken out of the habitat and the mining of a target area proceeds a test population must be created and placed into conditions that simulate that of the post-mining environment to be rehabilitated (Guerrant 1996). Such a test population

can make use of *Syncarpha recurvata* clones that have been multiplied efficiently either by air-layering or micropropagation.

The latter propagation method offers the same problem as direct propagation by achenes, as certain traits will not be able to be selected in the field. Air-layering and cuttage however allows plants with certain unique or desired traits to be cloned.

Micropropagation can however still be used to exploit unique traits. The embryo-derived callus can be multiplied, after which a small section can be regenerated to test the variant. This will however to a time consuming process. More efficient propagation desired traits may be achieved by using callus derived from the base of cuttings. Although this was not attempted in this study, the assumption is that it will be possible to obtain such callus, as callus formation is an essential step *de novo* in root formation.

This will enable the assessment of potential rehabilitation with minimum damage to the genetic diversity of the population. Such a test population should consist of at least 100 clones made from a few (~20) individuals from each sample unit. These clones should be raised similarly to transplants used for rehabilitation to a size suitable for transplantation (93 mm ± 3 S.E) after which they should be hardened for field conditions as explained later in detail for transplants used for restoration purposes.

The survival of these test populations in the post-mining habitat should be monitored until second generation plants are produced. If successful translocation and reproduction is observed the process of removing the plants from the population can begin.

Syncarpha recurvata shows a 98% transplantation success for plants with a height of 93 mm ± 3 S.E. The process of vegetation removal should start at least a year before mining so that individuals of this size can be gradually removed from the site to be

mined and so that other individuals that might be too small for removal can grow into this size class. As many individuals in the 93 mm ± 3 S.E. size class and as many mature flower heads as possible should be collected from the site at least every two months. Stocks should be clearly labelled so that age of the transplants and achenes as well as the sample unit and variant is known (Guerrant 1996). Flower heads should be collected in dry weather.

The removed individuals must be sprayed with a light mist before placing them in cooler boxes with ice bricks. The plants must then be transported to a nursery with suitable facilities (Given 1994). Such facilities must include areas covered with various grades of shade cloth and propagation tunnels such as the one described in this study and should also preferably have tissue culture facilities.

In this nursery, the plants should be planted in equally sized pots with a 1:2 mixture of freshly hydrated peat and calcareous soil collected from the study site and placed under a structure covered with shade cloth. The plants should be allowed to acclimate and root into the new substrate for at least 2 months.

Achenes should be sorted from the flower heads in the laboratory. The dark achenes must then be sorted out from the unviable achenes and stored in a cold and dry place. Not more that 50% of the collection should for rehabilitation purposes. Protocols should be established for periodical germination of so that the achene collection can function as a gene bank.

The achenes should be imbibed in a 1:100 smoke water dilution before germination on a 2:1 mixture of calcareous soil collected from the study site and peat that has been hydrated with 1:100 smoke water prepared fresh with boiling water. The soil should still be moist and warm when placing it in seed trays and planting the achenes on it. Achenes should only be covered with a sprinkle of powdered dried calcareous, sediment after which they should be sprayed with a light mist with a spray bottle filled

with a 1:100 smoke water solution. Seedlings should not be used as a primary method of rehabilitation as the average percentage germination is quite low (22.4%).

It would be most advantageous to remove the remaining plants by turfs. This can however be an expensive process and the plants might not be dense enough so that there will not be sufficient roots to hold the turf together. If this is not possible, the remaining plants should be removed before mining and treated like other transplants. Although percentage transplantation success will be far less than the plants with a height of 93 mm ± 3 S.E , this is better than just leaving them at the study site to be destroyed. Another option is to use these plants for cuttage, which might allow a greater amount of plant material to be recovered.

6.2.6.2 During mining process

The activities done while mining is in progress should include allowing the plant material to acclimate, replicating the plant material and hardening the replicated plant material to field conditions.

During the mining process, multiplication of the transplanted population should occur in the nursery. Such multiplication should occur through asexual and sexual propagation (Given 1994).

Asexual propagation can be done by rooting branches of *Syncarpha recurvata* through air layerage and cuttage and by micropropagation by regenerating plants from embryo derived callus.

In this study, the percentage rooting success of air-layered branches (32%) was lower than the percentage rooting success for cuttings (50%).

The use of air layered branches is less destructive. A branch that failed to root, which is still attached to the plant can be used as part of the original plant as a transplant or rooting can be attempted again at a later stage. Using cuttings will lead to a loss of

half of the plant material available and is therefore less suitable for the propagation of an endangered plant.

Air-layering should be done as described in this study and air-layered plants should be placed in propagation tunnels constructed in accordance with the tunnels used in this study. Plantlets should be removed from the mother plants after one month, planted in pots with a 2:1 mixture of calcareous soil and peat and placed under a shade cloth. The pots used should be same size as those in which the mother plants were planted. Achenes should also be germinated as described earlier to add genetic heterogeneity to the population.

After the newly cloned and germinated plants have grown to a size of 93 mm ± 3 S.E they should hardened to field conditions. This can be done by putting the plants under the cover of more transparent shade cloths over time, until the plant is able to grow in total sunlight. After this the plants should be given less water, until they are able to grow with only the water acquired from the environment (Davy 2002).

Plants that grow to a height significantly greater than 93 mm ± 3 S.E should be used for multiplication, after which their clones can be hardened when reaching this size class.

6.2.6.3 Post-mining

Transplants obtained from rooted branches, micropropagation and raised seedlings that have been acclimatised to field conditions can be planted at the rehabilitation site.

Transplanting should occur in late winter to early spring to aid transplantation success. Lower temperature during transplantation will prevent desiccation and the heat of spring will provide a more suitable environment for root growth.

