HYPERHYDRICITY OF *IN VITRO* CULTURED STURT'S DESERT PEA (*Swainsona formosa*) AND TECHNIQUES FOR ITS MINIMISATION

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Sturt's Desert Pea

(Swainsona formosa)

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Declaration

The work presented in this thesis contains no material which has been accepted for award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and my belief, contains no material previously published or written by another person, except where due reference is made in the text.

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Summary

Swainsona formosa (Sturt's desert pea) is the subject of this study. A member of the family Fabaceae, it has brilliant flowers of varied colour, and has excellent potential as a cut flower and flowering pot plant. Until now, propagation has been by seeds or cuttings. However, seedlings lack genetic uniformity, and cuttings require large numbers of stock plants and the method is time consuming.

Tissue culture techniques offer the most efficient method for producing large numbers of plants quickly, but have produced high rates of hyperhydricity (more than 50 % of cultures). Remedial techniques, such as increasing agar concentration, varying the support matrix, improving aeration and reducing cytokinin concentration have been examined in other species and have given satisfactory results.

In this study, the effects of agar concentration, support matrix, tube closure and 6- benzyl aminopurine (BAP) regime on hyperhydricity and other growth parameters were examined. A high gel concentration, and a tube closure with high gas exchange rate effectively minimised hyperhydricity and increased shoot vigour, but reduced the rate of proliferation. To overcome this problem, these treatments were tested together with different concentration of, and exposure time to, 6-benzylamino purine (BAP). The treatment combination that gave optimal growth and minimised hyperhydricity was 10 g/l agar (Sigma Chemical Company) together with low rate of BAP (1 - 5 μM) and semipermeable closures which allowed some gas and water vapour exchange (eg. cap with a hole fitted with a double layer of Whatman paper number 1).

To understand more clearly how hyperhydricity develops, the anatomy of hyperhydric shoots was studied. Abnormal tissue in between the two guard cells in the stomata, a low density of stomata, unclear differentiation of structural components of the meristem, and a large starch granule and reduction in numbers of thylakoid stacks in the chloroplast were found in hyperhydric shoots of Sturt's desert pea when compared to in vitro normal shoot.

This thesis describes, therefore, the establishment of *in vitro* tissue culture techniques for minimisation of hyperhydricity and maximisation of growth, together with an anatomical study of hyperhydricity in *Swainsona formosa*.

Chapter 1. Introduction and Literature Review

1.1. Introduction

Sturt's desert pea, *Swainsona formosa* (G. Don) J Thompson (syn. *Clianthus formosus)*, a plant native to Central Australia, from Western Australia through to New South Wales, is distributed in arid areas on sandy soil over limestone.

This plant trails up to 2 m in length with pinnate and glaucous leaves covered by silky hairs giving the plant a grey green colour. The large flower consists of an upper standard petal (or flag) with a shiny black boss and a lower keel housing the sexual organs. Besides the classic red flower with a black centre, the natural populations show broad colour variation (Kirby, 1990).

Sturt's desert pea is one of the world's most spectacular flower plants (Bridgeman, 1990) and has potential as a cut flower or as a flowering pot plant (Barth, 1990). Therefore, growers have given special attention to propagation of this flower commercially. However, difficulties have been found in the cultivation, production and marketing of the plant. Several laboratories have initiated studies to overcome these difficulties. Studies on the development of the plant for cut-flower and pot production are occurring at Black Hill Flora Centre (Botanic Garden of Adelaide), at Flinders University and at the University of New England (Jusaitis and Schmerl, 1993).

Propagation of Sturt's desert pea has been by seeds, cuttings and tissue culture. However, seed propagated plants were very variable in a range of characteristics, and limited vegetative material is available for propagation by cuttings. Therefore, propagation by tissue culture seems to offer the most efficient method of increasing plant number. However, research has found that hyperhydricity is a serious problem in tissue culture propagation of Sturt's desert pea (Jusaitis and Schmerl, 1993). One aim of this study was to develop suitable conditions to minimise hyperhydricity in Sturt's desert pea tissue culture propagation.

To understand more clearly how hyperhydricity develops, some researchers have looked at the anatomy of hyperhydric plants. Abnormal tissue structures such as

reduced meristematic zones in the shoot apex (Leshem, 1983a) and abnormal shape and size of stomata (Werker and Leshem, 1987) have been found in various species (see below, section 1.2.3). The second aim of this study was the anatomical examination of hyperhydric shoots of Sturt's desert pea cultures.

1.2 Literature Review

1.2.1 Propagation of Sturt's desert pea

1.2.1.1 Propagation by seed

Sturt's desert pea and other members of the Fabaceae produce seeds with a hard coat impermeable to water (McIntyre and Veitch, 1972). The impermeability of the coat is due to the presence of tightly packed layers which consist of malphigian or macroscleroid cells (Cavanagh, 1987). Because of impermeable seed coats, they need a long time to germinate in the natural state. Therefore, this hard coat needs to be weakened or broken. Many treatments have been tested to stimulate germination of seeds e.g., pricking the seed coat, rubbing seeds between two sheets of sand paper, soaking them in sulphuric acid or pouring boiling water over them.

McIntyre and Veitch (1972) investigated the influence of boiling water and other methods of scarification on the germination of Sturt's desert pea seeds. They found that scarification could yield almost 100% germination. This result was supported by Jusaitis and Schmerl (1993) who germinated seeds after scarifying with just-boiled water. They found that scarification could give almost 100% germination in less than 10 days. They suggested that manually nicking the seed coat with a sharp blade was the best method to stimulate seed germination if only a small quantity of seedlings were required. Soaking seeds in just-boiled water was the recommended technique for a large quantity of seeds. Rubbing the seeds lightly between two sheets of medium-grade sand paper was the method used by Bridgeman (1990) but in his he did not report the success of this method. All of these treatments above could be effective in breaking dormancy

in Sturt's desert pea. Scarification or rubbing, for instance, remove macroscleroid layers which are the main barrier for water infiltration required in germination. Moreover, soaking the seeds in just-boiled water separates macroscleroid cells and pops out the strophiole plug, permitting water to penetrate (Dell, 1980; Bewley and Black, 1978).

Because Sturt's desert pea is extremely variable in most of its characteristics when grown from seed, nurserymen have had difficulty producing uniform flowers for the cut flower trade. Therefore vegetative methods are required to produce uniform, clonal material. These techniques will be discussed in the next sections.

1.2.1.2 Propagation by cuttings

Propagation of Sturt's desert pea from cuttings allows the propagator to maintain stock material and propagate uniform plants from this stock. Williams and Taji (1987) used lengths of softwood stem two or three nodes long taken from pot grown seedlings. They found that 70% of cuttings pretreated with indolebutyric acid (IBA) + α-naphthaleneacetic acid (NAA) (20 + 20 mM) produced roots after 15 days compared to other treatments {10 mM IBA or IBA + NAA (10 + 10 mM)} which gave a poor root production. Moreover, Jusaitis and Schmerl (1993), found that a 5 second basal dip in IBA + NAA (5 + 5 mM) followed by incubation in perlite over bottom heat produced significantly more and longer roots after 4 weeks than did controls.

While cutting propagation overcomes some of the problems inherent in seed germination, in that uniform progeny are produced, its use as a breeding and selection tool is limited because vegetative cutting material is severely limited on a mature flowering plant, and the procedure is too slow to mass-produce large quantities of plants.

1.2.1.3 Propagation by tissue culture

1.2.1.3.1 Introduction

In vitro culture or tissue culture is a term used to describe the growth and manipulation of plant cells, tissues or organs under controlled nutrient conditions in an artificial environment (Scowcroft, 1984). Tissue culture of plant cells was first attempted by Haberlandt in 1902 (Thomas and Davey, 1975) to test ideas of cellular totipotency (capability of a single cell to regenerate a whole organism).

Since this initial experiment, various types of cells and organs from a number of plants have been grown *in vitro* using solid or liquid culture media (D'Amato, 1977). The steps involved in plant tissue culture were outlined by George and Sherrington (1984) and included preparation and selection of the mother plant, provision of a sterile culture, the production of suitable propagules, adjustment to the natural environment and transfer to the natural environment. Furthermore, they stated that plants could be produced in culture by multiplication of shoots from axillary buds or by formation of adventitious shoots, by either direct morphogenesis (plantlet formation from the differentiated cells of a piece of whole plant tissue, without proliferation of undifferentiated tissue) or indirect morphogenesis (explants are produced by an intermediate stage of callus formation).

One disadvantage of indirect morphogenesis is that genetic variability (somaclonal variation) may increase during the callus stage (Stafford, 1991). However, both techniques may be applied by tissue culturists depending upon their goals. A plant breeder aiming to broaden genetic variability for selection purposes may use indirect morphogenesis. On the other hand, plant propagators prefer to use direct morphogenesis from meristematic tissues to propagate their plant selections to ensure clonal stability. Stafford (1991) explained that the genetic stability of the meristem results from two factors. Firstly, the meristem has a higher efficiency of DNA repair mechanisms compared with disorganised tissues, and therefore the chance of mutation is very low.

Secondly, the axillary meristem consists of two or more multicellular layers which have a complex structure. Mutation of entire tissue layers is a rare event.

Many experiments have demonstrated that tissue culture offers the most efficient method of supplying a large volume of selected plant stock. In addition, by applying tissue culture propagation, stock material can be maintained, virus and other systemic pathogens can be eliminated, and plants produced regardless of season.

As well as a plant propagation and plant breeding tool, tissue culture has broader applications in plant pathology and in the production of secondary metabolites. Moreover, somatic hybridisation of sexually incompatible species and genetic engineering of plant cells could be performed on cells grown in culture (Thomas and Davey, 1975). Two major areas of biotechnology related to tissue culture which have commercial importance, are micropropagation and the production of plant biochemicals including enzymes and secondary metabolites (Allan, 1991). Increasingly, protoplast culture is also playing an important role in plant biotechnology in allowing the manipulation of individual cells through cell fusion, selection and transformation (Stafford, 1991). The production of transformed or transgenic plants using *Agrobacterium* vectors, for instance, depends upon *in vitro* technique (Walden, 1993).

1.2.1.3.2 Tissue culture of Sturt's desert pea

In vitro regeneration of legumes has been reported to be quite difficult. Media suitable for other plants do not necessarily extrapolate for use on legumes, which have been observed to require higher concentrations of cytokinins (Flick *et al.*, 1983). However, some success has been achieved in culture of some members of the Fabaceae family.

Rubluo and Kartha (1985), working with 3 species of *Phaseolus,* found that 65 % of *Phaseolus vulgaris* shoot apical meristem explants produced shoots when cultured on media supplemented with 6-benzylamino purine (BAP) + indoleacetic icid (IAA) or

IBA (10 μM + 10 μM). Moreover, they stated that *P. vulgaris, P. coccineus* and *P. lunatus* varied in their shoot proliferation responses. In other research, experiments with meristems of soybean, cowpea, bean, peanut and chick pea showed that these plants could be regenerated only when agar-solidified MS medium (Murashige & Skoog, 1962) was supplemented with BA + NAA (0.05 - 0.1 μM + 1 μM, 0.005 - 0.1 μM + 0.05 μM, 10 μM + 0 μM, 0.1 μM + 10 μM and 0.1 μM + 10 μM, respectively) (Kartha *et al.*, 1981). These results suggested that each plant contained different levels of endogenous hormone (auxin and cytokinin). Therefore, exogenous growth regulator requirements are quite specific for each plant species.

Tissue culture of Sturt's desert pea using different concentrations of growth regulators, it was found that highest callus formation was achieved from seedling hypocotyl explants cultured on half-strength De Fossard's medium plus 20 μM BAP (6-benzylamino purine) (Williams and Taji, 1987). Moreover, 20 μM NAA or NAA + IBA induced optimal root formation. Furthermore, a period of darkness increased rooting. Further experiments by Taji and Williams (1989) showed similar results, but after 30 days culture, 60% of cultures displayed the morphological and physiological disorder known as hyperhydricity (vitrification).

In vitro propagation of apical shoots of Sturt's desert pea seedlings which were grown in De Fosard medium solidified using 16 g/l agar (Difto Bacto) plus 1 μM kinetin and 1 μM BAP yielded the highest shoot proliferation rate (Matthews, 1991). Moreover, the proliferated shoots, which were treated with combination of 1 μM IAA, IBA, naphthoxyacetic acid (NOA), 2,4-dichlorophenoxyacetic acid (2,4-D) and parachlorophenoxyacetic acid (pCPA) in De Fossard medium solidified with 16 g/l agar (Difto Bacto), produced roots in 18% of cultures. Again, over 40% of cultures showed hyperhydricity (Matthews, 1991).

From a breeding point of view, the above experiments seem gave less benefit because all experiments used seedlings for explants. In this case, complete phenotypes cannot be known before the plant matures. Selection can only occur after a plant

matures, and therefore an explant from a mature plant (axillary bud) is required. Unfortunately, all new buds on adult plants are flower after flower initiation (Jusaitis and Schmerl, 1993), therefore only a few explants can be collected from each plant (from axillary buds below the first flower).

Propagation of Sturt's desert pea from axillary and apical buds using various concentrations of nutrients, auxin, cytokinin and growth factors showed that a low level of auxin and a high level of cytokinin in combination with a high level of nutrients and a low level of growth factor showed optimal shoot formation, but the tendency for hyperhydricity to occur increased with increasing time in culture (Jusaitis and Schmerl, 1993).

The experiments above show that hyperhydricity is a serious problem in propagation of Sturt's desert pea using tissue culture. Therefore, hyperhydricity will be discussed in detail in the next section.

1.2.2. Hyperhydricity

1.2.2.1 Introduction

Hyperhydricity (or vitrification, see Debergh *et al.*, 1992) is a physiological disorder frequently affecting herbaceous or woody plants during *in vitro* vegetative propagation (Kervers *et al.*, 1984; Gaspar, 1991).

Symptoms of hyperhydricity are not identical in all plants (Debergh *et al.*, 1992). Generally, shoots have shortened or non-existing internodes, and stem diameter may be enlarged. Leaves can be thickened, elongated, wrinkled and/or curled, brittle, and translucent with a reduced or hypertrophied surface (Debergh *et al.*, 1981).

Hyperhydric plants have been reported to have increased water content, decreased dry weight (Kevers *et al.*, 1984), reduced lignin, cellulose and soluble phenolic compounds content (Kevers *et al.*, 1984) and less chlorophyll (Gaspar, 1991). The additional water of hyperhydric tissues is located in the extra-protoplastic space

(Gaspar, 1991). The reduction of lignin content is associated with decreased activity of enzymes involved in the synthesis and polymerisation of lignin precursors. These enzymes are phenylalanine ammonia lyase (Kevers *et al.*, 1984), hydroxycinnamate CoA ligase, coniferyl alcohol dehydrogenase (Letouze and Daguin, 1987 in Gaspar, 1991), acidic and wall peroxidases (Kevers *et al.*, 1984), flavonone synthase and hydroxycinnamate CoA reductase (Hegedus and Phan, 1987 in Gaspar, 1991). The reduction of cellulose content is associated with a low C/N ratio favouring the synthesis of amino acids rather than sugar units for cellulose (Kevers *et al.*, 1984). Photosynthetic tissues of hyperhydric plants have less chlorophyll than normal plants, so are translucent and have a reduced photosynthetic capacity (Leshem, 1983b). Factors affecting hyperhydricity and ways to minimise hyperhydricity have been studied in various species.

1.2.2.2 Factors affecting hyperhydricity

While a number of factors affect hyperhydricity, some factors will only induce it if other conditions in the culture system are less than optimal (Debergh *et al.*, 1992). Factors involved in the induction and the control of hyperhydricity can be classified under the categories of explant, medium, container and environment (Debergh *et al.*, 1992).

Little information on the influence of explant type on hyperhydricity is available. According to Debergh *et al.* (1992), each plant species or cultivar has its specific susceptibility to hyperhydricity. Moreover, they stated that the way the explant was dissected, the position of the explant on the medium and damage during disinfection were important factors in influencing degree of hyperhydricity.

Hyperhydricity occurred more frequently in liquid than on solidified media and on solidified media with low rather than high concentrations of gelling agent (Debergh *et al.*, 1992). Increased agar concentration was effective in reducing hyperhydricity in

carnation meristem tip culture (Hakkaart and Versluijs, 1983; Ziv *et al.*, 1983), apple shoot tip culture (Pasqualetto *et al., 1988*), *Picea abies* adventitious shoot culture (Von Arnold and Eriksson, 1984) and globe artichoke culture (Debergh, 1983). However, the increased gel concentrations tended to reduce the number of adventitious buds and shoots and reduce the height of plantlets (Hakkaart and Versluijs, 1983). Furthermore, the brand of agar as well as its concentration clearly affected the solidity of the gels (Debergh, 1983).

