CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Vegetative propagation is the reproduction of plants identical in genotype to the source plant (Hartmann, Kester, Davies and Geneve, 1997). Hence, the objective is the formation of adventitious roots since it is the main regenerative process and the prerequisite for successful vegetative propagation. The main advantage of vegetative propagation is that growers are able to achieve genetic uniformity among the newly propagated plants, which is of utmost importance for cutflower producers. However, the difficulties in achieving root formation in certain plants, as well as the high costs of various propagation facilities such as mistbeds, fogging systems, temperature and light manipulation, are the disadvantages of vegetative propagation.

There are two types of adventitious roots: preformed and wound-induced roots (Hartmann, Kester, Davies and Geneve, 1997). Preformed root initials are normally dormant until the stems are made into cuttings and placed under environmental conditions which are conducive to emergence of the primordia as adventitious roots (Lovell and White, 1986). Wound-induced roots develop only after the cutting is made and is therefore formed *de novo* (Davies, Lazarte and Joiner, 1982). When a cutting is made, the stem responds to the wounding to protect the cut surface from desiccation and infections. These responses include: 1) the formation of a necrotic plate, a corky substance (suberin) seals the wound, and the xylem is plugged with gum; 2) cell division takes place and a layer of parenchyma cells (callus) is formed; 3) cells close to the vascular cambium and phloem start to divide to initiate *de novo* adventitious roots (Cline and Neely, 1983). The stages of development of *de novo* adventitious roots are: 1) dedifferentiation, 2) cell initiation to form root initials, 3) development of root initials into root primordia, and 4) the growth and elongation of the root primordia (Girouard, 1967).

A special adaptation of proteas to nutrient-deficient soils is the growth of unique roots known as proteoid roots (Vogts, 1982). Proteoid roots are dense clusters of fine rootlets that are produced by all cultivated species of protea (Lamont, 1986). Individual proteoid roots vary in length from a few millimetres to over 10 cm in length and can consist of up to thousands of hairy rootlets. The rootlets readily form a 2 – 5 cm thick mat at the soil surface in localized wet pockets of soil (Lamont, 1986). The primary function of proteoid roots is to enhance nutrient uptake, particularly in poor soils. It has been shown that proteoid root formation is suppressed in clayey soils, and the production of proteoid roots decreases markedly when soil nutrient availability is increased (Gardner, Parbery and Barber, 1982), indicating that the formation of proteoid roots is temporary, which takes place only when insufficient nutrients and moisture are present. Proteoid roots were not observed during the study of adventitious root formation in *P. cynaroides* (Chapter 5).

The first part of the following literature review deals with relationships between vegetative reproduction and carbohydrate, etiolation and phenolics. The second part describes alternative propagation methods that could be applied to *P. cynaroides*.

1.1.1 Carbohydrates

Reducing sugars such as glucose and fructose, non-reducing sugar such as sucrose, and starches (storage carbohydrates) are the most important carbohydrates for the rooting process (Haissig, 986). Carbohydrates are important in root formation because they are the basic building blocks of structural elements and are used as energy sources in the plant (Struve, 1981). The amount of carbohydrates required to fulfill this function has not been defined. Carbohydrates may regulate the number of roots to be supported and their subsequent growth, however, it is not necessarily a controlling mechanism (Veierskov, 1988). This may explain numerous apparent contradictory findings of positive correlations between carbohydrates and rooting in certain plant species, and negative correlations in others. Veierskov, Stummann and Henningsen (1982) reported a positive correlation in *Pisum sativum* plants, where a high carbohydrate content in the cuttings increased the number of roots formed. However, it has been proposed that a positive correlation between starch and rooting may be due to the supply of photosynthate being insufficient to support rooting (Veierskov, 1988).

Conversely, a negative correlation was observed in *Pinus sylvestris* by Hansen, Stromquist and Ericsson (1978), where an increase in carbohydrate content reduced the number of roots formed. Similarly, Nanda and Anand (1970) and Okoro and Grace (1976) showed that starch content was not related to rooting. Therefore, in this thesis (Chapter 5), the role of starch in the rooting of *P. cynaroides* was investigated to provide explanations to the low rooting rate of *P. cynaroides*.

1.1.2 Etiolation

Etiolation is defined as growing plants in total darkness (Bassuk and Maynard, 1987). Other practices related to etiolation are banding and blanching of the stem, which are both localized light exclusion techniques. Banding involves etiolating the entire plant until the new shoots have grown to a suitable length. Subsequently, shading is gradually reduced and the shoot is allowed to turn green, while an adhesive band is wrapped around the portion of the shoot that will become the cutting base. Blanching involves the plant being grown under the usual light conditions and once the normal development of new shoots has been completed, the future cutting base is banded with adhesive tape for several weeks (Bassuk and Maynard, 1987). Over the years, all the various light exclusion treatments, whether etiolation, shading, banding or blanching, have in most cases improved rooting of numerous plants. Of these species, many were difficult-to-root woody plants (Maynard and Bassuk, 1987).