In this study, it was observed that the soil in which plants had grown for 5 months was bound together quite strongly. The soil is bound not only by the calcareous nature of the soil, but also by the roots of the plants, as after 5 months the roots of the plants

have filled the pots. Roots probably penetrated hard soil as a result of the addition of peat. Plants must only be watered after transplantation. Plants must also not be watered the previous day. This will make transplantation of the plants easier.

Not more than 75% of the total number of cultivated plants should be planted in the first year of rehabilitation. This will reduce the risk of great losses as a result of a disaster. The plants that remain in the nursery after rehabilitation should include at least 100 individuals of each variant or sample unit. These plants should be used as mother plants and placed in an area separate from the clones. To minimise the contamination of these mother plants, they should ideally be maintained under dust, insect and disease free conditions (Ahloowalia, *et al.* 2002, Kyte and Kleyn 1996). These plants should also be grown under controlled conditions that allows active growth while maintaining a low relative humidity (Kane 2004). Drip irrigation should preferably be used as misting will facilitate contamination by wetting the foliage (Kane 2004).

The rehabilitation site should be mapped according to environments suitable for the variants identified to potentially be adapted to specific environmental parameters. The distribution and abundance of other variants in the population should mimic the original population at Grassridge (Davy 2002). The density of the individuals in the rehabilitation site should also be similar to the original population at Grassridge.

Once all these variables have been accounted for and a map of the new population has been constructed, holes should be made at the restoration site to fit the pots. Plants can then be transported to the rehabilitation site. Here plants can be taken out of the pots and planted as plugs in the holes at the study site.

The bases of these transplanted plants should be covered with some powdered calcareous sediment after which they should be watered thoroughly. The latter step can perhaps be avoided by selecting a series of rainy days for transplantation. If turfs were used, they should be placed in a microenvironment similar to that in which it was

found and also watered well. Watering once a week for a period after translocation may be necessary to allow the plants to establish their root systems (Guerrant 1996). When the plants become mature and flower, it might also be advantageous to water the soil with a 1:100 smoke water solution. This will result in greater percentage germination. This could however act as a selection process and it is perhaps important to allow phenotypes such as the two achenes that germinated under control conditions to be expressed *in situ*.

Plants that do not survive the translocation process should be removed from the rehabilitation site and be replaced by other plants from the nursery. Adjacent plant material that might compete with the growth of the transplants should also be removed (Primack 1996).

The reintroduction of a rare species is a complex and protracted process (Sutter 1996). The population should be monitored for weeks, months and years after translocation. A detailed program for pre-translocation monitoring should be constructed (Sutter 1996). The number of individuals within the population should be checked on a regular basis to establish whether new individuals are established in the population. The reproduction timing, achene production and viability and dispersing ability should be compared with that of the original Grassridge population (Primack 1996). Transects should be done and the study of Watson (2002) should be used to assess the success of rehabilitation at a community level.

Once the population achieves levels of establishment, reproduction and dispersal that is higher or equal to that of the population at Grassridge monitoring can occur at lower frequencies (~once a year).

6.3 Conservation of *Syncarpha recurvata*

6.3.1 Introduction

Syncarpha recurvata (L.f.) B. Nord. is listed in the red data book as a vulnerable (intermediate priority) species nationally and in the Eastern Cape (Hilton-Taylor 1996).

The conservation status was not evaluated at the time of this study (Campbell pers. comm.). *Syncarpha recurvata* seem to fit both in the endangered and vulnerable category because of various factors (IUCN Species Survival Commission 2000)

There has been a reduction in the size of *Syncarpha recurvata* populations. There has not been a reduction in population size as greater than 70% over the last 10 years or three generations as required by the criteria for endangered. A population size reduction of more than 50%, as is required by the criteria to be classed as vulnerable, is more probable.

A population size reduction of more than 50%, as is required by the criteria for endangered, could be inferred. A reduction of population size of more than 30%, as is required for it to be classed as endangered, can also is inferred. A population size reduction of more than 50% or more than 30% for the next ten years or over the next three generations as a result of mining is suspected.

Syncarpha recurvata has a restricted geographical range (500 km^2). The species exist in only four populations (Home and Campbell 1996). Populations are also in a continuing state of decline (Hilton-Taylor 1996).. The area of occupancy is also likely to be less than 500 km^2, as this estimation was made in 1995, almost 12 years ago by Campbell (1995).

No accurate estimations on the number of *Syncarpha recurvata* individuals are available and no quantitive analysis has been done to calculate the probability of extinction.

According the sets of criteria and conditions given by the IUCN Species Survival Commission (2000), *S. recurvata* can be classed as endangered or vulnerable.

A species is classed as vulnerable when the best available evidence indicates that it's status meets any set of criteria, which implies that the species is facing extinction risk in the wild (IUCN Species Survival Commission 2000). Species are classed as endangered when the best available evidence indicates that it meets any set of criteria and these species are considered to have a high risk of extinction (IUCN Species Survival Commission 2000).

Syncarpha recurvata can therefore be classed as either vulnerable or endangered according to the available evidence. If *S. recurvata* is placed in the endangered category, as is usually done in such cases, its red list category can be abbreviated according to the IUCN Species Survival Commission (2000) as:

EN A2cd+3cd; B1ab(iv)+2a

EN shows that the species is classed as endangered and the rest of the code states the reason why the species is classed as endangered. It is classed as endangered as a result of a reduction in population size (A) and a restricted geographical range (B).

A reduction of population size was concluded after a more than 50% reduction of the population size over the last 10 years (2) was observed. This conclusion is based on the observed decline in area of occupancy and occurence (c) and a rise in levels of exploitation (d). A reduction in population size of more that 50% can also be expected for the next 10 years or three generations (3). This conclusion is also based on the observed decline in area of occupancy and occurence (c) and a rise in levels of exploitation (d).

A restricted geographical range was concluded because of a geographical range of less than 5000 km^2 (1) and an area of occupancy of less than 500 km^2 (2).

The species fitted into (1) because its populations are severely fragmented and they exist at less than five locations (a) and a continuing decline has been observed and invered (b) with respect to its extent of occurence (i) and number of mature individuals (v).