Increased levels of cytokinin tended to increase the percentage of hyperhydricity in *Cucumis melo L.* cultures (Leshem *et al.*, 1988). A similar result was obtained in the culture of globe artichokes, where the frequency of hyperhydricity was decreased by lowering the cytokinin concentrations in the medium with a 6 g/l agar (Difto Bacto) content (Debergh, 1983). The interaction between cytokinins, gel concentrations and temperatures in hyperhydricity of *Olearia microdisca* shoot cultures was reported. The increase in gel concentrations or the reduction in temperatures with the presence of 20 μM BA reduced hyperhydricity (Williams and Taji, 1991). Other growth regulators such as auxin had only minor effect on hyperhydricity in *Camellia sinensis, Gerbera jamesonii, Malus domestica* and the hybrid *Populus tremula x P. alba* (Kataeva *et al.*, 1991).

Many studies show that the increase in gel concentration tends to reduce humidity in culture vessels and lowering cytokinin concentration tends to reduce the activity of cellular division. Both factors are postulated as important in controlling hyperhydricity. These reports were supported by Kataeva *et al.* (1991), who stated that there are two key factors involved in the induction of hyperhydricity. They are the physiological state of the shoot apex (active cellular division due to elevated content of internal cytokinins) and the high relative humidity in the culture vessel.

Screen rafts and water absorbent resins are other treatments used to reduce hyperhydricity. Filter paper rafts and screen rafts of 1 and 60 μm pore size were found to be suitable for lettuce growth in culture and completely normal shoots were achieved

by replacing agar with the water - absorbent resin Super Sorb (Teng and Liu, 1993). In research on *in vitro* cultures of carnation shoot apices, an increase in shoot proliferation and decreased translucency were achieved by culturing apices for 5 and 10 days on filter paper bridges in stationary liquid medium followed by subculture to 1.5 % agar (no specification) medium and 3 % sucrose (Ziv *et al.*, 1983). It seems that suitable humidities for culturing lettuce and carnation were achieved by placing screen and paper rafts on the top of media and replacing agar with resin in lettuce cultures and filter paper bridges followed by agar media in carnation cultures.

Another way of developing suitable conditions in culture vessels is by selecting or modifying the container. An aeration system using a Whatman filter paper covered hole in vessel lids has successfully minimised hyperhydricity in rare Australian plants which normally showed a high hyperhydricity rate (Rossetto *et al.*, 1992). Cotton wool, metal caps, aluminium foil and parafilm instead of normal lids have also been tested (Hakkaart and Versluijs, 1983). Cotton wool, metal caps and were effective in reducing hyperhydricity compared with aluminium foil and parafilm. These closures seemed to enhance gas exchange, thereby maintaining suitable humidity in the culture vessel.

Environmental conditions are amongst the most important factors controlling hyperhydricity. Cold treatment (10 days at 5^{o}C) was shown to reduce hyperhydricity from 68 to 22% in Sturt's desert pea cultures (Taji and Williams, 1989). In another experiment with *Olearia microdisca* (Williams and Taji, 1991), found that hyperhydricity could be reduced to about a third of that at room temperature by maintaining the culture at 5^{o}C for 6 weeks. Cold treatment seemed to produce its effect on hyperhydrity by reducing cytokinin uptake. Another possibility was that cold treatment promoted subsequent lignin synthesis, thus reducing hyperhydricity (Phan and Hegedus, 1986).

In further studies on the development of hyperhydricity and the causes of abnormal organ function, some researchers have investigated the relation between

structural changes and malfunction of hyperhydric tissue in various plant species. These studies will be discussed in the next section.

1.2.3 Anatomy of hyperhydric plants

Research on the anatomy of hyperhydric plants, mainly leaves and stems (including the shoot apex), has been reported in a number of species and various types of organs and tissues. In general, many of the alterations in structures were similar for all species. However, each species also exhibited different specific abnormalities in the same organs. The anatomy of the stoma, cuticle, trichome and mesophyll of hyperhydric leaves has been studied.

Studies of stomata, which are mainly responsible for transpiration and gas exchange (CO_2 and O_2) in plants (Salisbury and Ross, 1992), showed abnormalities in hyperhydric leaves in various species. Stomata in hyperhydric leaves of carnation, soybean and chicory were occluded, closed and distorted, respectively (Yassen *et al.*, 1992). Miguens *et al.* (1993) found three kinds of abnormal stomata on hyperhydric *Datura insignis* leaves. First, those where both guard cells had projections in the extremity of their external anticlinal walls that occlude half of the pore. Second are those with a round protuberance of external anticlinal cell wall in one or both guard cells. The third was where the projections occurred in the external periclinal side of guard cells. Stomatal abnormality seemed to be due to a deformation of the cell or erroneous deposition of the cell wall constituents. Stomata which did not respond to stimuli were reported in hyperhydric leaves (Ziv *et al.*, 1987). This was apparently due to abnormal properties of the guard-cell wall affecting the elasticity of guard cells and resulted in inefficient water regulation and transpiration (Yassen *et al.*, 1992). The density of stomata in hyperhydric plants reportedly varied greatly from one plant to another (Werker and Leshem, 1987) but Miguens *et al.* (1993) found no significant

difference in stomatal density between normal and hyperhydric leaves of *Datura insignis.*

The cuticle also plays a role in control of transpiration in plants (Salisbury and Ross, 1992). Studies of cuticles in tissue-cultured carnation found that the cuticle was thin and non-continuous (Leshem, 1983) and this may explain why hyperhydric shoots had low survival upon transplanting due to increased water loss and desiccation. Ziv *et al.* (1987) also suggested that two main causes for desiccation were the lack of cuticular waxes and the non-functional stomata. In contrast, Yassen *et al.* (1992) found that hyperhydric soybean leaves had more epicuticular waxes than normal leaves.

There is little information about the anatomical structure of trichomes in hyperhydric leaves. According to Miguens *et al.* (1993), there were no significant differences in trichome density and appearance between normal and hyperhydric leaves of *Datura insignis* .

Mesophyll, especially palisade parenchyma where chloroplasts are located, plays an important role in photosynthesis. An anatomical study of hyperhydric leaves of *Conostylis wonganensis* showed loosely connected mesophyll cells and poorly developed palisade layers (Rossetto *et al.,* 1992). Increases in intercellular spaces of mesophyll were also found in *Picea abies* (Von Arnold and Erriksson, 1984) and *Olearia microdisca* (Williams and Taji, 1991). Moreover, hyperhydric chestnut displayed a lack of clear differentiation between the palisade and spongy mesophyll tissue (Vieitez *et al.,* 1985). The abnormality of the mesophyll is apparently due to the excess water in the culture environment. It is known that the internal structure of leaves can vary in response to environment. The correlation between mesophyll and relative humidity can be seen in many hydrophytes which have undifferentiated mesophyll and aquatic plants which have aerenchyma in the mesophyll (Vieitez *et al.*, 1985).

Anatomical studies of the cambium, shoot apex and vascular bundles of hyperhydric plants have also been reported in many species. These are the regions responsible for growth and differentiation. Hyperhydric stems of *Olearia microdisca*

showed an enlarged cortex of irregular cells, and reduced cambial activity (Williams and Taji, 1991). Moreover, in a study of structural changes during hyperhydricity of carnation plantlets, a reduction in the period during which cells remained meristematic in the developing parts of the shoots was found to be associated with hyperhydricity. The early maturation of the meristem may be the cause of various structural changes, such as shorter stems (Werker and Leshem, 1987). Hyperhydric carnation plantlets showed fewer vascular bundles, which lacked the typical arrangement of normal shoots (Leshem, 1983). Moreover, reduced lignification was found in vascular bundles (both in xylem and phloem fibres) of *Olearia microdisca* (Williams and Taji, 1991). Less differentiated xylem and sclerenchyma tissue occurred in hyperhydric chestnut stems (Vieitez *et al.*, 1985). The reduction of lignification seems to be due to the reduction of enzyme activity (see section 1.2.2.1).

All of the above studies have shown abnormality in the anatomy of various hyperhydric tissues (e.g. abnormal stomata, thin cuticle layer, disorganised vascular bundles). Such conditions affect the function of organs and the growth of plants, and thus reduce the survival rate of hyperhydric shoots when transferred to soil.

1.2.4 Summary

The commercial propagation of Sturt's desert pea has historically been from seed or cuttings. However, uniform and large quantities of flowers required for commercial purposes could not be achieved by these methods. Tissue culture propagation seems to offer the most efficient method of providing uniform flowers in large quantities. However, hyperhydricity is a serious constraint in the propagation of Sturt's desert pea using the tissue culture method.

Factors involved in hyperhydricity were categorised into explant, medium, container and environment. Some researchers have attempted to minimise hyperhydriciy in various species by developing suitable media and containers for

culture. Varying gel concentrations, BAP concentrations, tube coverings and support matrices have been successful in minimising hyperhydricity in a range of species.

1.2.5 Research aims

The objectives of this project were two fold: firstly, to develop suitable conditions to minimise hyperhydricity in Sturt's desert pea with remidial treatments in developing suitable agar concentration, tube closure and 6 - benzyl aminopurine (BAP) regimes; and secondly, to investigate the anatomical changes which occur in hyperhydric explants.

Chapter 2. Materials and Methods

2.1 Tissue culture techniques

2.1.1 Plant material

Explants were taken from 10 weeks old Sturt's desert peas (*Swainsona formosa)* grown from seed in a temperature-controlled glass house at Black Hill Flora Centre (see Jusaitis & Schmerl (1993) for cultural conditions). The plants formed part of the fourth generation of breeding and selection trials.

2.1.2 Media and tissue culture conditions

The basal medium used in all experiments was full strength MS (Murashige and Skoog (1962) plus 12 g/l agar (Sigma Chemical Company), 30 g/l sucrose and 5 μM 6 benzylamino purine (BAP). The agar, sucrosa and BAP were edded to MS medium after MS medium solved in distilled water. Media were than adjusted to pH 5.5 before being transferred to 30 ml polycarbonate tubes (10 ml/tube). Loosely capped tubes were autoclaved at 121°C (1.2 kg cm^{-1}) for 20 minutes.

All cultures were maintained at a constant temperature of 23.5 ± 1 °C with a 16 hour photoperiod (cool white fluorescent light, 50 μMol m^{-2} s^{-1}). All tissue culture experiments were conducted in the laboratories at the Black Hill Flora Centre.

2.1.3 Preparation of explants

Meristems from axillary buds of stock plants were dissected under a microscope using fine forceps to remove leaflets, hairs and bracts surrounding the apical dome as described by Jusaitis and Schmerl (1993) for preparing explant stocks. The apical dome and adjacent leaf primordia were excised using a sharp, flattened, bent-tipped needle (sterilised in 70% ethanol and flamed) and deposited immediately onto the surface of the culture medium (one explant per vial). Using this technique, sterile explants could be

obtained without tissue disinfection, because the meristem was well protected by its surrounding leaves.

Contaminated explants were discarded after initial culturing on basal medium for one week, and uncontaminated explants were transferred to treatment media. All tissue transfers were done aseptically in a laminar flow cabinet.

2.1.4 Experiment 1: Agar concentrations and tube closure treatments

The aims of this experiment were to investigate the effects of agar concentration and tube closure on growth and hyperhydricity in Sturt's desert pea cultures. Four concentrations of agar (Sigma Chemical Company) (6, 8, 10 and 12 g/l) were used to solidify basal medium plus 5 μM BAP, and five types of tube closure (PWGS Vitafilm® [1], MW Vitafilm® [1], cotton wool, polycarbonate screw caps with holes (a 7 mm diameter single hole directed through the cap and fitted with a double layer of Whatman paper number 1) and polycarbonate screw caps) were applied in a factorial experimental design. All tube coverings, except PWGS Vitafilm®, and MW Vitafilm® were autoclaved at 121°C (1.2 kg cm^{-1}) for 15 minutes before being placed on the tubes. PWGS and MW Vitafilm® were not sterilised since they were rolled strongly and proven no contamination when were used without chemical sterilisation. The experiment was set up as a randomised complete block with 5 replications. One explant was used in each replication.

Cultures were assessed after 30 and 60 days, when callus width, shoot number, average shoot length (sum of the length of all shoots divided by the total number of shoots), vigour (rated visually using the scale 1 = dead, 2 = severely necrotic, 3 = chlorotic with some necrosis, 4 = slightly chlorotic and 5 = vigorous and green) and the

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1. Vitafilm is clear plastic film manufactured by the Goodyear Tyre and Rubber Company. PWGS and MW Vitafilm have different water vapour transmission rates (400 and 461 g / m^2/24 hours respectively) and gas transmission rates (O_2: 8,835 and 14,871 cm^2/m^2/24 hours respectively; CO_2: 63,906 and 168,704 cm^2/ m^2/24 hours).

number of hyperhydric shoots were assessed. All measurements were made non-destructively from outside the tubes, taking parallax error into consideration.

2.1.5 Experiment 2: Support matrices and tube closure treatments

The aims of this experiment were to investigate the effect of various support matrices and tube closures on growth and hyperhydricity in Sturt's desert pea cultures. Five support matrices, (Oasis Horticubes® [2], Oasis Rootcubes® [2], perlite, filter paper bridges[3] and 12 g/l agar) were used to support explants. Oasis Horticubes and Oasis Rootcubes (2.5 cm diameter and 3 cm length), perlite (1 g) and filter paper bridges (1.5 cm width) were placed in the tubes and basal medium were added to the tube. Tube closures tested were identical to those used in experiment 1. A factorial randomised complete block design was replicated five times. One explant was used in each replication.

All media with support matrices and tube coverings (except PWGS Vitafilm® and MW Vitafilm®) were autoclaved before use. After 30 and 60 days, the same five parameters as for experiment 1 were measured.

2.1.6 Experiment 3: BAP concentrations, gel concentrations and tube closure treatments.

The aims of this experiment were to investigate the interaction of BAP and gel concentration and tube closure on growth and hyperhydricity in Sturt's desert pea

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2. Oasis horticubes and oasis rootcubes were growing media produced by Smithers Oasis Company, Ohio. Horticube and Rootcube retain 65 % and 70 % water respectively.

3. Paper bridges were made from filter paper (Whatman paper number one) folded so that the lower ends were in the medium and the middle region was above the medium. Explants were placed on the surface of the paper bridge, above the liquid medium.

cultures. Four concentrations of BAP (0, 0.1, 1, 10 μM) were added to basal media, two agar concentrations (8 and 10 g/l) were used to solidify media and three types of tube closure (PWGS Vitafilm®, screw caps with holes and normal screw caps) were used to cover the tubes. The screw cap with a hole and the normal screw cap were autoclaved before use. This factorial experiment was replicated five times in a randomised complete block design. One explant was used in each replication.

After 30 and 60 days, the same five parameters as for experiment 1 were measured.

2.1.7 Experiment 4: Exposure time to BAP, gel concentration and tube closure treatments.

The aims of this experiment were to investigate the effect of exposure time to BAP and its interaction with gel concentrations and tube closure on growth and hyperhydricity in Sturt's desert pea cultures. Cultures were exposured to BAP (5 μM) for 5, 10, 15 and 30 days after which explants were then transferred to BAP-free media. Two agar concentrations (8 and 10 g/l), and three types of tube closures (PWGS Vitafilm®, screw caps with holes and normal screw caps) were also tested in a factorial arrangement using a randomised complete block design with 4 replications. One explant was used in each replication.

After 30 and 60 days, the same five parameters as for experiment 1 were measured.

2.1.8 Water content

The aim of this experiment was to investigate the water content of normal and hyperhydric shoots cultured on various media.

Cultures were treated with three levels of agar concentration, three types of tube closure, or three levels of BAP as follows: Three levels of agar (6, 10, 14 g/l) were used to solidify basal medium plus 5 μM BAP. Tube closures were PWGS Vitafilm®, a screw cap with a hole and a normal screw cap. All closures, except PWGS Vitafilm®, were autoclaved before being placed on the tubes and media plus 5 μM BAP were solidified with 8 g/l agar (Sigma). Finally, three BAP concentrations (0.1, 1.0 and 10 μM) were added to media solidified with 8 g/l agar. Normal scew caps were used in the experiment using various gel and BAP concentrations.

The treatments were set up as a randomised complete block and replicated three times. Each replication consisted of 10 plants. After 30 days, cultures were sacrificed for water content measurement.

2.1.9 Water content measurement

Water content was measured as follows: cultures from each treatment were grouped into normal and hyperhydric, and their fresh weights determined after removing excess agar and moisture. They were then dried in an oven at 80°C for 12 hours and reweighed. The percentage water content was calculated using the formula :

$$WC = \frac{(FW - DW)}{FW} \times 100$$

WC: percentage water content

FW: fresh weight (g)

DW: dry weight (g)

2.1.10 Measurement of water transmission rate of tube closures

The aim of this experiment was to investigate water transmission rates of five types of tube closures used in previous experiments.

Culture tubes containing 10 ml water were closed with various tube closures (as experiment 1). The tubes with water and closures were weighed before incubating

under the culture condition described in section 2.1.2. The tubes were reweighed 30 days later.