For example, Reid (1923) successfully rooted *Camphora* spp. cuttings, which is one of the earliest reports on the promotion of rooting by etiolation. Subsequently, the success of rooting of various plant genera through etiolation has been increased. These include *Acer* spp., *Betula papyrifera, Carpinus* spp., *Corylus americana*, *Pinus* spp. and *Quercus* spp. (Maynard and Bassuk, 1985), *Persea americana* (Frolich, 1961), *Hibiscus rosa-sinensis* and *Phaseolus vulgaris* (Herman and Hess, 1963), *Syringa vulgaris* (Patience and Alderson, 1985) and *Malus* spp. (Gardner, 1936; Delargy and Wright, 1978; Delargy and Wright, 1979; Sun and Bassuk, 1991).

Although the various etiolation techniques have resulted in numerous successes in promoting rooting of difficult-to-root species, the mechanisms of etiolation is still not fully understood. However, several anatomical and biochemical studies have led to

some elucidation. Less lignification was found in etiolated stem tissues than in light-grown tissues (Reid, 1923). A decrease in cell wall thickness and an increase in protoplasmic cell contents were observed which led to the idea that the ease of rooting in etiolated cuttings was due to a change in the mechanical properties of the stem. In addition, Gardner (1936) reported that blanched shoots had more undifferentiated tissues that may have led to easier root initiation. Furthermore, Frolich (1961) showed a negative relationship between total light duration and rooting of mung beans, where the longer the mung beans were exposed to light, the poorer was the rooting.

Plant growth regulators such as auxin and rooting co-factors may also play a role in the rooting of *P. cynaroides* cuttings. However, the focus of this study was on the changes of endogenous concentrations of starch and phenolic compounds during rooting of cuttings. No research on etiolation of *P. cynaroides* has been done, therefore blanching, which could be used as an etiolation technique for proteas, was investigated (Chapter 5).

1.1.3 Phenolic compounds

When cuttings are prepared for rooting, they are wounded when removed from the motherplant. This wounding leads to the release and oxidation of phenolics which were formerly contained in cell compartments. The roles of these phenolics could include inhibition of the growth of microbes and assisting in the formation of lignin around the wound to act as a physical barrier against diseases (Salin and Bridges, 1981).

Numerous research papers have suggested that endogenous phenolic compounds may play a role in the rooting ability of stems. For example, before the seasonal increase in the rooting ability of apple ‘M26’ shoots, the amount of phloridzin, which is a phenolic found in apple, increased in the xylem of those shoots (Roy, Roychoudhury, Bose and Basu, 1972). Phloroglucinol, which is also a well-known phenolic, has been shown to promote rooting in apple shoots (Jones and Hatfield, 1976; James and Thurbon, 1981; James, 1983) and in *Prunus* species (Jones and Hopgood, 1979). However, one should keep in mind that a compound could either be a promoter or inhibitor of root formation depending on its concentration. In addition, it is generally

agreed that a number of different factors and compounds work together during rooting, rather than a certain compound alone, whether phenolics, auxins or other endogenous promoters/inhibitors.

Interestingly, Spiegel (1954) found that the rooting of easy-to-root *Vitis* sp. was inhibited when supplied with leachates taken from the bases of difficult-to-root cuttings. Bioassay investigations carried out on extracts taken from various vines also showed that difficult-to-root species have relatively higher amounts of inhibitors than easy-to-root species. On the other hand, in *Hibiscus* extract bioassay results led Hess (1964) to conclude that a higher concentration of promotory substances are found in easy-to-root *Hibiscus* materials than in difficult-to-root species. The effects of phenolic compounds on the rooting of *P. cynaroides* have not been investigated. Therefore, phenolic compounds from *P. cynaroides* cuttings were isolated and identified. Allelopathic potential of these compounds and their effects on root formation were studied (Chapter 6).

1.2 *In vitro* propagation

The potential of *in vitro* propagation using tissue and organ culture for rapid mass propagation of Proteaceae has been researched relatively extensively (George, 1996). Most of the methods used involved using stem or leaf segments as explants, which are established, multiplied, rooted, acclimatized and planted out into *ex vitro* conditions. However, in terms of obtaining a propagation protocol that is relatively simple and reliable, which can be used in a commercial environment, it has still not been successful. This is evident in the fact that Proteaceae plants are still not being extensively mass-propagated using tissue culture techniques, which is often the standard procedure in numerous economically important cutflowers.