The species fitted into (2) because the populations are severely fragmented and exist in less than 5 locations (a).

Conservation can be aided by placing plant material in gene banks (Given 1994). Such gene banks include live plants, seed collections and preserved germplasm. Such gene banks should be replicated in as many localities as possible (Given 1994).

Gene banks have three essential functions (Given 1994). The first is to preserve rare or threatened material (Given 1994). The aim of this is to avoid possible extinctions of vulnerable or endangered species. The second is to make plant material available for research and applied purposes (Given 1994). This allows plant material to be obtained without damage to natural populations. The third is to build up banks of living seeds or other tissue. These banks should represent a large portion of the genetic diversity of the species in the wild (Given 1994).

6.3.2 Further studies needed for successful conservation

Achene longevity studies need to be done in order to determine the lifetime of seeds under various storage conditions. This knowledge will enable seeds to be germinated before they loose their viability.

A full micropropagation protocol is needed, describing the materials and methods for stage II, III and IV of micropropagation. This will enable plants to be regenerated from

callus after cryopreservation. A cryopreservation protocol should then be developed so that tissue can be stored for extended periods.

Climate change is already impacting plants and altering the structure of plant communities (Pounds and Puschendorf 2004). Species that are particularly vulnerable to climate change include those with limited ranges and dispersal abilities (Pitelka 1997). A study should also be done to predict the effects of climate change on the extant populations of *Syncarpha recurvata*.

The following protocol proposal is based on the assumption that these studies will be completed before it is considered.

6.3.3 Proposed conservation protocol

Plants of each possible variant should be replicated by air-layering or micropropagation. These should be planted in the NMMU botanical garden, botanical gardens of other institutions and some privately owned gardens. They should be hardened as in the rehabilitation protocol, before transporting them to these locations.

Viable achenes should be obtained and stored under suitable conditions. These achenes should be germinated when their age reaches their estimated life time. Seeds should be collected from the offspring of these achenes and placed under suitable storage conditions.

Because of the low average percentage germination and unknown longevity of the achenes, it is necessary to explore other ways of preserving and managing tissue of *Syncarpha recurvata*. Successful micropropagation of *S. recurvata* will ensure efficient propagation, through stem proliferation and artificial seed production, and make cryopreservation of *S. recurvata* tissue possible. Seeds and germplasm of each variant should also be sent to other institutions.

6.4 Horticultural propagation of *Syncarpha recurvata*

Syncarpha recurvata plants and flowers are sometimes exploited as everlastings by the public. The plants often die because they are planted in normal garden soil and potential genetic variability that undeveloped achenes within the flower would have added to the population is lost. Horticultural propagation of *S. recurvata* and marketing of its flowers as dried flowers will enable these members of the public to buy cultivated plants, seeds and flowers. The preparation and marketing of these three products is discussed.

6.4.1 Cultivated plants

Cultivated plants can be obtained by rooted air-layered or cut branches, regenerating tissue through micropropagation and raising seedlings. These plants should be sold with detailed information on their cultivation and calcareous sediment and peat should be available for purchase.

6.4.2 Seeds

A percentage germination of 22.4% was obtained in this study with a 1:100 smoke water treatment. The public should be informed of this low percentage germination and a smoke primer should be sold with the achenes. Instructions should be included to instruct the buyer on how to apply the smoke primer and how the achenes should be planted.

6.4.3 Flowers

The flowers of *Syncarpha recurvata* are exceptionally attractive. They are placed as ornamental dried flowers in vases by numerous members of the public. In the Eastern Cape, they have been used in bathrooms, entrance halls, dining rooms, bed rooms and kitchens.

Plants should be cultivated exclusively for floricultural purposes. These plants may be clones of plants that have a unique flower colour or form that is aesthetically pleasing. Studies should be done to elongate the flower stalk so that the flowers can be arranged with other plants such as dried grasses.

6.5 Conclusion

Much work needs to be done before rehabilitation, conservation and propagation studies can be concluded. The completion of these studies will enable the successful conservation of *Syncarpha recurvata* populations.

Acknowledgements

Appendix A: Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	N6(2-isopentenyl)-adenine
ABA	Abscisic acid
BA/BAP	6-Benzly-aminopurine
$CaCl_2$	Calcium Chloride
GA	Gibberellic acid
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butryric acid
Kinetin	6-Furfurylaminopurine
KOH	Potassium hydroxide
MS	Murashige and Skoog
NAA	Naphthaleneacetic acid
NaOH	Sodium hydroxide
Thidiazuron	N-phenyl-N-1,2,3,-thidiazol-5-ylurea
Zeatin	Trans-6-(4-hydroxyl-3-methylbut-2enyl) aminopurine
μM	Micromole
μm	Micrometer