The experiment was set up as a randomised complete block and replicated 10 times. Water transmission rates were calculated using the formula:

$$WTR = \frac{W0 - W30}{30}$$

WTR: water transmission rate (ml/vial/day)

W0 : tube weight at 0 day (g)

W30 : tube weight after 30 days (g)

2.1.11 Measurement of water evaporation rate of support matrices

The aim of this experiment was to investigate water evaporation rate from the support matrices used in experiment 2.

Tubes containing various support matrices (perlite, Oasis Rootcube®, Oasis Horticube® and filter paper bridges) as outlined in section 2.1.5, were supplied with 8 ml distilled water per vial and agar (12 g/l) was used as a control. Tubes were left uncovered, were weighed before incubating (as in section 2.1.2) and reweighed after 30 days.

The experiment was set up as a randomised complete block and replicated 10 times. Water evaporation rate was calculated using the formula:

$$WER = \frac{W0 - W30}{30}$$

WER : water evaporation rate (ml/vial/day)

WO : tube weight at 0 day (g)

W30 : tube weight after 30 days (g)

2.2 Anatomical study of hyperhydric shoots

2.2.1 Plant material

Shoot apices and leaf segments approximately 5 mm and 1 mm in length respectively were excised from 100% hyperhydric and normal shoots of Sturt pea cultured as outlined in section 2.1. Shoot apices were prepared for light microscopy and leaf segments were collected for scanning and transmission electron microscopy.

2.2.2 Scanning electron microscope study of leaf surfaces

The aim was to compare the structure of the leaf surface of normal leaves with that of hyperhydric leaves.

2.2.2.1 Fixation and dehydration

Hyperhydric and normal leaves were dissected over ice in a drop of fixative and immediately transferred to the fixative solution, placed in a vacuum for 10 minutes and finally transferred to a cold room (4^{o}C) for 24 hours. The fixative used was 3% glutaraldehyde in 0.025 M PIPES buffer pH 7. Following the glutaraldehyde fixation, the specimens were thoroughly washed in 0.025 M PIPES buffer and then postfixed for 3 hours at room temperature in 2 % osmium tetroxide in 0.025 PIPES buffer. The tissues were then washed in distilled water and dehydrated through a graded ethanol series (25, 40, 55, 70, 80, 95, 100%), each step taking 30 minutes. The samples were then critical point dried using liquid CO_2 and subsequently kept in a desiccator (Hayat, 1989).

2.2.2.2 Scanning the tissues

The dried tissue was sputter-coated with a thin layer of gold (50 nm thickness) using Emscope SC 500 Sputter Coater. The surface of the leaves and structure of the

stomata, were examined and photographs were taken using a Cambridge Stereoscan S250 scanning electron microscope.

2.2.3 Study of distribution of stomata using a light microscope

The aim was to compare the density of stomata in hyperhydric and normal leaves.

Study of distribution of stomata was done by painting both surfaces of hyperhydric and normal leaves with clear nail polish. When dry, the nail polish with the imprint of the leaf surface was stripped off and placed on a glass slide, with the imprint on the upper side. Stomata per unit area were counted under a Zeiss compound microscope (standard lab.16) using x 400 magnification.

2.2.4. Transmission electron microscopy of photosynthetic tissue

The aim was to study chloroplast structure in hyperhydric and normal photosynthetic tissue.

2.2.4.1 Fixation, dehydration, infiltration and embedding

Methods of fixation and dehydration were the same as those for the scanning electron microscope procedure above (section 2.2.2.1) with the addition of a further step. After the last stage of dehydration the tissues were thoroughly infiltrated through a series of ethanol : resin (London Resin Gold®, supplied by Probing & Structure) mixtures (3:1, 1:1, 1:3) followed by 100% resin. Each step was maintained for 12 hours.

The tissues were then embedded using resin (L. R. Gold®) polymerised with 0.5% benzoyl peroxidase for 24 hours (Hayat, 1989).

2.2.4.2 Sectioning

The resin containing the tissue was trimmed to size and positioned to ensure that tissues were correctly orientated in relation to the direction of sectioning. Embedded tissues were cut with the Reichert-Jung Ultracut-E microtome using a glass knife. The ribbons of sections were grouped in the middle of the trough by using a single hair and collected on the dull side of the grid by lowering the grid onto the sections. The grids containing sections were dried on filter paper (Hayat, 1989).

2.2.4.3 Staining

Grids with sections were stained with 5% uranyl acetate solution produced by adding the powdered uranyl acetate to distilled water in a lightproof container and storing over night. The solution was then filtered through Whatman filter paper. The staining procedure was as follows:

A drop of uranyl acetate solution was put onto a fresh square of Parafilm® placed on the bottom of a disposable Petri dish. The grids were touched section side down to the drop and quickly placed at the bottom of the drop, section side up. After 20 minutes, the grid was quickly removed from the drop of stain and immersed in a series of three beakers of distilled water and then blotted completely dry on filter paper before proceeding (Hayat, 1989).

2.2.4.4 Investigating the tissues

Cellular and chloroplast structures were observed under a Philips EM 400 transmission electron microscope and recorded photographically. Cellular and chloroplast structure was investigated on 5 sections from each of 5 leaves from hyperhydric and normal cultures. Data on the number of thylakoid layers per granum and the dimensions of starch granules were collected from chloroplasts in each section,

and these data then averaged for each leaf to comprise one replication. Four leaves from four plants were used in this experiment.

2.2.5 Light microscopy of leaf

The aim was to investigate the anatomy of hyperhydric and normal leaves. Two methods were used.

2.2.5.1 Light microscopy of fresh leaf

2.2.5.1.1 Embedding

The embedding medium (10% gelatine, 0.8% DMSO (dimethyl sulphoxide) and 2% glycerol) was dissolved in warm water with mixing, then chilled. Hyperhydric and normal leaves (approximately 2 mm long) were inserted into the embedding medium using a warm razor blade and quickly frozen on a freezing microtome.

2.2.5.1.2 Sectioning

Using a Lietz 1320 freezing microtome, embedded leaves were frozen and sectioned. Sections were collected in warm water to separate gel from tissues. The tissues were then transferred to a glass slide and cell structures were observed using a Zeiss compound microscope.

2.2.5.1.3 Photography

Photographs were taken using Kodak ASA 200 film in an Olympus OM 2 camera attached to a Zeiss compound microscope (standard lab. 16).

2.2.5.2 Light microscopy of embedded leaf

Methods of fixation, dehydration, infiltration, and embedding were the same as those for the transmission electron microscope procedure above (section 2.2.4)

2.2.5.2.1 Sectioning and staining

The resin with the tissue was trimmed and sectioned with Reichert - Jung Ultracut - E microtome using a glass knife. The sections were transferred to a glass slide and dried on a hotplate. After drying, the tissues were stained with 0.5 % toluidine blue by dropping the stain on the tissue. After 60 seconds, the tissues were washed with distilled water.

2.2.5.2.2 Photography

Photographs were taken using Kodak ASA 200 film in an Olympus OM 2 camera attached to a Zeiss compound microscope (standard lab. 16).

2.2.6 Light microscopy of shoot apex

The aimwas to investigate the development of apical meristem and procambium in the shoots of hyperhydric and normal plantlets.

Methods for preparing the tissues sectioning and photographing were the same as those for light microscopy of embedded leaf (section 2.2.5.2).

2.2.7 Statistical analysis

Results were analysed by regression analysis (orthogonal polynomial contrasts) or ANOVA as appropriate. Means were separated using least significant difference (LSD) at P=0.05.

Chapter 3. Results

3.1 Tissue culture experiments

3.1.1 Introduction

The objective of the first four experiments was to investigate the influence of certain aspects of culture on hyperhydricity. However, it was not enough to assess merely the proportion of hyperhydric shoots, because other culture attributes were also important for optimal shoot production; for example certain culture conditions may reduce hyperhydricity but also decrease shoot proliferation, growth and/or vigour. Therefore, callus width, shoot number, shoot length and shoot vigour were also measured.

All experiments were assessed twice (30 and 60 days of culture). The second assessment was done to ascertain whether there were any changes since the first assessment. As the results in each experiment did not vary significantly between these 2 assessments, all data presented here represent day 30 assessments only.

3.1.2 Experiment 1 : Effect of agar concentration and tube closure on growth and hyperhydricity in Sturt's desert pea culture.

3.1.2.1 Callus width

The ANOVA indicated that there was a significant (P=0.05) 2 - way interaction between agar concentration and tube closure (Table 3.1.2). Figure 3.1.2.1 shows that callus width decreased linearly with increasing agar concentration. However, the significant interaction effect meant that the response of callus width to agar concentration was not the same for all closure types. In fact, significantly less callus was produced when cotton wool closures were used than when vitafilm closures were used at 6 g/l agar, but at 12 g/l agar the difference between these closure types was not significant.

Table 3.1.2. ANOVA table for callus width, shoot number, shoot length and shoot vigour (experiment 1).

	F-Value			
Source	Callus width	Shoot number	Shoot length	Shoot vigour
Closure	7.0 ***	1.9 ns	1.0 ns	5.5 ***
Agar	30.0 ***	8.1 ***	0.8 ns	17.0 ***
Agar (L)	88.3 ***	23.9 ***	0.8 ns	44.9 ***
Agar (Q)	1.1 ns	0.3 ns	0.2 ns	2.7 ns
Residual	0.1 ns	0.1 ns	1.4 ns	3.3 ns
Cls x agar	1.9 *	0.9 ns	2.0 *	0.7 ns
Cls x Ag.(L)	3.3 *	1.4 ns	2.5 *	1.2 ns
Cls x Ag.(Q)	1.0 ns	0.7 ns	2.7 *	0.8 ns
Residual	1.5 ns	0.7 ns	0.7 ns	0.1 ns

ns, ***, **, * Nonsignificant or significant at P = 0.001, 0.01 or 0.05, respectively.

L: Linear, Q: Quadratic.

3.1.2.2 Shoot number

While there was no significant interaction effect nor a significant main effect of tube closure on shoot number, ANOVA showed a significant linear relationship between shoot number and agar concentration (Table 3.1.2). Therefore results were averaged over all levels of tube closure and the linear response plotted in Figure 3.1.2.2. The results showed a linear decline in shoot number as agar strength increased.

3.1.2.3 Shoot length

The statistical analysis indicated that there was a significant ($P < 0.05$) 2 - way interaction between agar concentration and tube closure (Table 3.1.2). No significant effects of agar or tube closure were observed on shoot length and hence it may be concluded that length was not affected significantly by the type of closure and the agar concentration used (Figure 3.1.2.3).

Figure 3.1.2.1: Effect of tube closure and agar concentration on callus width of Sturt's desert pea after 30 days culture.

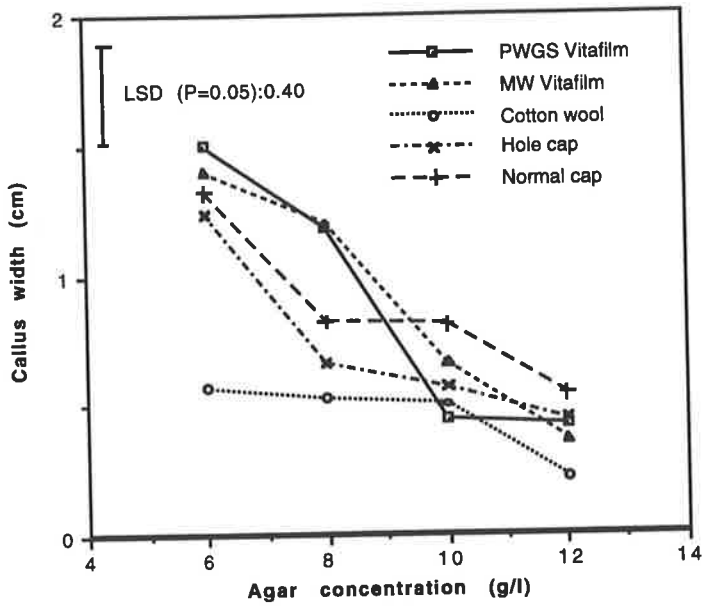


Figure 3.1.2.2: Effect of agar concentration on shoot number of Sturt's desert pea after 30 days culture. Results were averaged over all closure types.

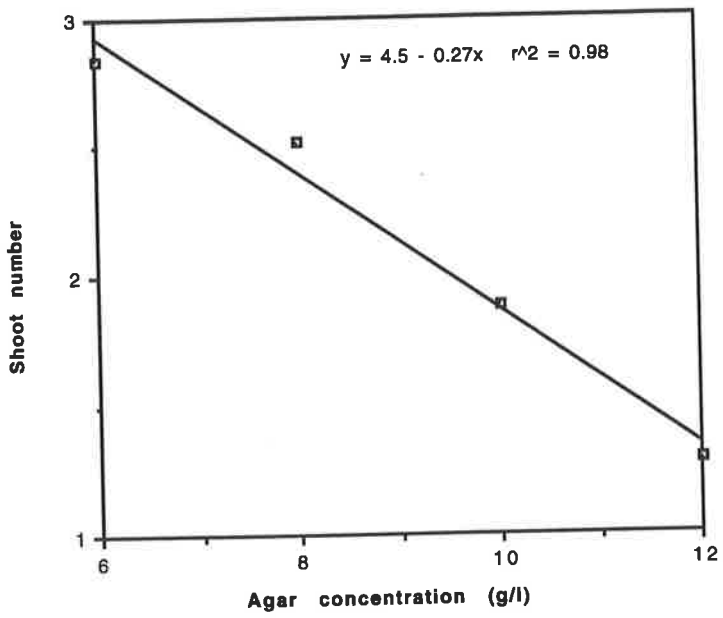
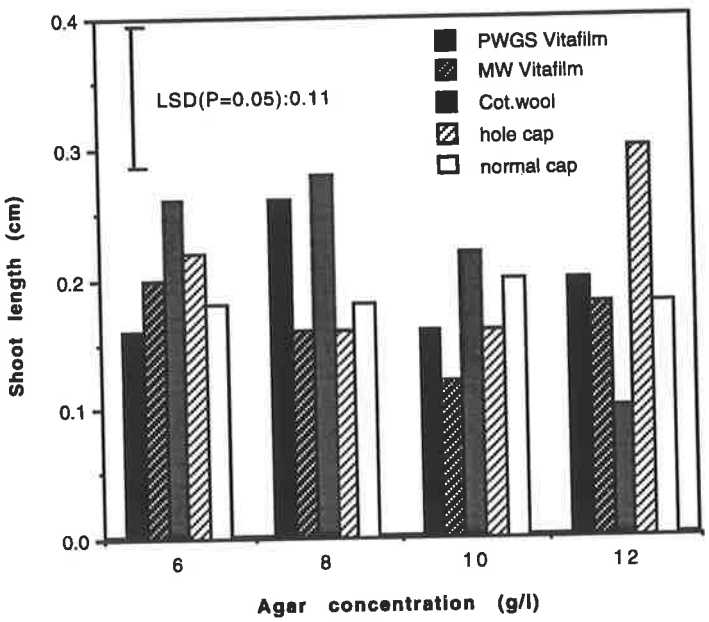


Figure 3.1.2.3: Effect of agar concentration and tube closure on shoot length of Sturt's desert pea after 30 days culture.



3.1.2.4 Shoot vigour

A significant linear main effect of agar concentration on shoot vigour ($P < 0.001$) and a significant main effect of tube closure on shoot vigour ($P < 0.001$) is shown in ANOVA Table 3.1.2.

Figure 3.1.2.4.a shows that mean shoot vigour increased linearly with agar concentration between 6 - 12 g/l agar. The effect of tube closure on shoot vigour is presented in Figure 3.1.2.4.b and shows that normal screw caps produced significantly less vigorous shoots than did those with other coverings. Tubes covered with screw cap with a hole proved to be outstandingly vigorous.

Figure 3.1.2.4.a: Effect of agar concentration on shoot vigour of Sturt's desert pea after 30 days culture (result averaged over all closure types).