It is surprising that even though *P. cynaroides* is probably the most popular and recognizable species in the Proteaceae family, very little research has apparently been done on its *in vitro* propagation. As a result, rooted *P. cynaroides* plantlets derived from tissue culture have yet to be achieved. Early work on *in vitro* establishment of *P. cynaroides* explants were reported by Ben-Jaacov and Jacobs (1986). Gibberellic acid

(GA_3) was found to be an essential growth regulator to start *P. cynaroides* in culture. More specifically, they concluded that the addition of 10 mg l^{-1} GA_3 and 2 mg l^{-1} BA (Benzyladenine) into Anderson medium (AND) (Anderson, 1975) was the most suitable combination for establishment. Following this, an alternative establishment medium was reported, where antioxidants were used as a pretreatment to reduce oxidative browning, resulting in the promotion of bud sprout (Wu and du Toit, 2004). These established explants were subsequently multiplied in a multiplication medium. However, rooting of these explants was not successful.

Research on *in vitro* propagation of other Proteaceae have also been published, such as *Protea obtusifolia* (Watad, Ben-Jaacov, Cohen, Tal and Solomon, 1992), where etiolated, multinodal shoot segments were established through bud sprouting and elongation, which were promoted by the addition of 2 mg l^{-1} GA_3 and 1 mg l^{-1} BAP (6-Benzylaminopurine). Similarly, Rugge (1995) also used multinodal explants to establish *Protea repens* on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) medium supplemented with 1 mg l^{-1} BA. Bud break was increased with the addition of 6 mg l^{-1} GA_3. Axillary shoot multiplication was achieved in *Leucadendron* when a mixture of 0.89 µM BA and 0.89 µM kinetin was added into the medium (Pérez-Francés, Expósito and Rodríguez, 1995).

In *Leucospermum*, shoots of the cultivar 'Red Sunset' were successfully established on AND liquid medium containing 2 mg l^{-1} BA (Ben-Jaacov and Jacobs, 1986), as well as using multinodal stem segments cultured on full strength MS medium with 2 mg l^{-1} kinetin (Rugge, Jacobs and Theron, 1989). Axillary shoots of *Leucospermum* 'Hawaii Gold' were induced to proliferate on modified MS medium supplemented with 0.2 mg l^{-1}, and rooted *in vitro* by dipping in 50 or 100 mg l^{-1} IBA (3-indolebutyric acid) (Kunisaki, 1989). Dwarf clones of *Leucospermum cordifolium* were also micropropagated using 1 mg l^{-1} GA_3, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA (1-Naphthalene acetic acid). Best *in vitro* rooting was achieved when 1 mg l^{-1} IBA was added to the medium (Tal, Solomon, Ben-Jaacov and Watad, 1992). Tal, Ben-Jaacov and Watad (1992) further reported that high light intensities and a low relative humidity are best suited for *in vivo* rooting of micropropagated *Leucospermum.*

In *Grevillea* spp., cultivars 'Robyn Gordon' and 'Crosbie Morrison' were propagated via tissue culture by adding 1 µM NOA (2-Naphthyloxyacetic acid) and 0.1 µM 2iP (N^6-(2-isopentyl)adenine) into the growth medium, and rooted *in vitro* with 10 µM IBA in the rooting medium. (Gorst, Bourne, Hardaker, Richards, Dircks and de Fossard, 1978). Ben-Jaacov and Dax (1981) proliferated shoot segments of *Grevillea rosmarinifolia* on half-strength MS medium containing 0.5 mg l^{-1} BA, which were rooted on paper bridges placed in liquid MS medium containing 0.1 mg l^{-1} NAA. Six other *Grevillea* species were established *in vitro* when cultured on half-strength MS medium with 1 mg l^{-1} BAP (Watad, Ben-Jaacov, Tal and Solomon, 1992). Highest rooting percentage was recorded with 1 mg l^{-1} NAA added into the rooting medium. The endangered *Grevillea scapigera* was micropropagated using leaf sections to obtain adventitious shoot growth (Bunn and Dixon, 1992). Shoot tips were initiated on filter paper placed in liquid Woody Plant Medium (WPM) (Loyd and McCown, 1981), supplemented with 20 µM zeatin riboside and 2 µM GA_3.