Appendix B: Photographs

Photograph 1: Embryo germinated on the control medium

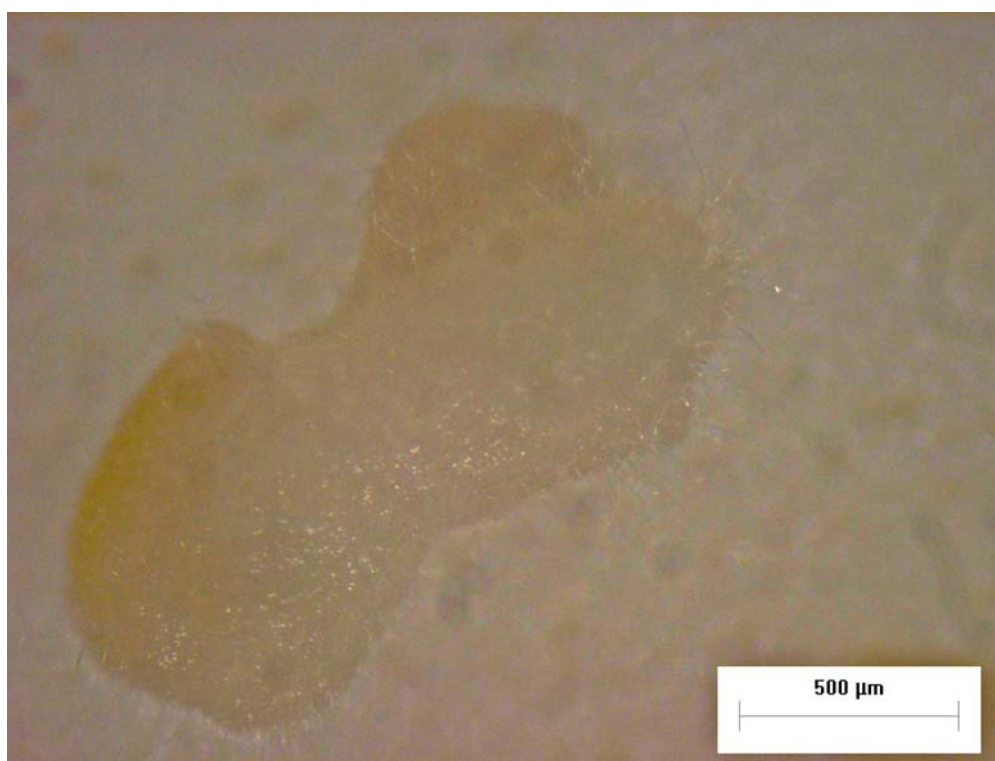**Photograph 2: Embryo germinated on a medium supplemented with 1 IAA**

Photograph 3: Embryo germinated on a medium with 5 IAA and 0.5 Zeatin
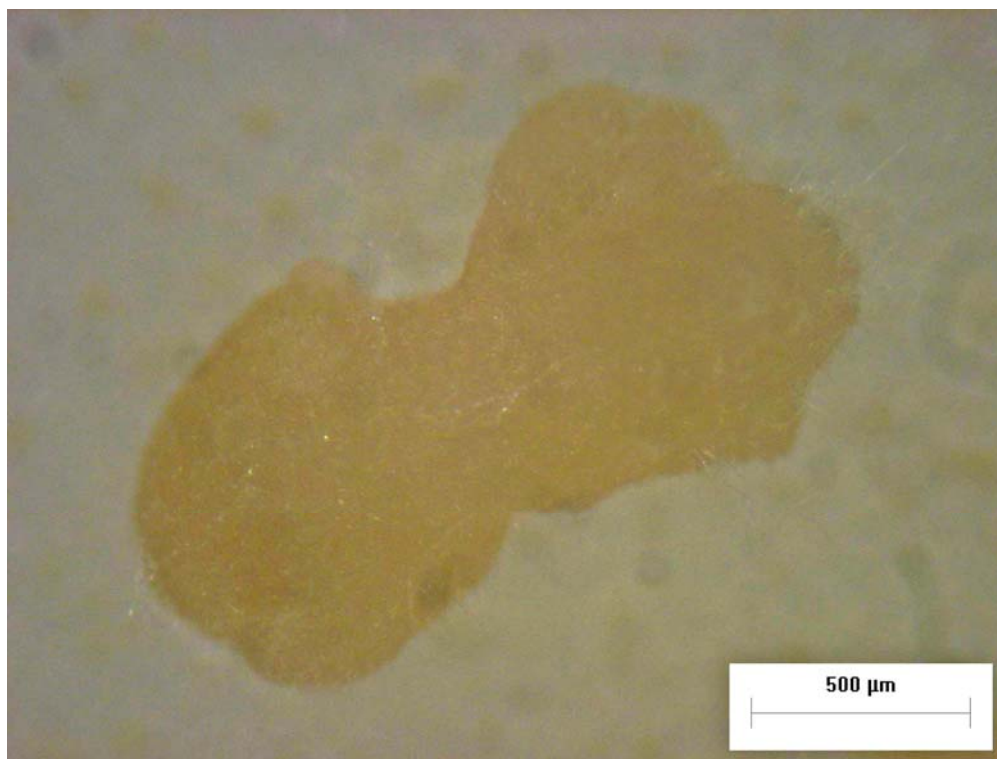