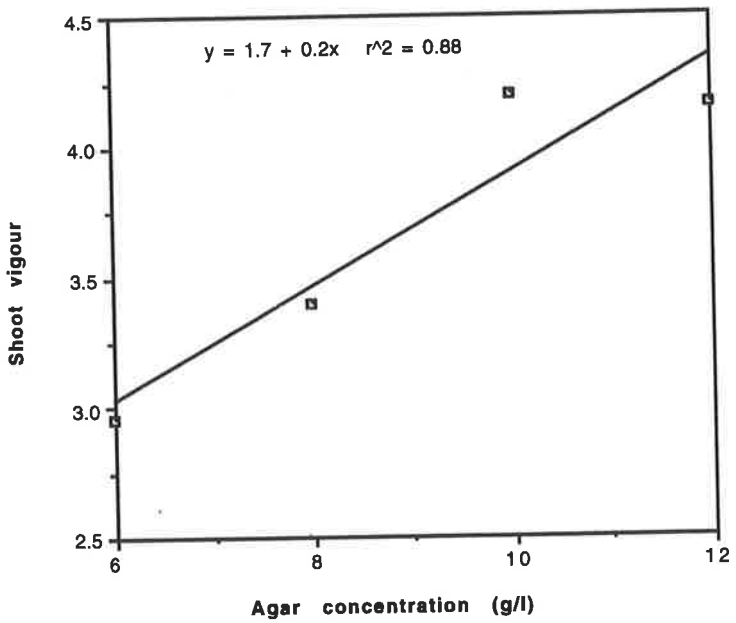
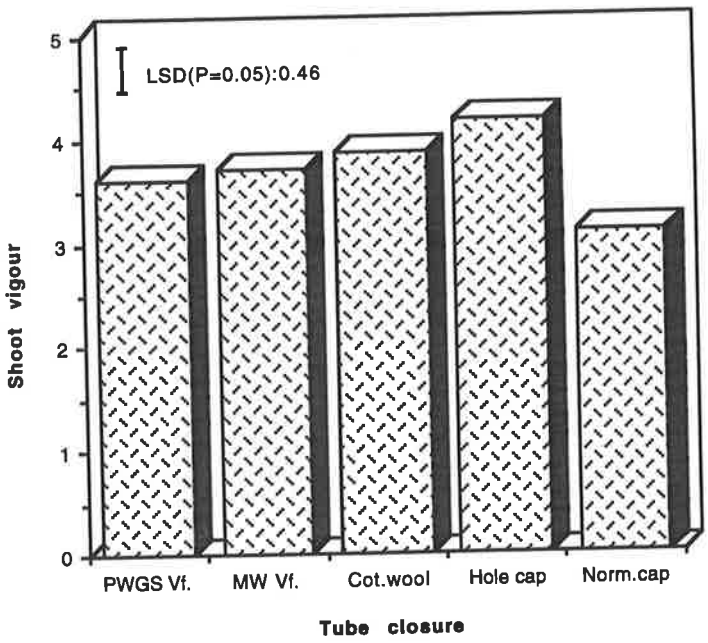


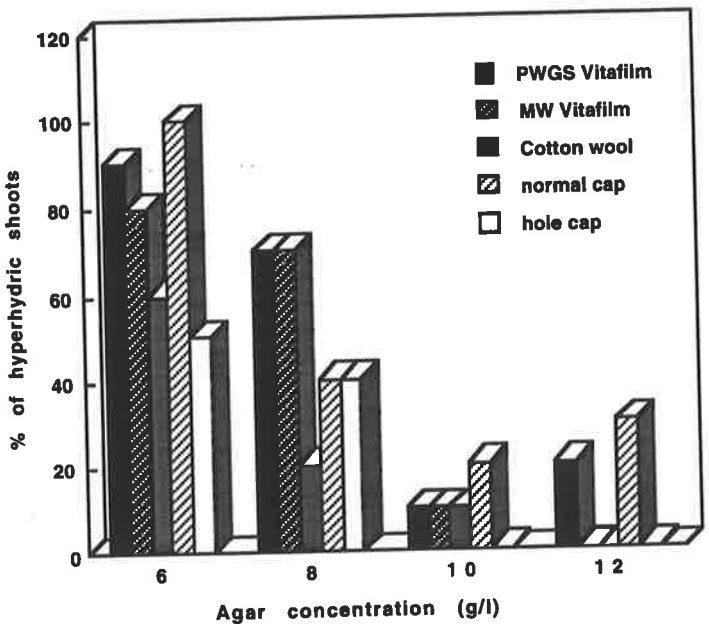
Figure 3.1.2.4.b: Effect of tube closure on shoot vigour (averaged over all agar concentrations) of Sturt's pea after 30 days culture.



3.1.2.5 Hyperhydricity

Since only a single explant (vial) was used per replicate per treatment and hyperhydricity was scored as present or absent, this parameter could not be statistically analysed. Figure 3.1.2.5 shows the percentage of hyperhydric shoots with various levels of agar concentration and various types of tube closure. Generally, hyperhydricity declined, irrespective of closure type, as agar strength increased. While tubes with normal screw caps appeared to maximise the incidence of hyperhydricity, screw cap with a hole consistently reduced its incidence at all agar concentrations.

Figure 3.1.2.5: Effect of agar concentration and tube closure on hyperhydricity of Sturt's desert pea after 30 days culture.



3.1.2.6 Summary

In summary, this experiment showed that closure type and agar concentration can influence growth, morphogenesis and hyperhydricity of Sturt pea cultures. Hyperhydricity generally declined as agar strength increased, and was minimised using closures with improved gas and water vapour exchange capacity, such as screw cap with a hole or cotton wool. Shoot proliferation was higher at lower gel strength, while vigour improved with higher agar strength and more permeable closures. Growth of cultures (length) was not markedly affected by closure or agar concentration, and callus production generally declined with increased agar strength and increased cap permeability.

3.1.3 Experiment 2 : Effect of support matrix and tube closure on growth and hyperhydricity in Sturt's desert pea culture.

3.1.3.1 Callus width

Data of callus width analysed statistically showed a significant ($P < 0.001$) 2 - way interaction between tube closure and support matrix (Table 3.1.3).

Figure 3.1.3.1 shows that generally 12 g/l agar and paper bridges produced larger callus than perlite, horticubes or rootcubes. PWGS Vitafilm and normal screw cap produced the highest callus width at 12 g/l agar, the lowest callus width occurred when cotton wool was used to seal tubes using either perlite or rootcube support materials. The general trend seen here supports the results of the previous experiment (Figure 3.1.2.1).

Table 3.1.3. ANOVA table for callus width, shoot number, shoot length and shoot vigour (experiment 2).

	F-Value			
Source	Callus width	Shoot number	Shoot length	Shoot vigour
Support matrix.	87.0***	60.2***	16.5***	28.3***
Closure	10.4***	15.6***	5.5***	13.3***
Supp. mtr. x Closure	3.3***	2.4**	1.7ns	3.5***

ns, ***, **, * Nonsignificant or significant at P = 0.001, 0.01 or 0.05, respectively.

3.1.3.2 Shoot number

The analysis of variance of shoot number indicated a significant (P = 0.1) 2 - way interaction between support matrix and tube closure (Table 3.1.3).

Figure 3.1.3.2 shows that 12 g/l agar and paper bridges yield more shoots than other support matrices. Shoot numbers were highest with normal caps on both agar and paper

bridges. The lowest shoot number was produced by the combination of perlite and cotton wool or by rootcube and cotton wool. Rootcubes were the poorest matrix in terms of shoot production. The significant effect of closure type in this experiment contrasted with its lack of effect on shoot number in the previous experiment (Table 3.1.2).

Figure 3.1.3.1: Effect of support matrix and tube closure on callus width of Sturt's desert pea after 30 days culture.

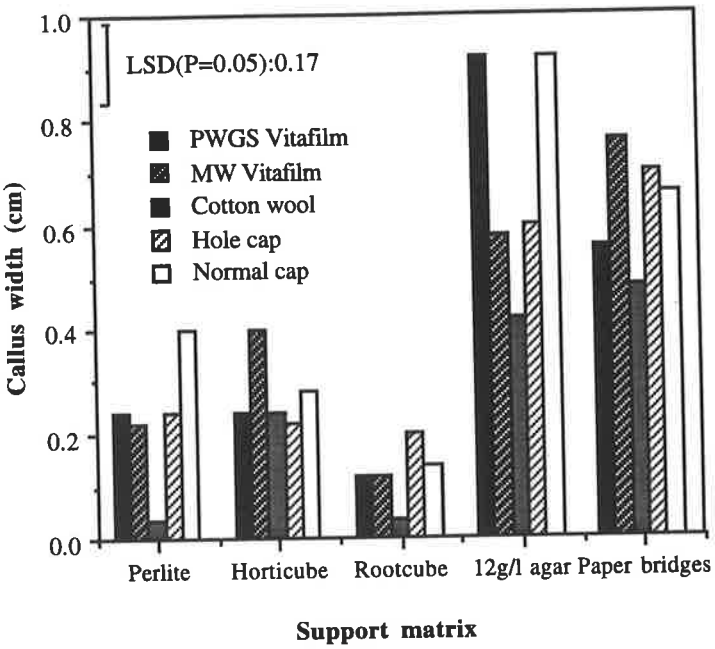
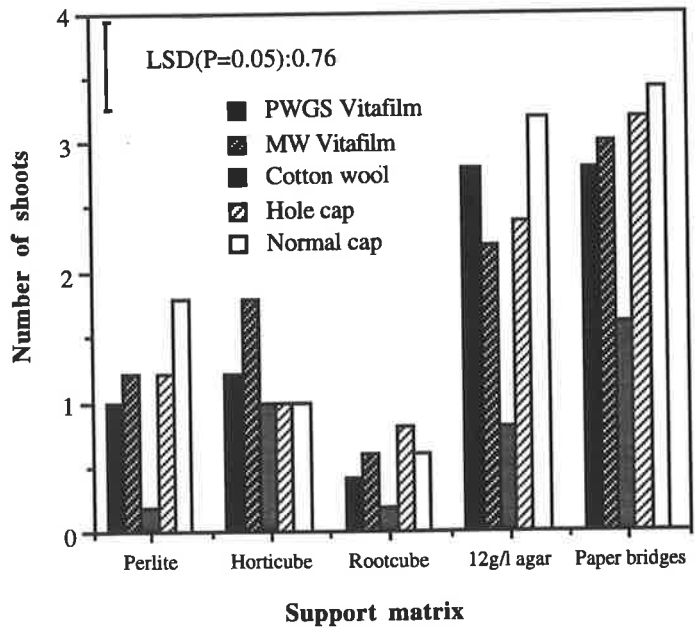


Figure 3.1.3.2: Effect of support matrix and tube closure on shoot number of Sturt's desert pea after 30 days culture.



3.1.3.3 Shoot length

While there is no significant interaction between agar concentration and tube closure (Table 3.1.3), the statistical analysis revealed significant main effects of support matrix and tube closure ($P < 0.001$) on shoot length, again contrasting with the previous experiment where closure type did not produce a significant effect on shoot length. Rootcubes produced significantly less shoot growth and paper bridges significantly more than the other support matrices, which were roughly comparable (Figure 3.1.3.3.a). Figure 3.1.3.3.b shows that all closure types (but for cotton wool which reduced shoot length) resulted in equivalent shoot growth.

3.1.3.4 Shoot vigour

A significant 2 - way interaction between support matrix and tube closure ($P < 0.001$) is shown in ANOVA of data for shoot vigour (Table 3.1.3).

Figure 3.1.3.4 shows that shoot vigour was consistently higher using agar medium across all closure types, and PWGS Vitafilm and normal caps consistently improved shoot vigour for most support matrices, while cotton wool proved to be the poorest closure in this regard. In contrast, rootcubes produced lowest shoot vigour with all tube closures.

Figure 3.1.3.3.a: Effect of support matrix on shoot length (result averaged over all closure types) of Sturt's desert pea after 30 days culture.

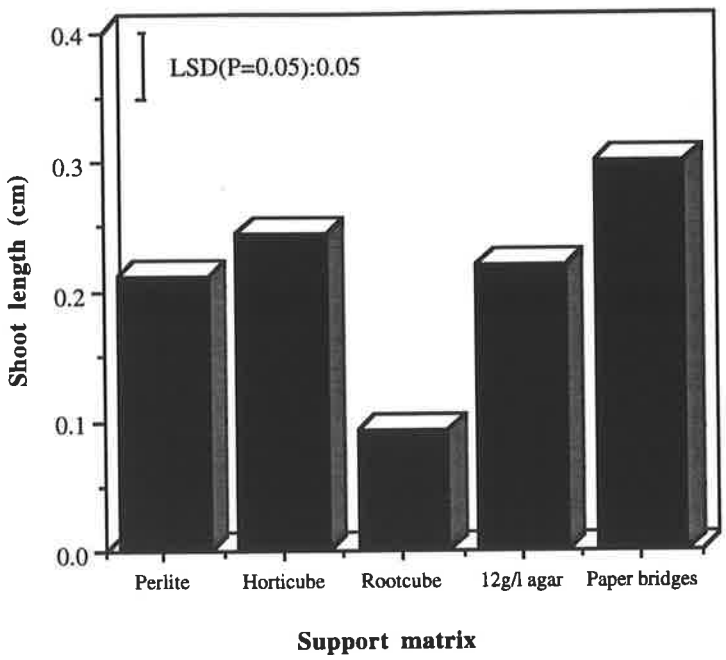


Figure 3.1.3.3.b: Effect of tube closure on shoot length (averaged over all support matrices) of Sturt's desert pea after 30 days culture.

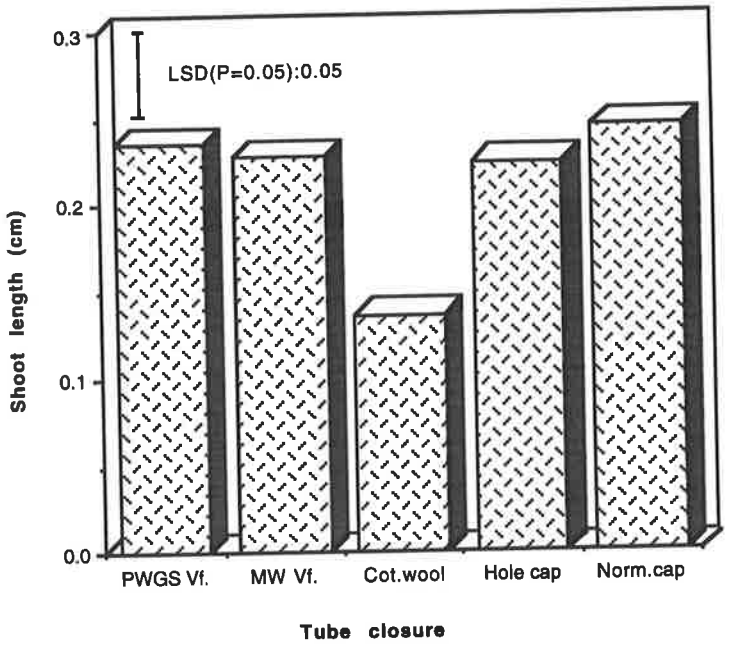
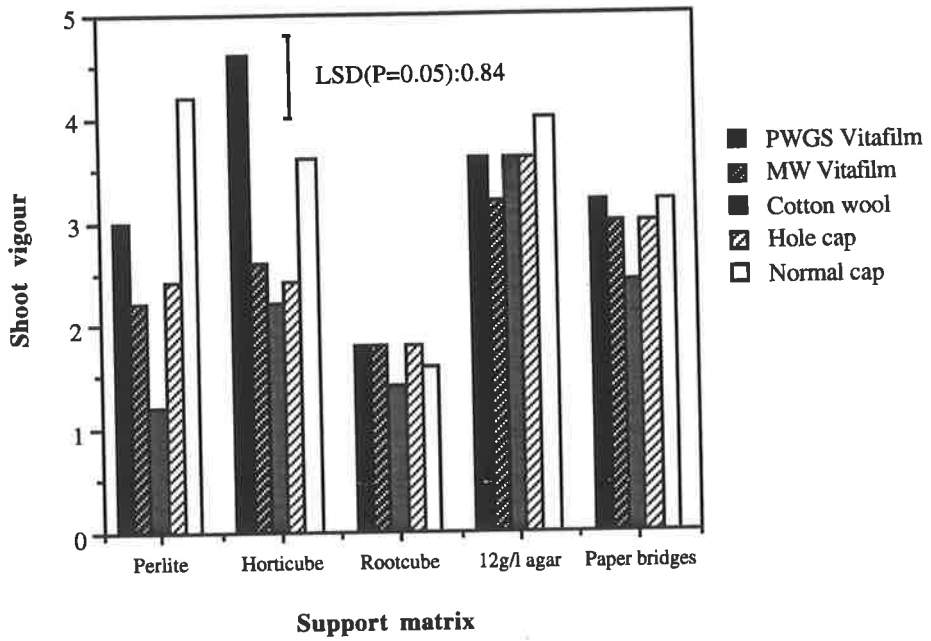


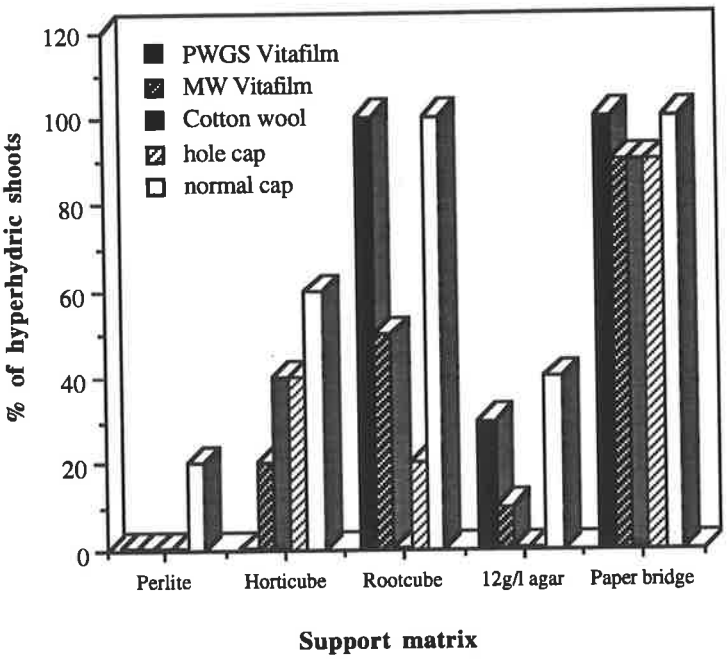
Figure 3.1.3.4: Effect of support matrix and tube closure on shoot vigour of Sturt's desert pea after 30 days culture.