Single-node explants of *Telopea speciosissima* were established in culture on modified MS medium (Seelye, Butcher and Dennis, 1986). Shoots then proliferated when grown on medium with 0.05 mg l^{-1} IBA, 0.3 mg l^{-1} BA and 2 mg l^{-1} GA_3. Similarly, Offord, Campbell and Mullins (1992) established *Telopea speciosissima* on a modified MS medium, with microshoot proliferation achieved with 1.25 µM BA and 1 µM GA_3 added to the medium. Microshoots were then subsequently rooted *in vitro* on agar, filter paper bridges and crushed quartz–sand (Offord and Campbell, 1992). Best rooting was obtained on the crushed quartz-sand containing 50 µM IBA.

Other *Protea* species that have been micropropagated include *Serruria florida*, where shoot proliferation was achieved on AND, MS and WPM liquid media (Ben-Jaacov and Jacobs 1986). Furthermore, numerous side shoots formed when the explants were subcultured onto MS medium containing 5 mg l^{-1} BA. Shoot tips of *Stirlingia latifolia* were successfully multiplied on half-strength MS medium supplemented with 5 µM 2iP or 0.5 µM BA (Bunn and Dixon, 1992). These microshoots were subsequently rooted *in vitro* in rooting medium containing IBA. *Alloxylon flammeum* was best grown via tissue culture on 0.6 µM BA, which produced the most number of usable shoots, whilst 3 – 25 µM 2iP gave the longest shoots (Donovan, Offord and Tyler, 1999).

1.2.1 Embryo culture

Embryo culture is defined as the *in vitro* isolation and growth of an immature or mature embryo to obtain a viable plant (Pierik, 1987). In the culture of immature embryos, the embryos are excised and cultured to avoid abortion. This procedure is commonly referred to as 'embryo rescue'. However, failure in culturing these immature embryos is generally high. Success rates depend largely on the development stage of the immature embryo. In addition, the nutritional requirements of immature embryos are complex, thus, composing a suitable nutrient medium is difficult (Pierik, 1987). Immature embryo cultures are often used by plant breeders for biochemical studies and interspecific hybridization, where a particular useful character from wild species, such as disease resistance, is transferred into cultivated species (Hadley and Openshaw, 1980). Embryo culture is also useful for determining seed viability and studying nutritional and physical requirements for embryonic development (Razdan, 1993). Mature (zygotic) embryos are easier to culture, where the use of simple medium is generally sufficient, although low temperatures, absence of light, and gibberellic acid, which is known to promote germination, are commonly applied during embryo culture. Mature embryo culture is mainly used to overcome seed dormancy, improve germination rates and shorten the breeding cycle, since chemical inhibitors are not present after the removal of the seed coat. This is particularly advantageous in plant species where germination is extremely slow, or does not occur at all, when using conventional germination techniques. From a commercial point of view, for highly-sought after seedlings that do not germinate easily under conventional propagation methods, embryo culture may be used as an alternative method for mass production.

Embryo culture was first described in the early 20th century, and subsequently, numerous studies have been reported over the years. The first publication on embryo culture was by Hannig (1904), who isolated *Cochleria* and *Raphanus* embryos, which were subsequently grown into transplantable seedlings. Following Hannig's work, Brown (1906) studied the nutritional requirements of excised barley embryos. *Linum* embryos were also one of the first to be successfully cultured (Laibach, 1929), where hybrid plants were raised from an interspecific cross, which in nature, failed to germinate. Other early embryo culture research were reported in *Prunus* spp. (Tukey,

1933; Tukey, 1934; Tukey, 1938), *Datura stramonium* (Van Overbeek, Conklin and Blakeslee, 1942), *Iris* spp. (Randolph and Cox, 1943), *Hordeum vulgare* (Kent and Brink, 1947), *Phaseolus* spp. (Honma, 1955), *Musa balbisiana* (Cox, Stotzky and Goos, 1960), and *Cocos* sp. (De Guzman, del Rosario and Eusebio, 1971). Zygotic papaya embryos were first cultured by Phadnis, Budrakker and Kaulgud (1970). This was followed by the successful embryo rescue of the interspecific cross between the incompatible *C. papaya* and *C. cauliflora* (Khuspe Hendre, Mascarenhas and Jagannathan, 1980). Similarly, Chung and Kim (1990) produced interspecific hybrids between *Glycine max* and *G. tomentella* through embryo culture. Self-sterile *Litchi chinensis* embryos, which were excised and cultured successfully, produced up to four axillary shoots each when the cotyledons and shoot tips were cultured separately (Amin, Razzaque and Anisuzzaman, 1996). Embryos of cross-bred seeds of peach (*Prunus persica*), which did not germinate under conventional methods, germinated *in vitro* after excision and stratification (Chopra, Kanwar, Gosal, Dhaliwal and Chanana, 1996).