Photograph 4: Embryo germinated on a medium with 5 IAA and 0.5 Zeatin at higher magnification
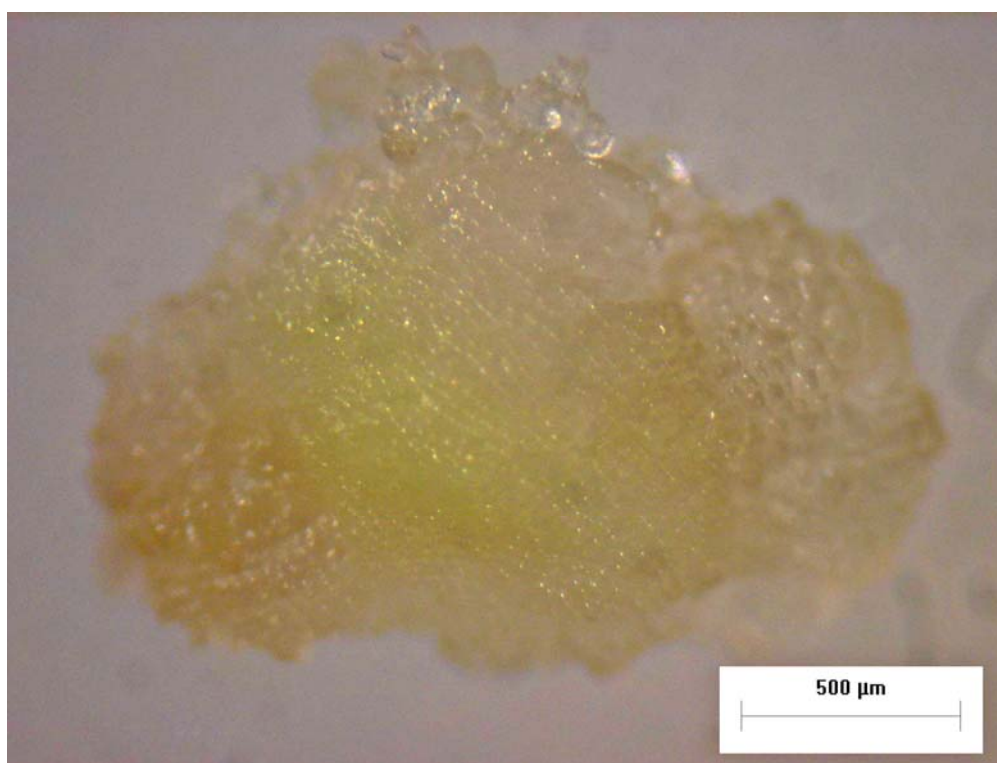
Photograph 5: Embryo germinated on a medium supplemented with 5 IAA and 0.5   Zeatin one week after germination was first observed
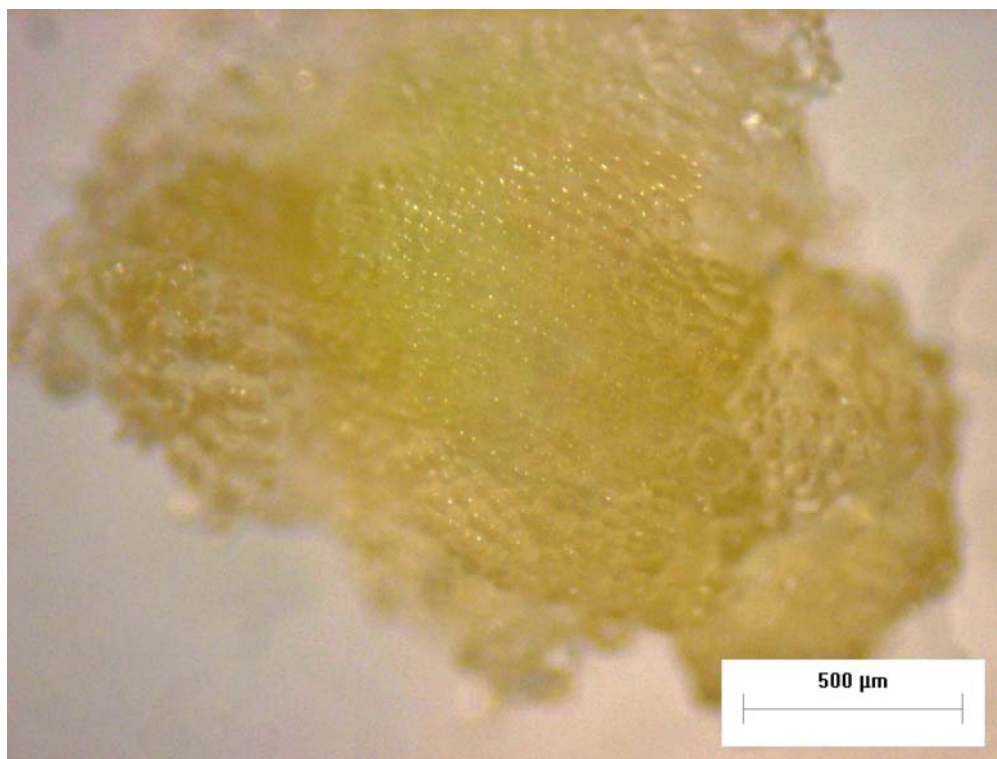

Photograph 6: Callus induced on a medium with 0.5 IAA and 1 Zeatin

Photograph 7: Callus induced on a medium with 0.5 IAA and 1 Zeatin a week after callus induction

Photograph 8: Callus induced on a medium with 5 IAA

Photograph 9: Callus induced on a medium with 5 IAA a week after callus induction

Literature Cited

Ahloowalia, B. S. and J. Prakash. 2002 Proceedings of a Technical Meeting organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, pp. 17-28.

Ahloowalia, B. S., J. Prakash, V. A. Savangikar and C. Savangikar. 2002 Proceedings of a Technical Meeting organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, pp. 3-10.

Band, S. R. and G. A. F. Hendry. 1993. General procedures and methodologies ed. Trans. In: J. P. Grime and G. A. F. Hendry (eds) Methods in comparative plant ecology: A laboratory manual. Chapman & Hall, London, pp. 252.

Baskin, C. C. and J. M. Baskin. 1998. Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination. Vol. Academic Press, San Diego, 666 pp.

Bayer, R. J., C. F. Puttock and S. A. Kelchner. 2000. Phylogeny of South African Gnaphalieae (Asteraceae) based on two noncoding chloroplast sequences. American Journal of Botany 87**:** 259–272.

Bewley, J. D. and M. Black. 1982. Physiology and biochemistry of seeds in relation to germination. Vol 2. Springer-Verlag, Berlin, 375 pp.

Bewley, J. D. and M. Black. 1994. Seeds: Physiology of Development and Germination. Vol. Plenum Press, New York, 445 pp.

Beyl, C. A. 2005. Getting started with tissue culture: Media preparation, sterile technique and laboratory equipment ed. Trans. In: R. N. Trigiano and D. J. Gray (eds) Plant development and biotechnology. CRC Press LLC, Florida, pp. 19-37.

Blank, R. R. and J. A. Young. 1998. Heated substrate and smoke: Influence on seed emergence and plant growth. Journal of Range Management 51**:** 577-583.

Bleasdale. 1973. Plant physiology in relation to horticulture. Vol. Macmillan Press Ltd., London, 144 pp.

Brown, J. T. 1990. The initiation and maintenance of callus cultures ed. Trans. In: J. W. Pollard (eds) Plant cell and tissue culture. Vol VI. Humana Press, Clifton, pp. 57-63.

Brown, J. T. and B. V. Charlwood. 1990. Organogenesis in callus culture ed. Trans. In: J. W. Pollard (eds) Plant cell and tissue culture. Vol VI. Humana Press, Clifton, pp. 65-70.