3.1.3.5 Hyperhydricity

Figure 3.1.3.5 shows the percentage of hyperhydric shoots with various kinds of support matrices and tube closures. In general, perlite and 12 g/l agar were most effective in minimising hyperhydricity. Tubes with cotton wool or screw cap with a hole produced 100 % normal shoots when coupled with either of these support matrices. Horticube with MW or PWGS Vitafilm yielded high proportions of normal shoots. Similarly, a holed cap or cotton wool plug used over a rootcube matrix yielded 20 % and 0 % hyperhydricity respectively. In contrast, paper bridges produced almost total hyperhydricity with all tube closures.

Figure 3.1.3.5: Effect of support matrix and tube closure on hyperhydricity on Sturt's desert pea culture after 30 days culture.



3.1.3.6 Summary

From the above results, it is evident that support matrices and tube closures influence both hyperhydricity and growth of Sturt desert pea culture. Perlite and 12 g/l agar minimised hyperhydricity, as also did the more permeable closures (eg. cotton wool, screw cap with a hole). Shoot production was maximised on 12 g/l agar or filter paper bridges although these treatments also produced big callus width. Callus width decreased as closures become more permeable. Shoot vigour was consistently higher on agar medium, and when PWGS or normal closures were used. Paper bridges produced optimum shoot length and rootcubes the lowest. Cotton wool closures produced shorter shoots than other closures. In summary, agar medium was the overall best support matrix for optimisation of shoot growth.

3.1.4 Experiment 3: Effect of BAP concentration, agar concentration and tube closure on growth and hyperhydricity in Sturt desert pea cultures.

3.1.4.1: Callus width

The analysis of variance showed a significant 2 - way interaction between agar concentration and BAP concentration (quadratic) ($P= 0.005$), and the main effects of tube closure on callus width ($P < 0.001$) (Table 3.1.4).

Figure 3.1.4.1.a shows the effect of BAP and its interaction with agar concentration on callus width. Callus width increased linearly with BAP concentration at both agar strengths, but the higher agar strength generally resulted in lower callus production. The main effect of tube closure on callus width was that normal screw caps produced more callus than screw cap with a hole (Figure 3.1.4.1.b).

Table 3.1.4 ANOVA table for callus width, shoot number, shoot length and shoot vigour of experiment 3.

	F value			
Source	Callus width	Shoot number	Shoot length	Shoot vigour
Closure	25.0 ***	4.6 *	22.2 ***	1.1 ns
Agar	17.9 ***	3.8 ns	0.0 ns	4.9 *
BAP	124.2 ***	29.1 ***	127.5 ***	8.4 ***
BAP (L)	277.6 ***	63.2 ***	244.0 ***	4.7 *
BAP (Q)	92.4 ***	21.7 ***	120.6 ***	8.5 **
Residual	2.6 ns	2.4 ns	17.7 ***	12.0 ***
Closure x agar	0.7 ns	3.5 *	4.4 *	3.4 *
Closure x BAP	0.7 ns	1.5 ns	4.0 **	1.4 ns
Closure x BAP (L)	0.0 ns	1.6 ns	2.4 ns	3.5 *
Closure x BAP (Q)	2.1 ns	2.3 ns	7.7 ***	0.2 ns
Residual	0.2 ns	0.4 ns	1.8 ns	0.5 ns
Agar x BAP	3.5 *	3.4 **	18.7 ***	1.3 ns
Agar x BAP (L)	0.0 ns	9.4 *	2.9 ns	3.3 ns
Agar x BAP (Q)	8.4 *	0.6 ns	2.3 ns	0.0 ns
Residual	2.2 ns	0.1 ns	50.8 ***	0.6 ns
Closure x agar x BAP	2.2 ns	1.8 ns	6.2 ***	1.4 ns
Closure x agar x (L)	2.7 ns	0.2 ns	1.5 ns	2.4 ns
Closure x agar x (Q)	2.4 ns	3.2 *	4.8 *	0.6 ns
Residual	1.4 ns	2.0 ns	12.2 ***	1.4 ns

ns, ***, **, * Nonsignificant or significant at P = 0.001, 0.01 or 0.05, respectively.

L: Linear, Q: Quadratic

3.1.4.2 Shoot number

A significant (P = 0.05) 3 - way interaction between tube closure, agar concentration and BAP concentration (Quadratic) is shown in Table 3.1.4.

Figure 3.1.4.2 shows the result in graphical form. On average, increase in BAP concentration increased shoot number. However, the interaction between tube closure, agar concentration and BAP concentration means that trends differed between agar levels and closure types and definitive conclusions could not be drawn about the effect of these other factors. A broader range of agar concentrations and tube closure permeability might give a more defined trend.

Figure 3.1.4.1.a: Effect of agar and BAP concentrations on callus width of Sturt's desert pea after 30 days culture.

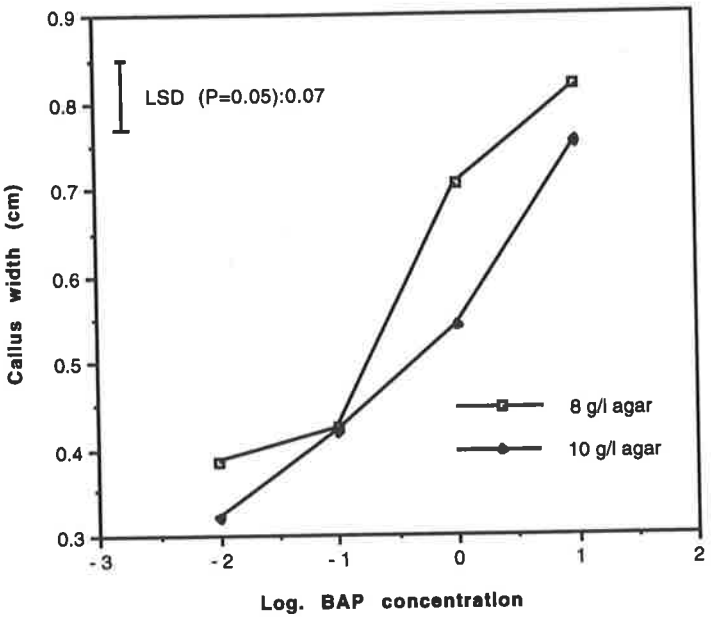


Figure 3.1.4.1.b: Effect of tube closure on callus width of Sturt's desert pea after 30 days culture.

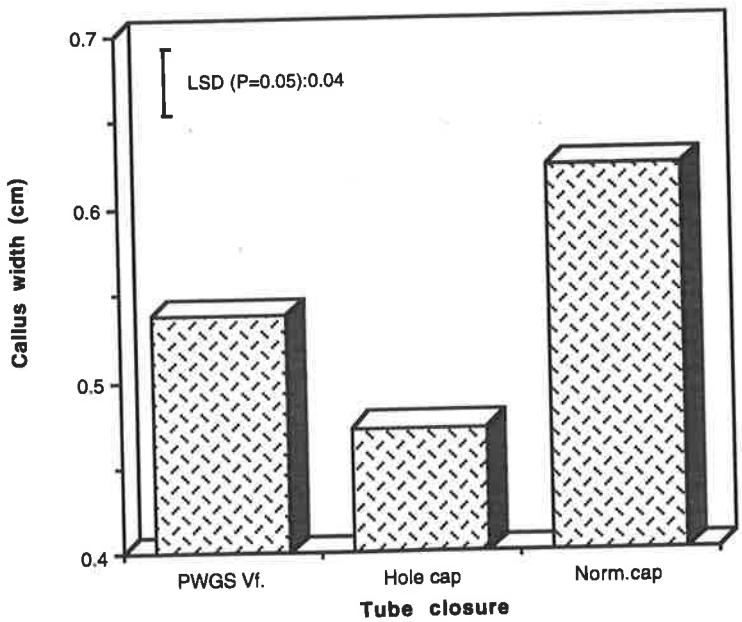
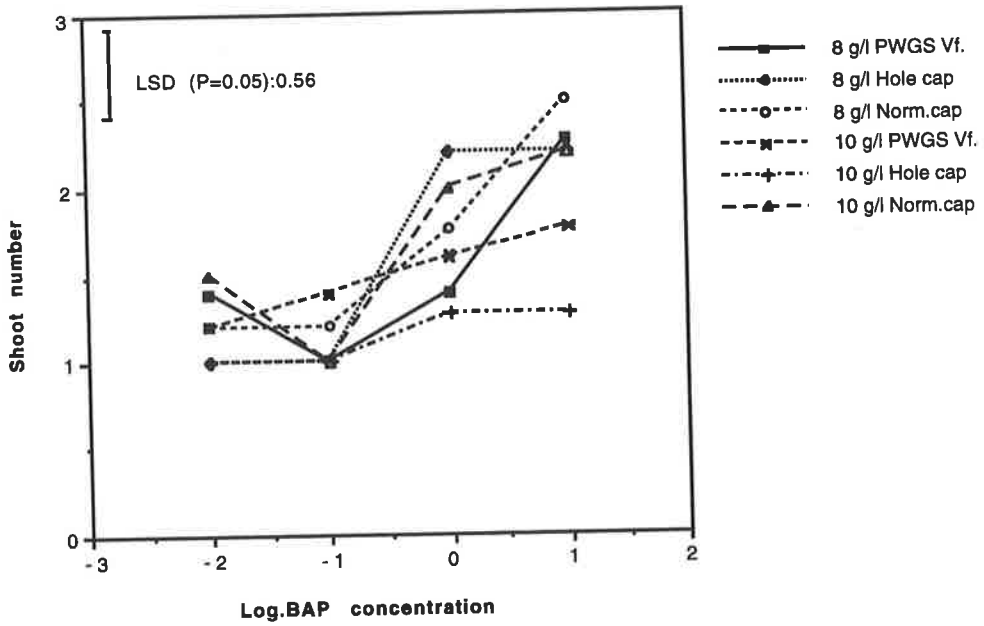


Figure 3.1.4.2: Effect of BAP concentration, agar concentration and tube closure on shoot number of Sturt's desert pea after 30 days culture.

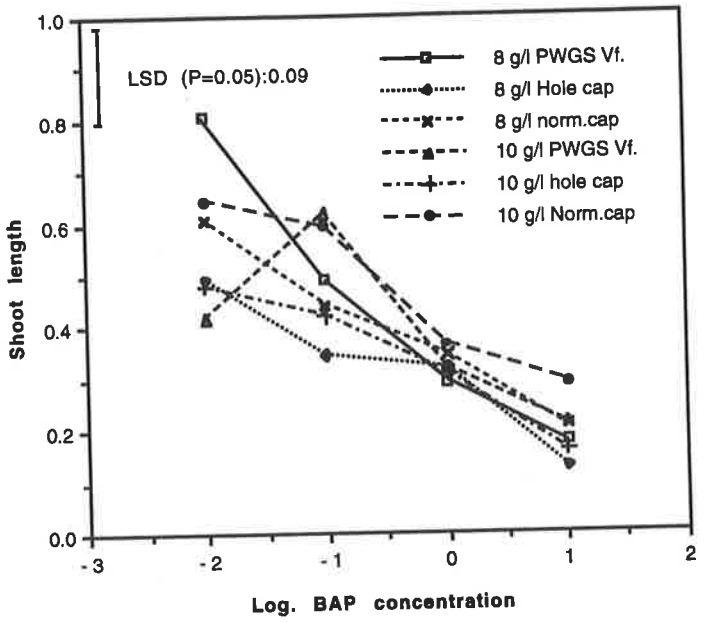


3.1.4.3 Shoot length

After statistical analysis of data for shoot length, ANOVA indicated that there was a highly significant 3 - way interaction between agar concentration, tube closure and BAP concentration ($P < 0.001$) (Table 3.1.4).

Figure 3.1.4.3 shows that shoot length declined with BAP concentration with a significant linear trend (Table 3.1.4). However, the effect of agar concentration was not significant. The significant interactions, however, meant that the response to BAP was not consistent between tube closures.

Figure 3.1.4.3: The effect of BAP concentration in the interaction with agar concentration and tube closure on shoot length of Sturt's desert pea after 30 days culture.



3.1.4.4 Shoot vigour

Data for shoot vigour showed significant 2 - way interactions between agar concentration and tube closure ($P < 0.05$), and between tube closure and BAP concentration (linear) ($P < 0.05$) (Table 3.1.4).

Figure 3.1.4.4.a shows that the combination of PWGS Vitafilm and 10 g/l agar significantly reduced shoot vigour compared with all other combinations, which did not differ significantly from each other. The interaction between tube closure and BAP concentration is shown in Figure 3.1.4.4.b, and demonstrates that for PWGS and screw cap with a hole closures, vigour declined with BAP concentration. However, this effect was not as clear when normal screw caps were used.

Figure 3.1.4.4.a: Effect of agar concentration and tube closure on shoot vigour of Sturt's desert pea after 30 days culture.

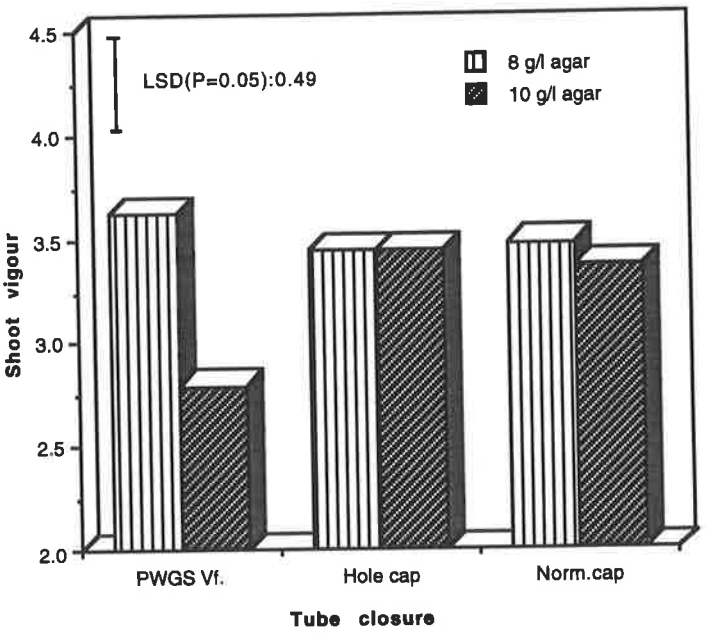
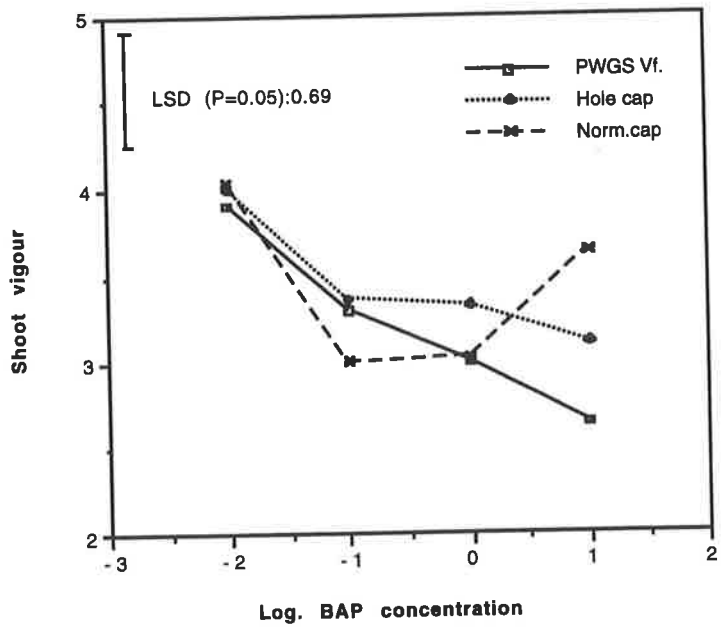


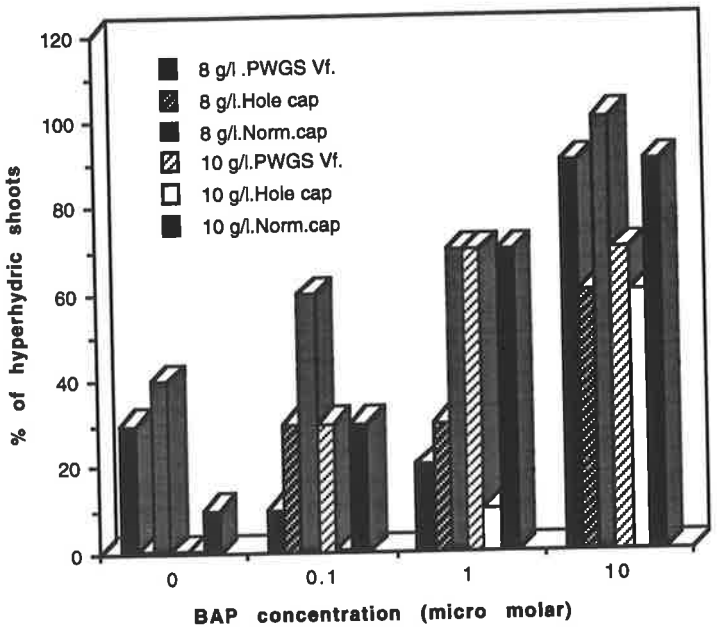
Figure 3.1.4.4.b: Effect of BAP concentration and tube closure on shoot vigour of Sturt's desert pea after 30 days culture.