In vitro germination of zygotic embryos has been studied in several Proteaceae species. Excised embryos of *Protea compacta*, *Leucadendron daphnoides L. cordifolium* (Van Staden and Brown, 1973), *Protea magnifica* (Deall and Brown, 1981) and *Leucadendron tinctum* (Brown and Dix, 1985),) were germinated in Petri dishes. In other studies, whole seeds of *P. compacta, P. barbigera*, *L. cordifolium* and *L. daphnoides* (Brown and van Staden, 1971) were used. *In vitro* germination of *P. cynaroides* excised embryos has not been reported in literature. Factors affecting *in vitro* germination of excised embryos of *P. cynaroides* and the transfer to the *ex vitro* environment are reported in Chapter 2.

1.2.2 Micrografting

Micrografting or *in vitro* grafting was first successfully carried out by Murashige, Bitters, Rangan, Roistacher and Holliday (1972). Newly-germinated seedlings are often used as the rootstock, although *in vitro*-rooted microshoots can also be used. The top of the rootstock seedling is cut off and the desired scion is grafted on. Depending on scion, different micrografting techniques are used. In the case where relatively large shoots are used, their bases are cut into a wedge (‘V’) shape, and

inserted into a vertical cut on the rootstock. If meristem tips are used as the scion, it is usually placed directly onto the cambium or cortex on the cut surface of the rootstock (George, 1996).

Problems encountered during micrografting include tissue blackening and death of the scion caused by oxidation of the cut surface, incompatibility between scion and rootstock, and desiccation of the graft area. Procedures to remedy tissue blackening have been developed. These include soaking the scion in a growth regulator or antioxidant solution such as ascorbic acid, thiourea, cysteine, chlorhydrate, sodium diethyl-dithiocarbamate (DIECA), or placing a drop of the solution onto the severed rootstock before inserting the scion (George, 1996). In *Citrus*, where micrografting has been used extensively to eliminate viruses, Edriss and Burger (1984) placed the shoot tips into a solution containing 10 mg l^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid), which doubled the number of successful micrografts. Similarly, Starrantino and Caruso (1988) soaked both the microscion and the tip of the rootstock in 0.5 mg l^{-1} BAP for 20 minutes before micrografting. Alternatively, an agar block, which contained mineral salts with or without hormones, was placed at the graft area between the scion and rootstock, helped in preventing dehydration of the scion (Pliego-Alfaro and Murashige, 1987). Furthermore, in order to improve the success rates, scions have been pre-cultured for a short period of time before being micrografted. Jonard, Hugard, Macheix, Matinez, Mosella-Chancel, Poessel and Villemur (1983) reported that pre-culturing the scion on medium containing 0.1 mg l^{-1} zeatin, encouraged rapid shoot formation after the micrograft has been successful.

Micrografting has been reported in numerous plant species. These include: *Persea americana* (Pliego-Alfaro and Murashige, 1987), *Anacardium occidentale* (Ramanayake and Kovoor, 1999; Mneney and Mantell, 2001; Thimmappaiah, Puthra and Anil, 2002), *Citrus* spp. (Murashige *et al*., 1972; Navarro, Roistacher and Murashige, 1975; Jonard *et al*., 1983), *Opuntia* spp. (Estrada-Luna, López-Peralta and Cárdenas-Soriano, 2002), *Picea* spp. (Ponsonby and Mantell, 1993), *Pistacia vera* (Abousalim and Mantel, 1992), *Prunus* spp. (Deogratis, Lutz and Dosba, 1986) and *Sequoia sempervirens* (Huang, Luis, Huang, Murashige, Mahdi and van Gundy, 1992).

Micrografting has mainly been used in fruit species to eliminate viruses or to overcome incompatibility between the microscion and rootstock. Micrografting has not been studied in *P. cynaroides*. Investigation into the use of micrografting as a technique to obtain rooted plantlets is reported in Chapter 3.

1.2.3 Somatic embryogenesis

Somatic embryogenesis is a process whereby a single cell or a group of cells initiate a developmental pathway that leads to reproducible regeneration of embryos ('embryoids'), which are capable of germinating and growing into complete plants (Razdan, 1993). The earliest work on plant regeneration was reported by Levine (1950), who after removing indole-acetic acid (IAA) from the growth medium, obtained roots and shoots from carrot callus. Wiggans (1954) also observed carrot plantlet regeneration when tissue was transferred from a medium containing adenine sulphate to a medium lacking it. However, it was Steward, Mapes and Mears (1958) who first described proembryo-like stages in carrot plantlet regeneration. This was followed by Reinert (1959) who proposed that carrot plantlets grew from bipolar embryos that were derived from single cells. Kato and Takeuchi (1963), Nakajima (1963) and Wetherell and Halperin (1963) also demonstrated that embryos formed in cultures were derived from mature organs of carrot plants.