Brown, N. A. C. 1993. Seed germination in the Fynbos fire ephemeral, *Syncarpha vestita* (L.) B.Nord. is promoted by smoke, aqueous extracts of smoke and charred wood derived from burning the ericoid-leaved shrub, *Passerina vulgaris* Thoday. International Journal of Wildland Fire 3**:** 203-206.

Brown, N. A. C. and J. van Staden. 1997. Smoke as a germination cue: a review. Plant Growth Regulation 22**:** 115-124.

Campbell, E. E. 1995. Vegetation and soils ed. Trans. In: L. E. McGwynne (eds) Specialist Reports on the Vegetation and Soils, Geology and Palaeontology of the Proposed Mining Area on the Farms Grassridge 190, 225 and 227 & 228. Vol C30. Institute for Coastal Research, Port Elizabeth, pp. 7-30.

Davy, A. J. 2002. Establishment and manipulation of plant populations and communities in terrestrial systems ed. Trans. In: M. R. Perrow and A. J. Davy (eds) Handbook of ecological restoration. Vol 1. Cambridge University Press, Cambridge, pp. 223-241.

Day, J. S. 2000. The influence of seed pre-treatments and soil conditions on the emergence of sesame seedlings. Australian Journal of Experimental Agriculture 40**:** 843–848.

de Lange, J. H. and C. Boucher. 1990. Autecological studies on *Autouinia capitata* (Bruniaceae). I. Plant-derived smoke as a seed germination cue. South African Journal of Botany 56**:** 700-703.

Deka, P. and M. C. Kalita. 2005. *In vitro* clonal propagation and organogenesis in *Spilanthes acmella* (L) Murray: A herbal pesticidal plant of North-East India. J. Plant Biochemistry & Biotechnology 14**:** 69-71.

Duncan, D. R. and J. M. Widholm. 1990. Measurements of viability suitable for plant tissue cultures ed. Trans. In: J. W. Pollard (eds) Plant cell and tissue culture. Vol VI. Humana Press, Clifton, pp. 29-37.

Eastwood, C. B. 1998. Population structure and propagation potential of *Syncarpha recurvata* (L. f.) B. Nord at Grassridge. 3rd year project. University of Port Elizabeth. 14 pp.

Edmond, J. B., T. L. Senn and F. S. Andrews. 1964. Fundamentals of Horticulture. 3rd ed., Vol. McGraw-Hill, Inc., New York, 476 pp.

Egerton-Warburton, L. M. 1998a. A smoke-induced alteration of the sub-testa cuticle in seeds of the post-fire recruiter, *Emmenanthe penduliflora* Benth. (Hydrophyllaceae). Journal of Experimental Botany 49**:** 1317–1327.

Egerton-Warburton, L. M. 1998b. A smoke-induced alteration of the sub-testa cuticle in seeds of the post-fire recruiter, *Emmenanthe penduliflora* Benth. (Hydrophyllaceae). Journal of Experimental Botany 49**:** 1317–1327.

Finer, J. J. 1995. Direct somatic embryogenesis ed. Trans. In: O. L. Gamborg and G. C. Phillips (eds) Plant cell, tissue and organ culture: Fundamental methods. Springer-Verlag, New York, pp. 91-101.

Flematti, G. R., E. L. Ghisalbert, K. W. Dixon and R. D. Trengove. 2004. Molecular weight of a germination-enhancing compound in smoke. Plant and Soil 263**:** 1–4.

Gaba, V. P. 2004. Plant growth regulators in plant tissue culture and development ed. Trans. In: R. N. Trigiano and D. J. Gray (eds) Plant development and biotechnology. CRC Press, New York, pp. 97-99.

Gamborg, O. L. and G. C. Phillips. 1995a. Sterile techniques ed. Trans. In: O. L. Gamborg and G. C. Phillips (eds) Plant cell, tissue and organ culture: Fundamental methods. Springer-Verlag, New York, pp. 35-42.

Gamborg, O. L. and G. C. Phillips. 1995b. Sterile techniques ed. Trans. In: O. L. Gamborg and G. C. Phillips (eds) Plant cell, tissue and organ culture: Fundamental methods. Springer-Verlag, New York, pp. 35-41.

Geldenhuys, D. A. 1997. The age and ditribution of the Coega population of *Syncarpha recurvata* (L. f.) B. Nord. 3rd year project. University of Port Elizabeth. 14 pp.

Giovannini, A., M. Amoretti, M. Savona and B. Ruffoni. 2001 First international symposium on acclimation and establishment of micropropagated plants, Sani-Halkidiki, Macedonia, Greece, pp. 339-342.

Given, D. R. 1994. Principles and practice of plant conservation. Vol. Chapman & Hall, London, 292 pp.

Gledhill, E. 1981. Veldblomme van Oos-Kaapland. Vol. Creda Press, Kaapstad, 275 pp.

Glenny, D. 1997. A revision of the genus *Anaphalioides* (Asteraceae: Gnaphalieae). New Zealand Journal of Botany 35**:** 451-477.

Golding, J. 2002. Southern African Plant Red Data Lists. Southern African Botanical Diversity Network Report, Vol 14. National Botanical Institute, Pretoria, 237 pp.

Goyal, K., L. J. Walton and A. Tunnacliffe. 2005. LEA proteins prevent protein aggregation due to water stress. Biochemical Journal 388**:** 151–157.

Gray, D. J. 2005. Propagation from nonmeristematic tissues: Nonzygotic embryogenesis ed. Trans. In: R. N. Trigiano and D. J. Gray (eds) Plant development and biotechnology. CRC Press, New York, pp. 187-200.

Guerrant, E. O. 1996. Designing populations: Demographic, genetic, and horticultural dimensions ed. Trans. In: D. A. Falk, C. I. Millar and M. Olwell (eds) Restoring diversity: Strategies for reintroduction of endangered plants. Island Press, Washington D.C., pp. 171-207.