3.1.4.5 Hyperhydricity

Figure 3.1.4.5 shows the percentage of hyperhydric shoots with various BAP concentration, agar concentration and various types of tube closure. BAP seems to play an important role in onset of hyperhydricity. In general, increasing BAP concentration tended to increase hyperhydricity. Hyperhydricity was lowest at all cytokinin concentrations when tubes were closed with screw cap with a hole, and was reduced to zero at the two lowest levels of cytokinin. Hyperhydricity appeared to be slightly less at the higher concentration of agar.

Figure 3.1.4.5: Effect of BAP concentration in the interaction with agar concentration and tube closure on hyperhydricity of Sturt's desert pea after 30 days culture.



3.1.4.6 Summary

BAP played an important role in growth, morphogenesis and hyperhydricity. Hyperhydricity generally increased as BAP concentration increased. However, this tendency was minimised with high strength agar and high permeability closures. Proliferation rate and callus production also increased linearly with BAP concentration but again less callus production was achieved in high agar strength and increasingly permeable closures. Growth and vigour of cultures generally declined as BAP concentration increased.

3.1.5 Experiment 4: Effect of exposure time to BAP, agar concentration and tube closure on growth and hyperhydricity in Sturt desert pea cultures.

Methods for this experiment are outlined in section 2.1.7. The experiment was designed to investigate the effect of exposure time to 5 μM BAP, agar concentration and tube closure on growth and hyperhydricity of cultures. The result from each of the parameters were analysed using orthogonal polynomial contrasts and ANOVA (Table 3.1.5).

3.1.5.1 Callus width

Data for callus width showed significant 3 - way interactions between agar concentration, tube closure and exposure time to BAP (linear) ($P < 0.01$) (Table 3.1.5). Individual effects of closure, agar and BAP exposure were highly significant.

Figure 3.1.5.1. shows that in general, the increase in exposure time to BAP increased callus width although there was some tendency to level off as exposure time increased beyond 15 days. The concentration of agar and the type of tube closure also played important roles in the growth of callus, which was largely minimised as agar strength increased or with closures than were more permeable to gases.

3.1.5.2 Shoot number

The ANOVA of shoot number indicated that there was a main effect of exposure time to BAP on shoot number ($P < 0.001$), but neither closure type nor agar strength yielded significant responses of this parameter (Table 3.1.5). The number of shoots was highest following 10 days exposure to BAP (Figure 3.1.5.2). As exposure time increased beyond 10 days, shoot number decreased significantly. As observed in earlier experiments, tube closure and agar strength produced no significant effect on shoot number.

Table 3.1.5. ANOVA table for callus width, shoot number, shoot length and shoot vigour of experiment 4.

	F value			
Source	Callus width	Shoot number.	Shoot length	Shoot vigour
Closure	25.1 ***	2.2 ns	9.1 **	0.4 ns
Agar	10.8 ***	0.5 ns	0.4 ns	2.3 ns
Days	30.0 ***	7.6 ***	60.5 ***	19.9 ***
Days (L)	70.7 ***	10.2 **	117.8 ***	52.9 ***
Days (Q)	19.3 ***	0.0 ns	61.9 ***	6.6 *
Residual	0.0 ns	12.7 ***	1.7 ns	0.3 ns
Closure x agar	8.9 ***	0.6 ns	8.6 ***	7.2 ***
Agar x days	3.1 *	1.3 ns	7.3 ***	1.9 ns
Agar x days (L)	1.3 ns	0.2 ns	9.6 **	4.7 *
Agar x days (Q)	7.5 *	0.6 ns	11.5 ***	0.1 ns
Residual	0.3 ns	3.0 ns	0.9 ns	0.9 ns
Closure x days	1.0 ns	1.6 ns	4.4 ***	1.7 ns
Closure x days (L)	0.5 ns	1.0 ns	0.7 ns	0.6 ns
Closure x days (Q)	1.4 ns	0.3 ns	5.1 **	3.0 ns
Residual	1.1 ns	3.6 *	7.6 ***	1.7 ns
Closure x agar x days	2.1 ns	2.2 ns	1.6 ns	1.4 ns
Closure x agar x (L)	5.1 **	2.3 ns	1.5. ns	1.4 ns
Closure x agar x (Q)	1.1 ns	0.2 ns	0.9 ns	0.2 ns
Residual	0.1 ns	4.2 *	2.3 ns	2.7 ns

ns, ***, **, * Nonsignificant or significant at P = 0.001, 0.01 or 0.05, repectively

L: Linear, Q: Quadratic

Figure 3.1.5.1: Effect of BAP concentration, agar concentration and tube closure on callus width of Sturt's desert pea after 30 days culture.

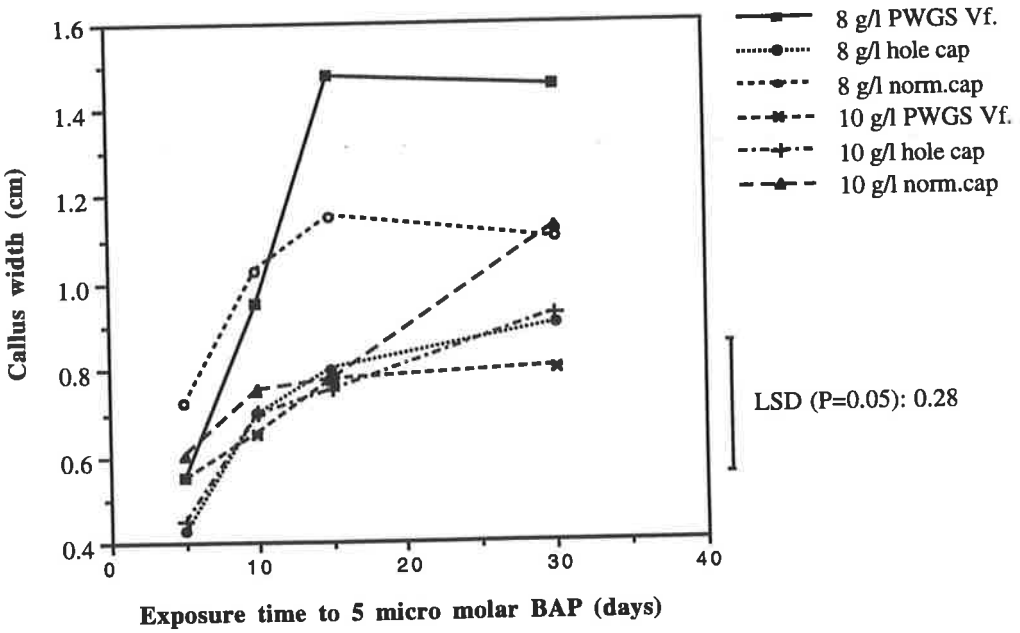
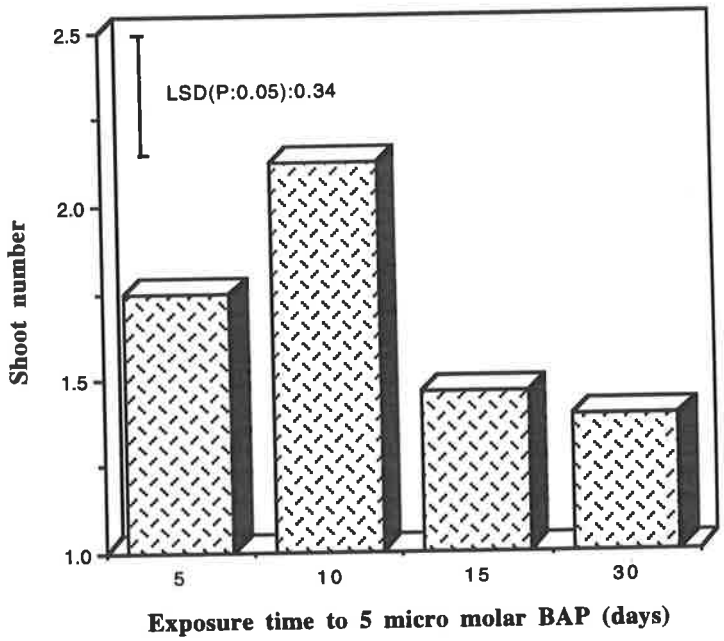


Figure 3.1.5.2: Effect of exposure time to BAP (averaged over all agar concentrations and tube closures) on shoot number of Sturt's desert pea after 30 days culture.



3.1.5.3. Shoot length

While the overall effect of agar strength on shoot length was not significant, closure type and BAP exposure did produce significant effects (Table 3.1.5). Furthermore, significant 2 - way interactions between agar concentration and tube closure ($P < 0.001$), between agar concentration and exposure time to BAP ($P < 0.001$) and between tube closure and exposure time to BAP ($P < 0.001$) were observed.

Shoot length was not markedly affected by tube closure or agar strength, except that a cap with a hole closure resulted in significantly heiger shoot growth at 8 g/l agar than other treatments (Figure 3.1.5.3.a).

Figure 3.1.5.3.b shows the interaction of agar concentration with exposure time to BAP. Both linear and quadratic effects of BAP exposure were significant in this interaction. The figure shows that the shoot length decreased with the increase in exposure time to BAP until 30 days at both concentrations of agar. Agar concentration affected shoot length at a short time of exposure to BAP (5 days) but not at longer times (15 - 30 days).

The interaction between tube closure and exposure time to BAP (Figure 3.1.5.3.c) again showed a decrease in shoot length with increasing time of exposure to BAP. The effects of closure were not consistent at all BAP exposure times; while normal screw caps or PWGS Vitafilm produced maximal shoot length at 5 days BAP exposure, screw cap with a hole produced maximal response after 10 days BAP exposure. Longer periods of exposure to BAP resulted in very short shoots, and differences between types became non significant.

Figure 3.1.5.3.a: Effect of agar concentration and tube closure on shoot length of Sturt's desert pea after 30 days culture.

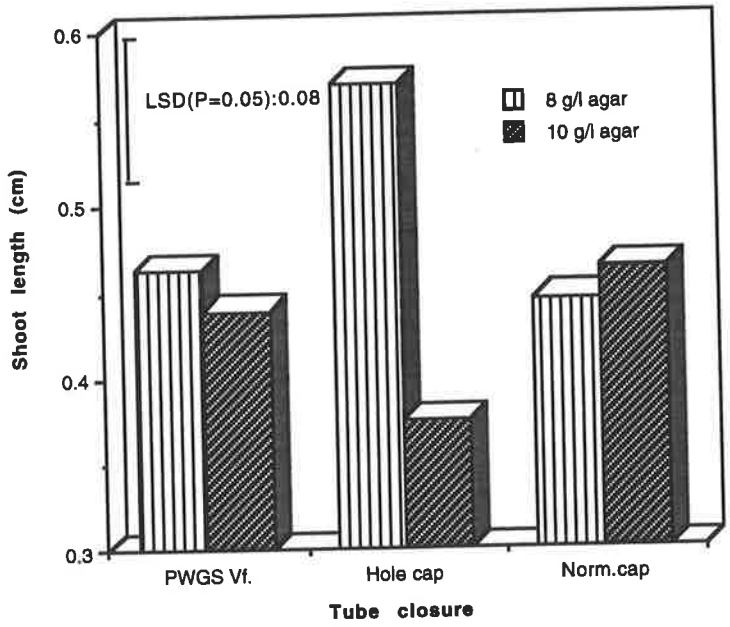


Figure 3.1.5.3.b: Effect of agar concentration and exposure time to 5µM BAP on shoot length of Sturt's desert pea after 30 days culture.

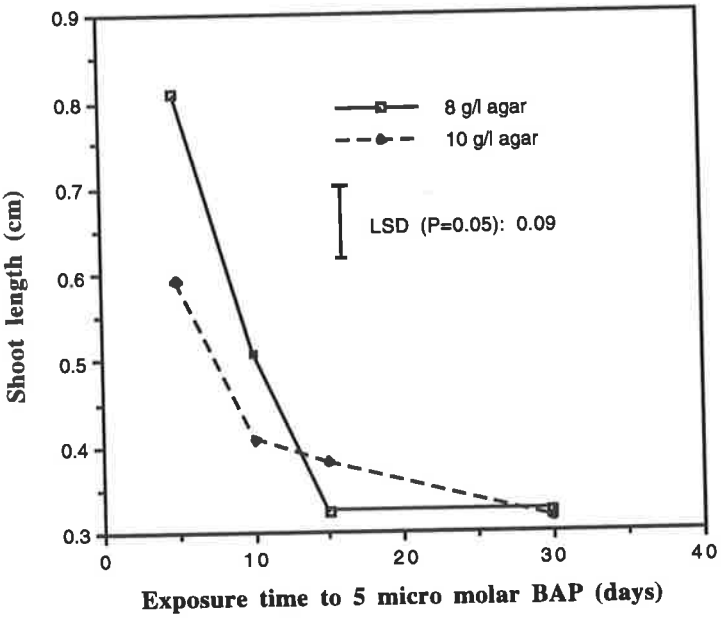
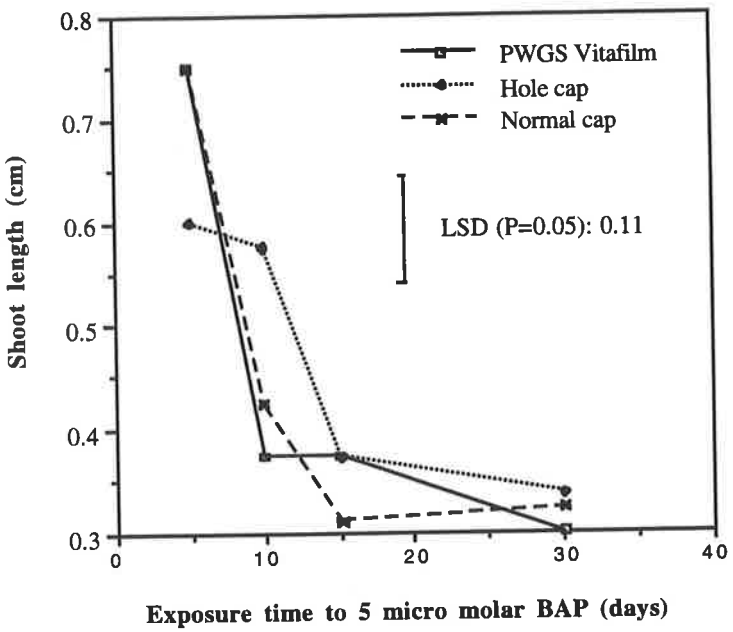


Figure 3.1.5.3.c: Effect of tube closure and exposure time to 5 μM BAP on shoot length of Sturt's desert pea after 30 days culture.



3.1.5.4 Shoot vigour

While neither closure type nor agar strength produced significant effects on vigour, significant 2 - way interactions were observed between agar concentration and tube closure ($P = 0.001$), and between gel concentration and exposure time to BAP ($P < 0.05$) (Table 3.1.5).

Figure 3.1.5.4.a shows the interaction between agar concentration and tube closure. Shoot vigour seemed largely independent of tube closure and agar strength, except that the combination of 10 g/l agar and normal screw cap produced significantly higher shoot vigour than other combinations.

Shoot vigour decreased linearly with exposure time to BAP ($P<0.001$), but the rate of decrease differed between agar concentrations ($P<0.05$) (Figure 3.1.5.4.b). The higher agar strength (10 g/l) produced higher shoot vigour after 30 days exposure to BAP than did 8 g/l agar, but resulted in similar shoot vigour after shorter exposure times to BAP.

Figure 3.1.5.4.a: Effect of agar concentration and tube closure on shoot vigour of Sturt's desert pea after 30 days culture.