According to Sharp, Sondahl, Caldas and Maraffa (1980), Evans, Sharp and Flick (1981) and Sharp, Evans and Sondahl (1982), somatic embryogenesis is initiated by either 'pre-embryogenic determined cells' (PEDC) or by 'induced embryogenic determined cells' (IEDC). In the PEDC pathway, direct embryogenesis occurs, without an intervening callus phase, from cells which are predetermined to become embryo-producing. Nutrient media and other *in vitro* conditions only serve to enhance the process. Physiologically, explants from which direct embryogenesis is most likely to occur, are juvenile. These include zygotic embryos, young seedlings, pollen microspores within the anther, tissues of all or part of the ovary, or ovules (George, 1993). On the other hand, in the IEDC pathway, indirect embryogenesis requires the differentiated cells of an explant to be induced to divide as undifferentiated callus, and then for certain cells to be re-determined to the embryogenic pathway, normally by exposure to growth hormones. Once the embryogenic state has been reached, plantlets

are produced. Direct somatic embryogenesis is less widely observed than indirect somatic embryogenesis. This is mainly due to the fact that conditions to obtain direct embryogenesis can be more critical than those required to produce embryogenic callus (George, 1996).

1.2.3.1 Stages in the development of somatic embryos

It is sometimes difficult to distinguish between plantlets that have been grown from embryos and adventitious shoots. However, detailed anatomical studies of somatic embryos will reveal a shoot and root pole (i.e. bipolar), a shoot axis and cotyledons (in dicotyledons). In addition, unlike axillary or adventitious buds, somatic embryos have no vascular connections with the underlying parental tissue (George, 1996). Somatic embryos and zygotic embryos are structurally similar, although somatic embryos do not have an orderly pattern of cell division, which is probably due to the different environmental conditions the cells are exposed to. Stages through which dicotyledonous somatic embryos develop are described as follows (George, 1996):

1) Pro-embryo stage: Small cluster of meristematic cells.
2) Globular stage: These are larger groups of cells, which are yet to have a definite embryo-like shape.
3) Heart stage: The cotyledonary initials are separated from the root pole.
4) Torpedo stage: An elongated form of the heart shape.
5) Cotyledon stage: Small seedling with cotyledons and root.

1.2.3.2 Selection of explant

Various parts of a plant can be used to induce somatic embryogenesis in culture. Direct embryogenesis has been induced on explants derived from seedlings or more mature organs, and zygotic embryos or their component parts. Examples of explants derived from young seedlings include: Petioles in *Apium graveolens* (Zee and Wu, 1980), cotyledonary node of *Corylus avellana* (Pérez, Fernandez and Rodriguez, 1983) and cotyledons of *Manihot esculenta* (Stamp, 1987). Embryogenesis induced on explants derived from mature organs include: leaf discs of *Amaranthus hypochondriacus* (Flores, Thier and Galston, 1981), mechanically isolated mesophyll

cells of *Asparagus officinalis* (Urigami, Sakai and Nagai, 1990), leaf midribs of *Dendranthema grandifolia* (May and Trigiano, 1991), and mesophyll protoplasts of *Medicago sativa* (Dijak and Simmonds, 1988). Examples of direct embryogenesis from zygotic embryos and their component parts include: Scutellum of *Sorghum bicolor* immature embryos (Thomas, King and Potrykus, 1977), immature zygotic embryo of *Brassica napus* (Pretova and Williams, 1987), cotyledons from *Camellia japonica* embryo (Kato, 1989), immature zygotic embryo of *Anacardium occidentale* (Gogate and Nadguada, 2003), and mature zygotic embryo of *Hyoscyamus niger* (Tu, Sangwan and Sangwan-Norreel, 2005).

Indirect embryogenesis has been successfully induced in numerous plant species such as: the immature zygotic embryos of *Mangifera indica* (Litz, Knight and Gazit, 1984) and *Glycine max* (Finer and Nagasawa, 1988), immature zygotic embryos of *Quercus* (Gingas and Lineberger, 1989), suspension cultures of *Pinus strobus* (Finer, Kriebel and Becwar, 1989), cell suspensions of *Ipomoea batatas* (Chée and Cantliffe, 1989), cell cultures of *Betula pendula* (Kurtén, Nuutila, Kauppinin and Rousi, 1990), shoot apices of *Pisum sativum* (Kysely and Jacobsen, 1990), and cell suspension cultures of *Prunus Persica* (Raj Bhansali, Driver, and Durzan, 1991).