Hammet, K. R. W. 1973. Plant Propagation. Vol. Hugh Keartland, Ltd., Johannesburg, 63 pp.

Hartmann, H. T. and D. E. Kester. 1965. Plant propagation: Principles and Practices. 2nd ed., Vol. Prentice-Hall, Inc., New Jersey, 702 pp.

Heydecker, W. 1977. Stress and seed germination: an agronomic view ed. Trans. In: A. A. Khan (eds) The physiology and biochemistry of seed dormancy and germination. North-Holland, New York, pp. 238-282.

Hilton-Taylor, C. 1996. Red Data List of Southern African Plants. Strelitzia, Vol 4. National Botanical Institute, Pretoria, 117 pp.

Hodgson, J. G. and K. Thompson. 1993. Size ed. Trans. In: J. P. Grime and G. A. F. Hendry (eds) Methods in comparative plant ecology: A laboratory manual. Chapman & Hall, London, pp. 252.

Home, I. F. and E. E. Campbell. 1996. Propagation potential of *Syncarpha recurvata* (L.f.) B. Nord. Unpublished report commissioned by the PPC, University of Port Elizabeth. pp.

Hu, C. Y. and M. H. B. Zanettini. 1995. Embryo culture and embryo rescue for wide cross hybrids ed. Trans. In: O. L. Gamborg and G. C. Phillips (eds) Plant cell, tissue and organ culture: Fundamental methods. Springer-Verlag, New York, pp. 129-140.

Huylenbroeck, J. M., A. Piqueras and P. C. Debergh. 1998. Photosynthesis and carbon metabolism in leaves formed prior and during ex vitro acclimatization of micropropagated plants. Plant Science 134**:** 21–30.

Huylenbroeck, J. M., A. Piqueras and P. C. Debergh. 2000. The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated *Calathea* plants. Plant Science 155**:** 59–66.

Jager, A. K., M. E. Light and J. van Staden. 1996. Effects of source of plant material and temperature on the production of smoke extracts that promote germination of light-sensitive Lettuce seeds. Environmental and Experimental Botany 36**:** 421-429.

Jann, R. C. and R. D. Amen. 1977. What is germination? ed. Trans. In: A. A. Khan (eds) The physiology and biochemistry of seed dormancy and germination. North-Holland, New York, pp. 7-28.

Kamra, S. K. 1972. Comparative studies on germinability of *Pinus silvestris* and *Picea abies* seed by the indigo carmine and x-ray contrast methods. Masters. Royal College of Forestry and Institute of Botany. 21 pp.

Kane, M. E. 2004. Shoot culture procedures ed. Trans. In: R. N. Trigiano and D. J. Gray (eds) Plant development and biotechnology. CRC, New York, pp. 145-157.

Koller, D. 1972. Environmental Control of Seed Germination ed. Trans. In: T. T. Kozlowski (eds) Seed Biology. Vol 2. Academic Press, Inc., New York, pp. 447.

Kyte, L. and J. Kleyn. 1996. Plants from test tubes: An introduction to micropropagation. 3rd ed., Vol. Timber Press, Inc., Portland, 240 pp.

Lawrence, E. 2000. Henderson's dictionary of biological terms. 12 ed., Vol. Prentice Hall, Harlow, 719 pp.

Leadem, C. L. 1984. Quick Tests for Tree Seed Viability. 18. B.C. Ministry of Forests Research Laboratory, Victoria

Light, M. E., M. J. Gardner, A. K. Jäger and J. van Staden. 2002. Dual regulation of seed germination by smoke solutions. Plant Growth Regulation 37**:** 135–141.

Marion-Poll, A. 1997. ABA and seed development. TRENDS in Plant Science 2**:** 447-448.

Mayer, A. M. 1977. Metabolic control of germination ed. Trans. In: A. A. Khan (eds) The physiology and biochemistry of seed dormancy and germination. North-Holland, New York, pp. 357-384.

Mineo, L. 1990. Plant tissue culture techniques ed. Trans. In: C. A. Goldman (eds) Tested studies for laboratory teaching. Vol 11. ABLE, Pennsylvania, pp. 151-174.

Minorsky, P. V. 2002. The Hot and the Classic. Plant Physiology 128**:** 1167–1168.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum 15**:** 472-497.

Nikolaeva, M. G. 2004. On criteria to used in studies of seed evolution. Seed Science Research 14**:** 315–320.

Nowak, J. and K. Pruski. 2002 Proceedings of a Technical Meeting organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, pp. 69-82.

Oliveira Dragovic, M. J., S. G. Ferreira and M. C. Neves. 2000. Preliminary studies on propagation of *Helichrysum obconicum* DC. Acta Horticulturae (ISHS) 541:**:** 273-275.

Oliveira Dragovic, M. J., S. G. Ferreira and M. C. Neves. 2005. Utilization of *Helichrysum obconicum* DC. as ornamental plant: preliminary sudies on pot adaptation. Acta Horticulturae (ISHS) 683**:** 181-188.

Pavlik, B. M. 1996. Defining and measuring success ed. Trans. In: D. A. Falk, C. I. Millar and M. Olwell (eds) Restoring diversity: Strategies for reintroduction of endangered plants. Island Press, Washington D.C., pp. 127-155.

Peng, J. and N. P. Harberd. 2002. The role of GA-mediated signalling in the control of seed germination. Current Opinion in Plant Biology 5**:** 376–381.

Phillips, G. C., J. F. Hustenberger and E. E. Hansen. 1995. Plant regeneration by organogenesis from callus and cell suspension cultures ed. Trans. In: O. L. Gamborg and G. C. Phillips (eds) Plant cell, tissue and organ culture: Fundamental methods. Springer-Verlag, New York, pp. 35-42.

Pierik, R. L. M. 1997. *In vitro* culture of higher plants. Vol. Kluwer academic publishers, Dondrecht, 348 pp.