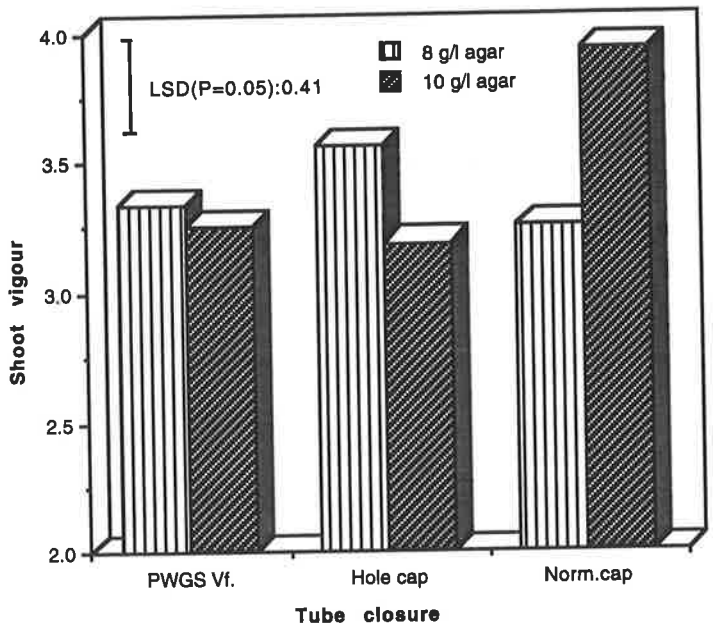
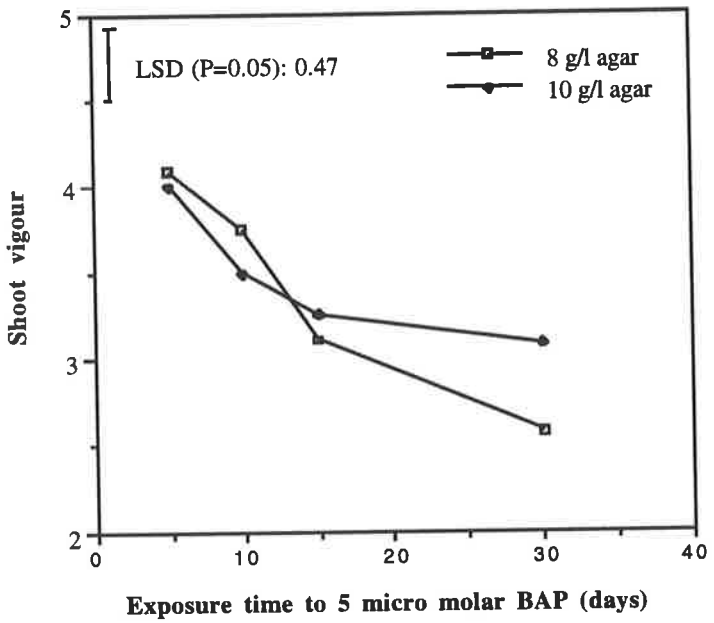


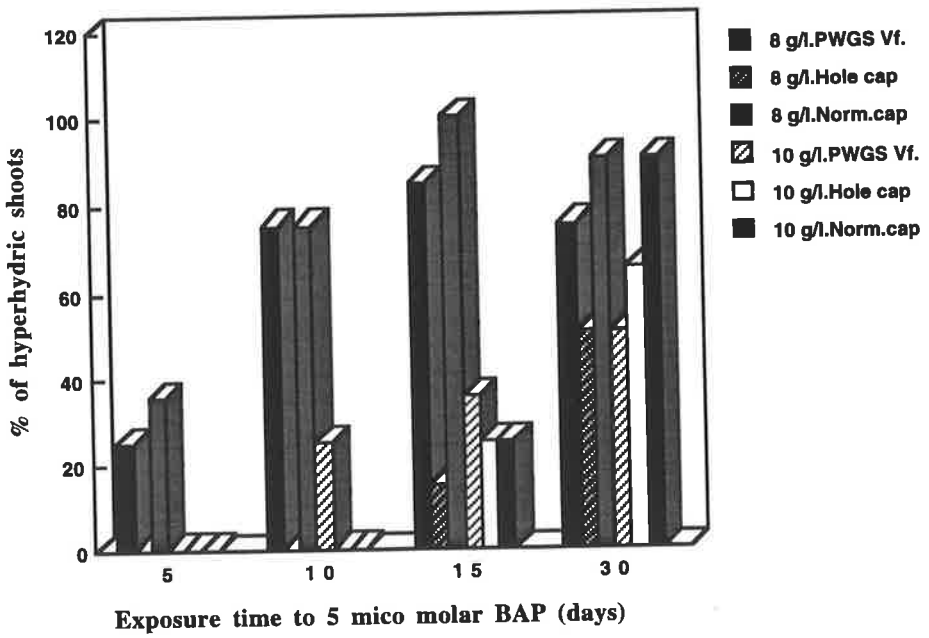
Figure 3.1.5.4.b: Effect of exposure time to BAP and agar concentration on shoot vigour of Sturt's desert pea after 30 days culture.



3.1.5.5 Hyperhydricity

The percentage of hyperhydric shoots with various agar concentrations, different BAP exposure time and tube closure is shown in Figure 3.1.5.5. Hyperhydricity increased with exposure time to BAP, regardless of closure or agar strength. The higher concentration of agar (10 g/l) reduced hyperhydricity at BAP exposure times up to 15 days when compared with 8 g/l agar. PWGS Vitafilm and normal screw caps consistently increased hyperhydricity, while screw cap with a hole minimised hyperhydricity in most treatment combinations, possibly due to its high level of gas and water vapour exchange.

Figure 3.1.5.5: Effect of exposure time to 5 µM BAP, agar concentration and tube closure on hyperhydricity of Sturt's desert pea after 30 days culture.



3.1.5.6 Summary

Time of exposure to 5 μM BAP strongly affected growth, morphogenesis and hyperhydricity in this experiment. Hyperhydricity increased as exposure time to BAP increased but decreased with increasing agar strength and more permeable closures. Callus growth also increased with the increase in BAP exposure time but this was largely minimised as agar strength increased or with closures more permeable to gases. Nevertheless, 10 days exposure to BAP seemed to be the optimum exposure in terms of propagation rate, as the number of shoots decreased following exposures of greater than 10 days. Shoot length and vigour declined as duration of BAP exposure increased and, as before, these parameters remained largely unaffected by agar concentration or closure type.

3.1.6 Tissue water content

This experiment was designed to investigate the relationship between tissue water content and hyperhydricity in Sturt pea cultures.

Analyses of variance of the data for percentage water content indicated that there was a significant difference ($P<0.001$) between hyperhydric and normal shoots. Figure 3.1.6.1 shows that water content of hyperhydric shoots was significantly higher than that of normal shoots at each agar concentration. Water content of hyperhydric or normal shoots did not differ significantly between agar strengths.

Similar correlations between hyperhydricity and water content were observed when tissues were cultured using different closures or different BAP concentration (Figure 3.1.6.2 and 3.1.6.3). In both cases, water content was significantly higher ($P<0.001$) in hyperhydric than in normal shoots. Furthermore, water content of hyperhydric or normal shoots did not differ significantly between closure treatments or between BAP treatments, suggesting that the effect on water content was correlated with hyperhydricity of the tissue rather than with the treatment to which the cultures were subjected.

Figure 3.1.6.1 The water content of hyperhydric and normal shoots of Sturt's desert pea cultured at different agar concentrations after 30 days culture.

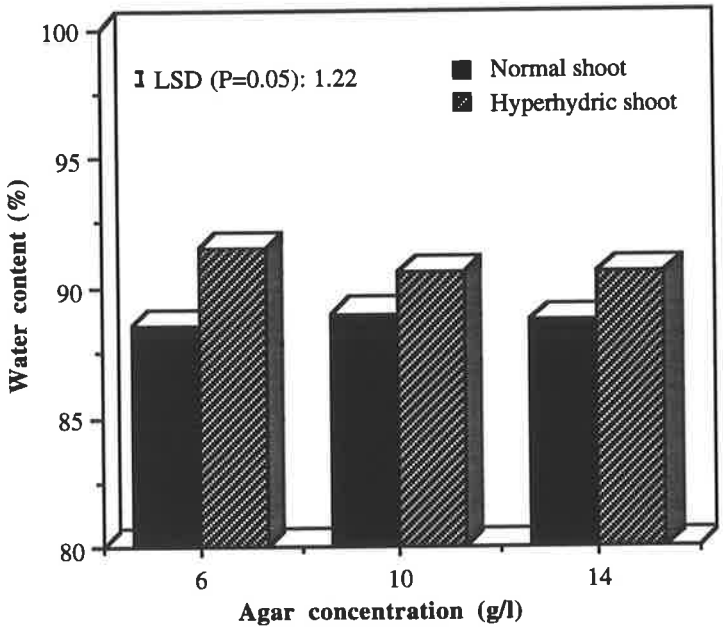


Figure **3.1.6.2** The water content of hyperhydric and normal shoots of Sturt's desert pea cultured with different tube closures after 30 days culture.

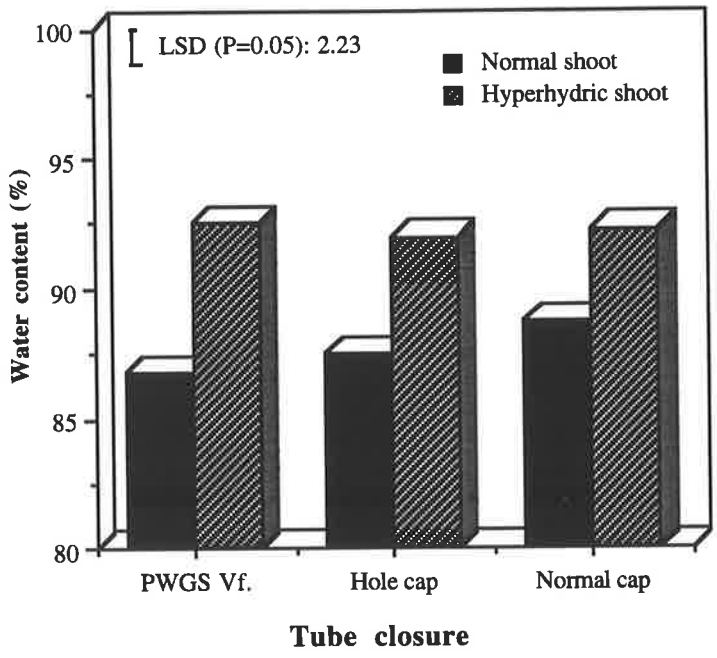
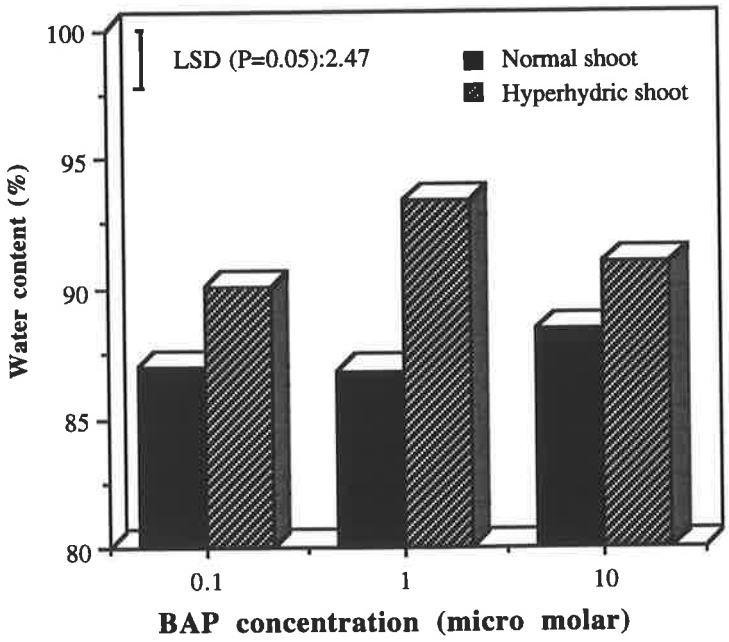


Figure 3.1.6.3 The water content of hyperhydric and normal shoots of Sturt's desert pea cultured at different BAP concentrations after 30 days culture.



3.1.7 Water transmission rate of tube closure and water evaporation rate of support matrix

The rates of water transmission from tube closures and the water evaporation rate from support matrices were tested in the following experiments.

Analyses of variance of water transmission rate data indicated that there were significant differences ($P<0.001$) between the five types of tube closure used in these experiments (Figure 3.1.7.a). Cotton wool had the highest water transmission rate followed by a cap with a hole, MW Vitafilm®, PWGS Vitafilm® and then normal screw cap.

Data for water evaporation rate of five types of support matrix showed a significant difference ($P<0.001$) between the five types of support matrix. As expected, agar had the lowest water evaporation rate, while other support matrices lost water significantly more quickly than agar. Differences in water loss between the other support matrices were non significant (Figure 3.1.7.b).

Figure 3.1.7.a Water transmission rate through five types of tube closure

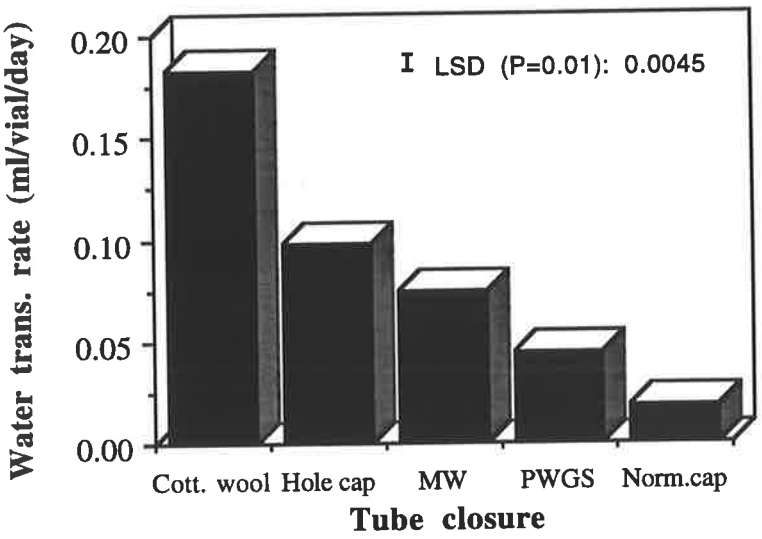


Figure 3.1.7.b Water evaporation rate from five types of support matrix

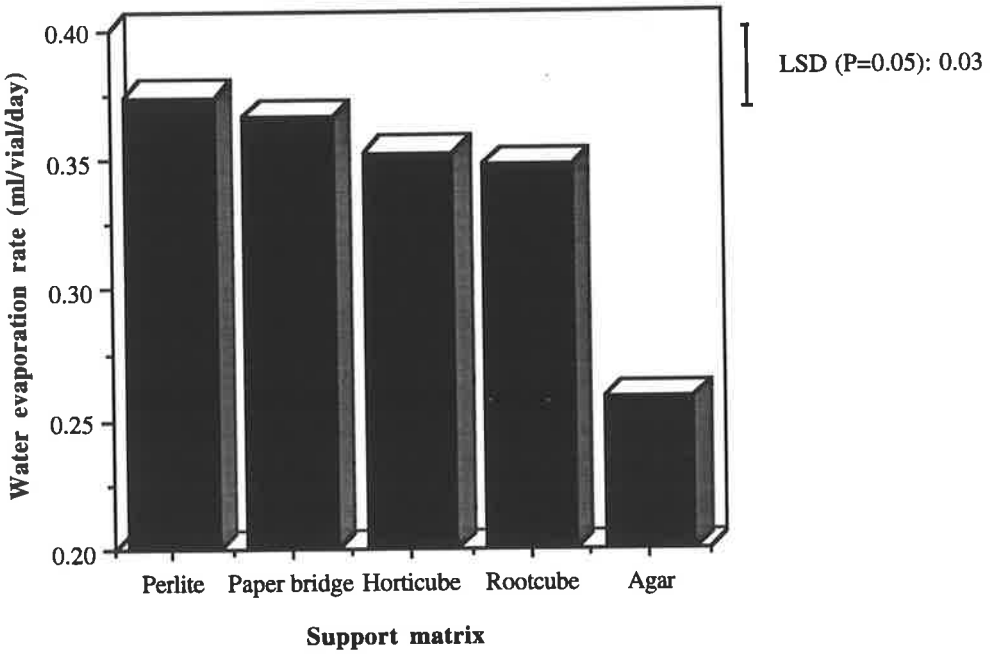


Table 4.1: Summary of results of tissue culture experiments on *Swainsona formosa*

Parameter	Agar concentration	Support matrix	Tube closure	BAP concentration	BAP exposure.
Hyperhydricity	-Declined as agar strength increased	-12 g/l agar & perlite minimised hyperhydricity and paper bridges maximised it	-High permeability reduced hyperhydricity	-Increased as BAP concentration increased	-Increased as BAP exposure increased
Shoot number	-Reduced with increases in agar concentration	-12 g/l and paper bridge the highest and rootcube, the lowest	- Low permeability increased shoot number	-Increased as BAP concentration increased	-Reduced beyond 10 days BAP exposure
Shoot length	-No effect	-Paper bridge the highest & rootcube, the lowest	-Low permeability reduced shoot length	-Reduced with increases in BAP concentration	-Reduced as BAP exposure increased
Shoot vigour	-Increased with agar concentration	-12 g/l agar gave the highest	-Less permeability reduced vigorous	-Reduced with increases in BAP concentration	-Reduced with increases in BAP exposure
Callus width	-Declined with increases in agar strength	-Maximal on12 g/l agar and paper bridge	-Maximal at low permeability closure	-Increased with increases in BAP concentration	-Increased up to 15 days BAP exposure

3.2 Anatomical studies

3.2.1 Anatomical study of shoot apices using a light microscope

Techniques for all anatomical studies of hyperhydric and normal shoots of Sturt's desert pea are outlined in Section 2.2. In this study, 5 normal and 5 hyperhydric shoots were used as replicates and 5 slides were made from each shoot.