Somatic embryos have also been obtained from haploid organs such as the pollen, anther and unfertilized ovules. Research has shown that pollen and anther culture can be induced to give rise to vegetative cells instead of pollen grains (George, 1996). The normal development pattern of the pollen is changed, and instead of the pollen producing gametes and a pollen tube, microspores are produced, which are capable of forming callus tissue and haploid pro-embryos. Formation of plantlets from pollen microspores is called androgenesis. Guha and Maheshwari (1964) regenerated haploid plants from pollen of *Datura innoxia* using intact anthers. Nitsch and Nitsch (1969) subsequently obtained pollen-derived embryos in *Nicotiana*. Batty and Dunwell (1989) reported that more pollen-derived embryos were produced when potato anthers were cultured in maltose than in sucrose.

Another method of obtaining haploid plants is to use unfertilized ovules, ovaries or flower buds. Such a method is referred to as gynogenesis (George, 1996). Gynogenesis was achieved in *Beta vulgaris* using unfertilized ovules (Doctrinal,

Sangwan and Sangwan-Norreel, 1989). Somatic embryos derived from unfertilized ovules were also obtained in onion (*Allium cepa*), where young developing embryos sprouted from split ovules (Campion and Alloni, 1990). Other examples of haploid plantlets produced include: ovules of *Gerbera jamesonii* (Sitbon, 1981; Meynet and Sibi, 1984), ovules of *Beta vulgaris* (Hosemans and Bossoutrot, 1983) and ovaries of maize (Truong-Andre and Demarly, 1984),

1.2.3.3 Growth media, hormone supplements and culture conditions

From extensive research carried out on somatic embryogenesis on a wide range of plants, a few general rules for the induction of somatic embryos have been written and are often applied. Nitrogen, usually in reduced form such as ammonium salts, is needed during embryo initiation and maturation (Razdan, 1993). Other nutritional additives, which contain various forms of nitrogen, such as coconut milk, casein hydrolysate and amino acid are also used in the growth medium. Potassium is also known to promote embryogenesis, particularly if nitrogen is restricted (Pierik, 1987). Although numerous types of basal media have been used to induce somatic embryogenesis, the MS medium or modified versions thereof, are the most commonly used media (George, 1996). The presence or the lack of certain macronutrients in the media is often essential for the induction and development of somatic embryos. For example, Reynolds (1990) reported that calcium was needed to induce somatic embryos in horsenettle pollen. Also, Walker and Sato (1981) showed that alfalfa embryos did not develop in the absence of ammonium or nitrate.

The most common type of auxin used to induce somatic embryogenesis is 2,4-D, although other auxins such as NAA, IBA and IAA are also used (George, 1996). The type of auxin and the concentrations used vary greatly, depending on the type of plant species. In direct embryogenesis, where explant tissues are embryogenically determined, it may not be necessary to add growth regulators to obtain embryos. In addition, in the case where spontaneous somatic embryos have been formed directly from *Citrus sinensis* nucellus tissues, it is thought that the nucellus tissue is auxin-habituated, since the addition of auxin depressed embryogenesis (Button, Kochba and Bornman, 1974). Furthermore, in certain cases the competence of cells, which become embryogenically determined, is increased with the addition of auxin.

Numerous papers have shown that direct embryogenesis is induced without any growth hormones: *Camellia reticulata* (Plata and Vieitez, 1990), *Citrus* (Rangan, Murashige and Bitters, 1968; Gmitter and Moore, 1986), *Daucus carota* (Smith and Krikorian, 1988), and *Mangifera indica* (Litz, Knight and Gazit, 1982). Many reports state that auxin is required for the induction of indirect somatic embryogenesis. Generally, auxin is required in the growth medium to induce embryogenic callus. In addition, it has also been established that continuous exposure of embryos to auxin can be detrimental to their development (Merkle, Parrott and Flinn, 1995). Halperin and Wetherell (1964) first observed that maintaining carrot embryos at the globular stage in 2,4-D inhibited their development and led to abnormal growth. Similar observations were made in *Cronilla varia* (Dusková, Opantrny, Sovová and Dusek, 1990).

Other common growth hormones used in somatic embryogenesis include cytokinin, ABA (abscisic acid) and GA_3. Cytokinins such as kinetin are often added with auxin to the media for the induction of somatic embryogenesis (George, 1996). In embryogenic-determined explants, the addition of cytokinins only is sometimes sufficient to induce somatic embryos. For example, only the addition of BAP was required to induce somatic embryos in *Pelargonium* (Marsolais, Wilson, Tsujita and Senaratna, 1991) and *Trifolium* (Maheswaran and Williams, 1985; Maheswaran and Williams, 1986). Abscisic acid is normally used in the later stages of indirect embryogenesis for maturation and germination of embryos. In most experiments, ABA has been reported to inhibit somatic embryo formation. However, Qureshi, Kartha, Abrams and Steinhauer (1989) reported that ABA promoted the production of embryogenic callus and suppressed precocious germination in zygotic embryos of wheat. Gibberellic acid has also, in most cases been found to suppress embryogenesis, although Shekhawat and Galston (1983) and Mehra and Sachdeva (1984) used gibberellic acid to induce embryogenesis in *Vigna aconitifolia* and *Malus domestica*, respectively.