Pitelka, L. F. 1997. Plant migration and climate change. American Scientist 85**:** 464-474.

Pounds, J. A. and R. Puschendorf. 2004. Clouded futures. Nature 427**:** 107-109.

Prakash, S., M. I. Hoque and T. Brinks. 2002 Proceedings of a Technical Meeting organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, pp. 29-40.

Primack, B. P. 1996. Lessons from ecological theory: dispersal, establishment, and population structure ed. Trans. In: D. A. Falk, C. I. Millar and M. Olwell (eds) Restoring diversity: Strategies for reintroduction of endangered plants. Island Press, Washington D.C., pp. 209-233.

Raghavan. 1976. Experimental embryogenesis in vascular plants. Experimental Botany (J. F. Sutcliffe).(eds), Vol 10. Academic Press, London, 603 pp.

Raveh, E., J. Weiss, A. Nerd and Y. Mizrahi. 1993. Pitayas (Genus *Hylocereuhs*): A new fruit crop for the Negev desert of Israel ed. Trans. In: J. Janick and J. E. Simon (eds) New crops. Wiley, New York, pp. 491-495.

Reed, S. M. 2005. Embryo rescue ed. Trans. In: R. N. Trigiano and D. J. Gray (eds) Plant development and biotechnology. CRC Press, New York, pp. 235-239.

Roberts, A. V., E. F. Smith and J. Mottley. 1990. The preparation of micropropagated plantlets for transfer to soil without acclimatization ed. Trans. In: J. W. Pollard (eds) Plant cell and tissue culture. Vol VI. Humana Press, Clifton, pp. 227-236.

Schwartz, O. J., R. A. Sharma and R. M. Beaty. 2004. Propagation from nonmeristematic tissues: Organogenesis ed. Trans. In: R. N. Trigiano and D. J. Gray (eds) Plant development and biotechnology. New York, CRC Press, pp. 159-172.

Scott, B. and C. L. Gross. 2004. Recovery directions for monoecious and endangered *Bertya ingramii* using autecology and comparisons with common *B. rosmarinifolia* (Euphorbiaceae). Biodiversity and Conservation 13**:** 885–899.

Slater, A., N. W. Scott and M. R. Fowler. 2003. Plant Biotechnology: The Genetic Manipulation of Plants. Vol. Oxford University Press, New York, 346 pp.

Smith, R. H. 2000. Plant Tissue Culture: Techniques and Experiments. 2nd edition ed., Vol. Academic Press, London, 231 pp.

Sparg, S. G., M. G. Kulkarni, M. E. Light and J. van Staden. 2005. Improving seedling vigour of indigenous medicinal plants with smoke. Bioresource technology 96**:** 1323-1330.

Stephan-Sarkissian, G. 1990. Selection of media for tissue and cell culture ed. Trans. In: J. W. Pollard (eds) Plant cell and tissue culture. Vol VI. Humana Press, Clifton, pp. 13-26.

Stephan-Sarkissian, G. and D. Grey. 1990. Growth determination and medium analysis ed. Trans. In: J. W. Pollard (eds) Plant cell and tissue culture. Vol VI. Humana Press, Clifton, pp. 13-27.

Sutter, R. D. 1996. Monitoring ed. Trans. In: D. A. Falk, C. I. Millar and M. Olwell (eds) Restoring diversity: Strategies for reintroduction of endangered plants. Island Press, Washington D.C., pp. 235-264.

Swart, P. A. 2005. The effect of smoke water on the germination and growth of selected species across five families of flowering plants of agricultural importance. BSc Honours project. Nelson Mandela Metropolitan University. 60 pp.

Taylor, J. L. S. and J. van Staden. 1998. Plant-derived smoke solutions stimulate the growth of *Lycopersicon esculentum* roots *in vitro.* Plant Growth Regulation 26**:** 77-83.

Teixeira da Silva, J. A. 2003. Chrysanthemum: advances in tissue culture, cryopreservation, postharvest technology, genetics and transgenic biotechnology. Biotechnology Advances 21**:** 715–766.

Tian, X., A. D. Knapp, L. R. Gibson, R. Struthers, K. J. Moore, E. C. Brummer and T. B. Bailey. 2003. Response of Eastern Gamagrass Seed to Gibberellic Acid Buffered below Its pKa. Crop Science 43**:** 927–933.

Todkill, W. B. 1996. Propagation and aspects of the population ecology of *Syncarpha recurvata* (L. f.) B. Nord. 3rd year project. University of Port Elizabeth. 26 pp.

Urbanska, K. M. and J. C. Chambers. 2002. High-elevation ecosystems ed. Trans. In: M. R. Perrow and A. J. Davy (eds) Handbook of ecological restoration. Vol 2. Cambridge University Press, Cambridge, pp. 376-400.

Wareing, P. F. and I. D. J. Phillips. 1978. The control of growth and differentiation in plants. Vol. Pergamon Press, Oxford, 347 pp.

Watson, J. J. 2002. Bontveld ecosystem functioning, and rehabilitation after strip mining. PhD Thesis. University of Port Elizabeth. 257 pp.

Weatherall-Thomas, C. R. 2005. Germination requirements of two Eastern Cape endemics, *Syncarpha recurvata* (L.f.) B. Nord. and *Cyclopia pubescens* Eckl. & Zeyh. BSc Honours project. Nelson Mandela Metropolitan University. 40 pp.

Welch, H. J. 1973. Mist Propagation and Automatic Watering. Vol. Faber and Faber Ltd., London, 172 pp.

Withers, L. A. 1990. Cryopreservation of plant cells ed. Trans. In: J. W. Pollard (eds) Plant cell and tissue culture. Vol VI. Humana Press, Clifton, pp. 39-48.

Yasmin, S., B. Ahmed and R. Soomro. 2003. Influence of ABA, Gibberellin and Kinetin on IAA induced adventitious root development on hypocotyl cuttings of mungbean. Biotechnology 2**:** 37-43.