The growth condition requirements of somatic embryos are dependent on the plant species (George, 1996). However, light is generally needed to promote embryogenesis, although various levels of irradiance ranging from low to total darkness have been reported to be critical in some species. Similarly, the temperature

requirements for the growth of somatic embryos are also species-dependent. Although high temperatures are normally favourable for embryogenesis, certain types of cultures, such as anther cultures, require a cold shock to initiate the formation of embryos (Pierik, 1987).

Somatic embryogenesis has not been reported in *P. cynaroides*. In other proteas, very few research papers have been published. An early study by Van Staden and Bornman (1976) obtained initiation and growth of *Leucospermum cordifolium* callus. In addition, callus and proteoid rootlet formation were obtained on cotyledonary explants of *Protea neriifolia*, but attempts to induce adventitious root and shoot development were unsuccessful (Van Staden, Choveaux, Gililand, McDonald and Davey, 1981). Somatic embryogenesis was, however, achieved in *P. repens*, where somatic embryos formed directly on the base of shootlets and callus, of which some developed into plantlets (Rugge, 1995). Chapter 4 reports the regeneration of *P. cynaroides* plantlets from cotyledon and zygotic explants through direct somatic embryogenesis.

1.2.4 Organogenesis

Various parts of intact plants grown *in vitro* on nutrient media can form new shoots, root and even flower initials without prior growth of callus tissue, i.e. direct organogenesis (George, 1996). Juvenile tissues of explants derived from the germination of zygotic embryos often form shoots readily. However, organogenesis is highly dependent on the morphogenetic potential of the plant species concerned, as well as the organ part from which organogenesis takes place. Adventitious shoot buds are often derived from cotyledons, leaves, roots and stems. Some explants have the capacity to regenerate shoots directly, without the addition of growth regulators, although the addition of these growth regulators usually increases the regeneration rate. In most cases, growth regulators are essential for the formation of adventitious shoots and roots. Examples of direct organogenesis where shoot formation were obtained include: *Glycine max* where cotyledonary nodes were used (Saka and Cheng, 1980), and leaf segments of *Petunia* sp. (Economou and Read, 1981). Adventitious shoots have also been reported to derive from root explants. For example, *Rubus* sp.

(Borgman and Mudge, 1986) and *Nicotiana* spp. (Zelcer, Soferman and Izhar, 1983) were induced to form shoots from root cultures.

Indirect organogenesis can occur from callus tissue that has been maintained in culture for a prolonged period of time. As in the case with direct organogenesis, the effective concentrations of growth regulators such as auxin and cytokinin vary from one plant species to another. There are two ways in which indirect organogenesis can arise (George, 1996): 1) On callus tissue which has been produced on the original explant, or 2) on callus tissue in which morphogenesis capacity has been induced, which forms plant organs once it is transferred to another medium.

Root initiation can occur simultaneously with shoot formation, although shoots are often excised from the original explant or callus, and rooted separately in rooting media. Generally, adventitious root formation is promoted when the ratio between auxin and cytokinin is greater than one. However, the capacity of tissues to produce endogenous hormones, or to metabolize exogenous growth regulators, differs. Therefore, certain callus tends to produce shoots and not roots, and *vice versa*. Indirect organogenesis have been reported in *Citrus grandis*, where seedling stem and leaf tissues were induced to form callus, which were subcultured to new media to form adventitious shoots (Chaturvedi and Mitra, 1974). Similarly, hypocotyls of young *Brassica* spp. were continuously subcultured to form callus tissue, on which adventitious shoots were initiated (Dietert, Barron and Yoder, 1982). Shoot apical meristems of *Chrysanthemum morifolium* (Sangwan and Harada, 1977), ovaries of *Freesia hybrida* (Bach, 1987) and stem segments of *Camellia* hybrids (Tosca, Pandolfi and Macchi, 1992), were induced to form callus and shoots.

Currently, methods to multiply *P. cynaroides* explants are limited. Preliminary studies of plantlet regeneration through direct multiple shoot-bud development of *P. cynaroides* explants are reported in Chapter 4, with the view to promote an alternative propagation method.

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