A longitudinal section of a normal shoot apex of tissue cultured Sturt's desert pea (Figure 3.2.1 A) shows the basic anatomical structure of this plant. In this section the tunica, which develops into the epidermis, and the corpus, which becomes the cortex and stele, are perfectly differentiated. Tunica and corpus were distinguished based on the orientation of cell division. Tunica cells divide only with the anticlinal wall, but the corpus, covered by the tunica, can divide in any direction. Therefore, corpus cells grow in three dimensions (Mauseth, 1988; Pandey, 1982). In contrast, tunica and corpus layers were not as readily differentiated in a longitudinal section of a hyperhydric shoot (Figure 3.2.1.B). In fact, all tissues were parenchymous. The lack of meristem at the dome of the shoot indicates lack of differentiation and directional growth in this part of the shoot apex.

3.2.2 Scanning electron microscope study of leaf surface

Studies of leaf surfaces using the scanning electron microscope were replicated five times. Five slides were made for each replication.

The scanning electron micrograph shows that the stomata of normal leaves have kidney-shaped guard cells with an outer ledge over the stomatal pore and external periclinal cell walls without deformation (Figure 3.2.2.A).

Figure 3.2.2.B and C show the appearance of stomata from hyperhydric leaves. Projections of the cell wall of guard cells that occlude part of the pore were formed in the stomata in hyperhydric leaves, which was considered to be abnormal (Figure 3.2.2.B). Unknown tissue which plugged the pore of the stoma was the other abnormal character of hyperhydric leaves of Sturt's desert pea (Figure 3.2.2.C).

Normal and hyperhydric leaves of Sturt's desert pea are amphistomatous (stoma are located in both surface of leaf) (Figure 3.2.3 E and F). The stomata have two kidney - shaped guard cells and there are no obvious subsidiary cells, the guard cells appear to be embedded in ordinary epidermal cells (anomocytic type) (Mauseth, 1988) (Figure 3.2.3.A).

3.2.3 Anatomical study of leaves using light microscope

The size of the stomata in the normal and hyperhydric leaves magnified 400 times are shown in Figure 3.2.3.a and b respectively. From these figures, it can be seen that stomata in hyperhydric leaves are larger than in normal leaves . In addition, counts of stomatal numbers per field of vision showed that the density of stomata in hyperhydric leaves was lower than that of normal leaves (Table 3.2.3). Stomates on both normal and hyperhydric leaves were more dense on their abaxial surfaces.

Transverse sections of hyperhydric leaves (400 x magnification) showed that palisade parenchyma and spongy parenchyma were loosely connected. This caused great difficulty in cutting the sections. Palisade parenchyma and spongy parenchyma separated more easily when sections were cut from hyperhydric leaves than from normal leaves. In addition, intercellular spaces were larger in hyperhydric leaves than in normal leaves (Figure 3.2.3 C and D). Sections from embedded leaves (Figure 3.2.3 E and F) showed that the cells of hyperhydric leaves contained fewer chloroplasts than normal leaves.

Table 3.2.3 The density of stomata from hyperhydric and normal leaves of Sturt's desert pea per field of vision (400X magnification).

Type of leaf	Abaxial	adaxial
Hyperhydric leaves	68.1	40.3
Normal leaves	142.1	102.0
LSD (P = 0.05)	15.3	

3.2.4 Transmission electron microscopy of chloroplasts

Ultrastructural analysis of leaves showed that chloroplasts of hyperhydric leaves had larger starch granules and fewer thylakoid layers than normal leaves. (Figure 3.2.4 and Table 3.2.4).

Table 3.2.4 Number of thylakoid layers and length and width of starch granules in the cells of normal and hyperhydric leaves of Sturt's desert pea (± standard error)

Type of leaf	Number of thylakoid layers in each grana	starch granule	
		length (μm)	width (μm)
Hyperhydric	4.82 ± 0.34	156.65 ± 38.56	42.16 ± 7.3
Normal	10.20 ± 0.77	48.89 ± 7.12	25.19 ± 4.83

± was counted from 5 replications.

Figure 3.2.1 Anatomy of Sturt's desert pea shoot apex

A. Longitudinal section of normal shoot apex (400 x).

B. Longitudinal section of hyperhydric shoot apex (160 x).

t. tunica layers c. corpus layers l. leaf primordia

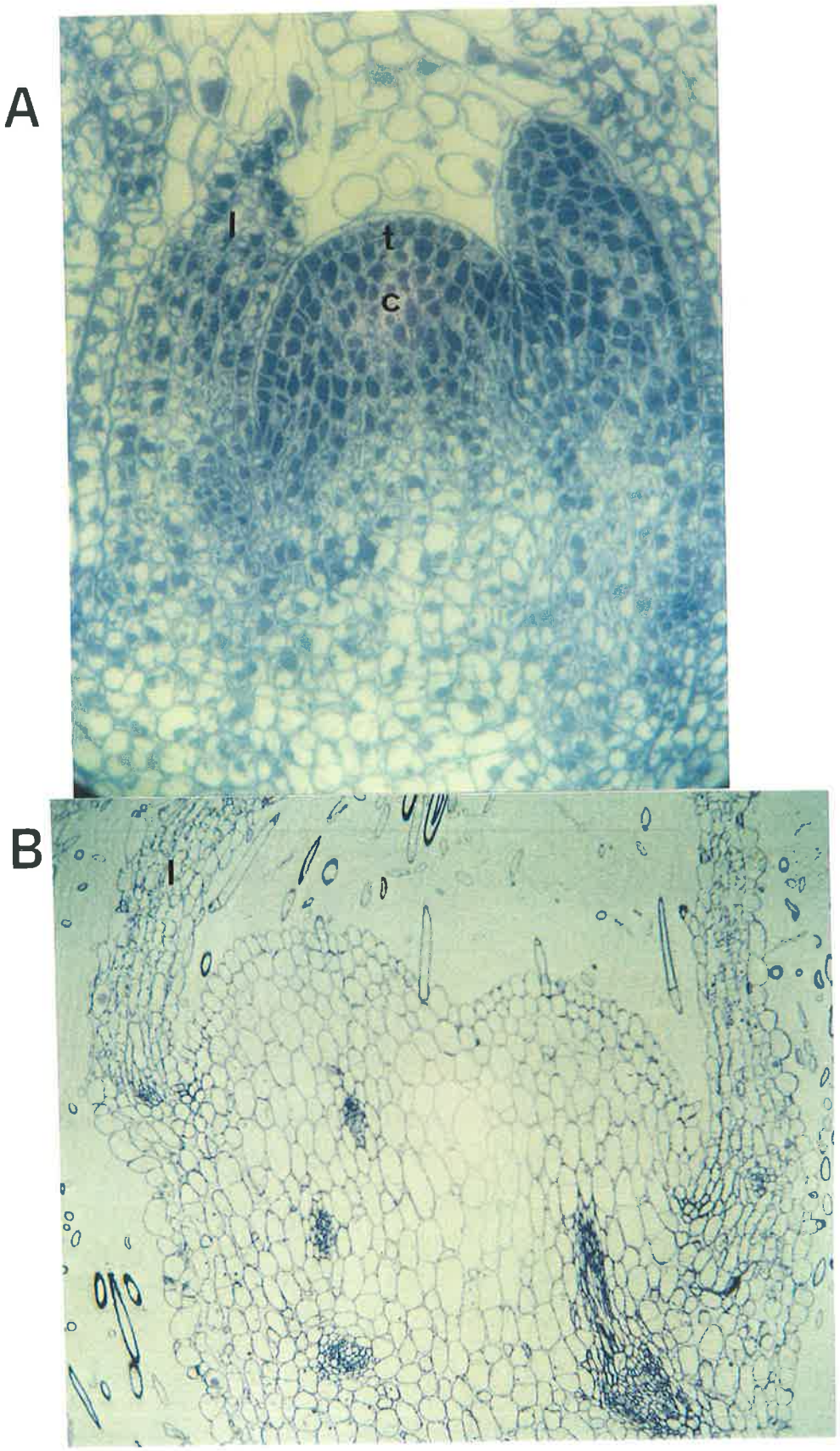

A
l
t
c
B
l

Figure 3.2.2 Scanning electron micrograph of Sturt's desert pea stoma

(Bars indicate 4 μM at figure A, 20 μM at figure B and 10 μM at figure C).

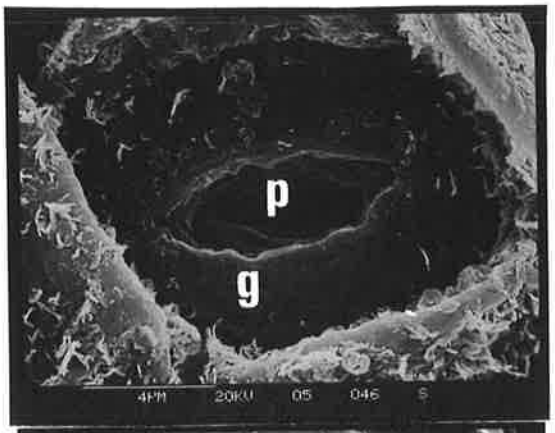
A. Stoma of normal leaf

B. Stoma of hyperhydric leaf

C. Stoma of hyperhydric leaf

g. guard cell p. pore

A



B

C

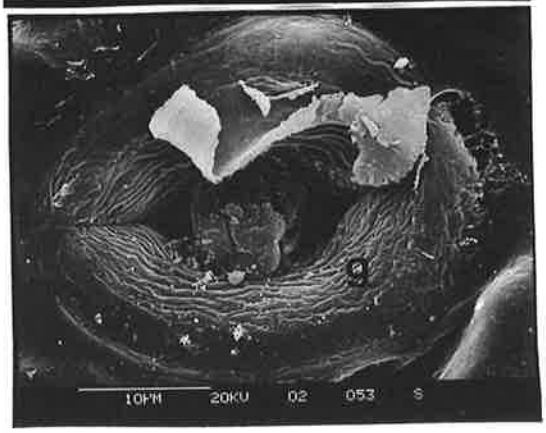


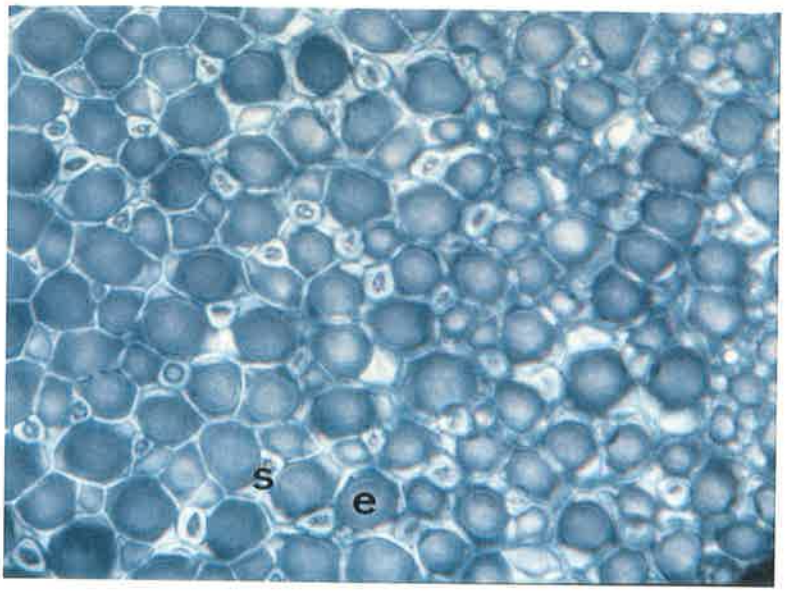
Figure 3.2.3.A and B. The imprint of surface of Sturt's desert pea leaf.

A. The imprint of normal leaf surface (400 x)

B. The imprint of hyperhydric leaf surface (400 x)

s. stoma e. epidermis cells

A



B

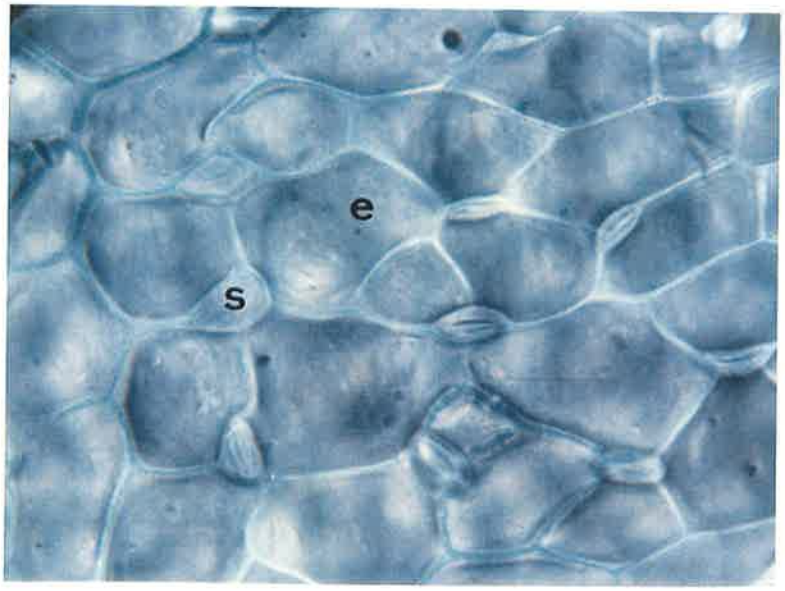


Figure 3.2.3.C - F. Transverse section of Sturt's desert pea leaves

(all photographs of comparable scale)

C. Transverse section of fresh normal leaf (400 x)

D. Transverse section of fresh hyperhydric leaf (400 x)

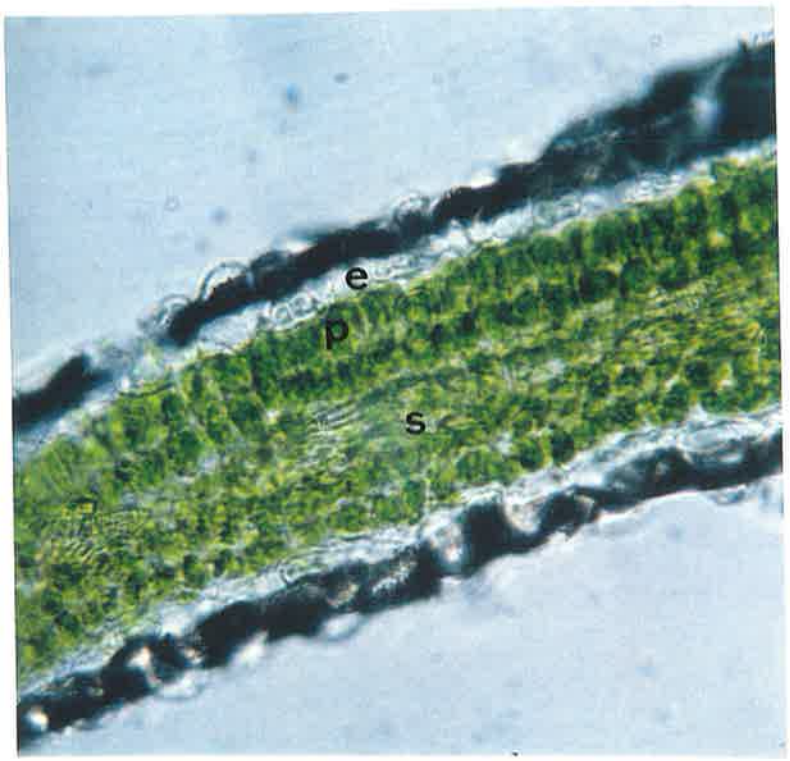
E. Transverse section of embedded normal leaf (400 x)

F. Transverse section of embedded hyperhydric leaf (400 x)

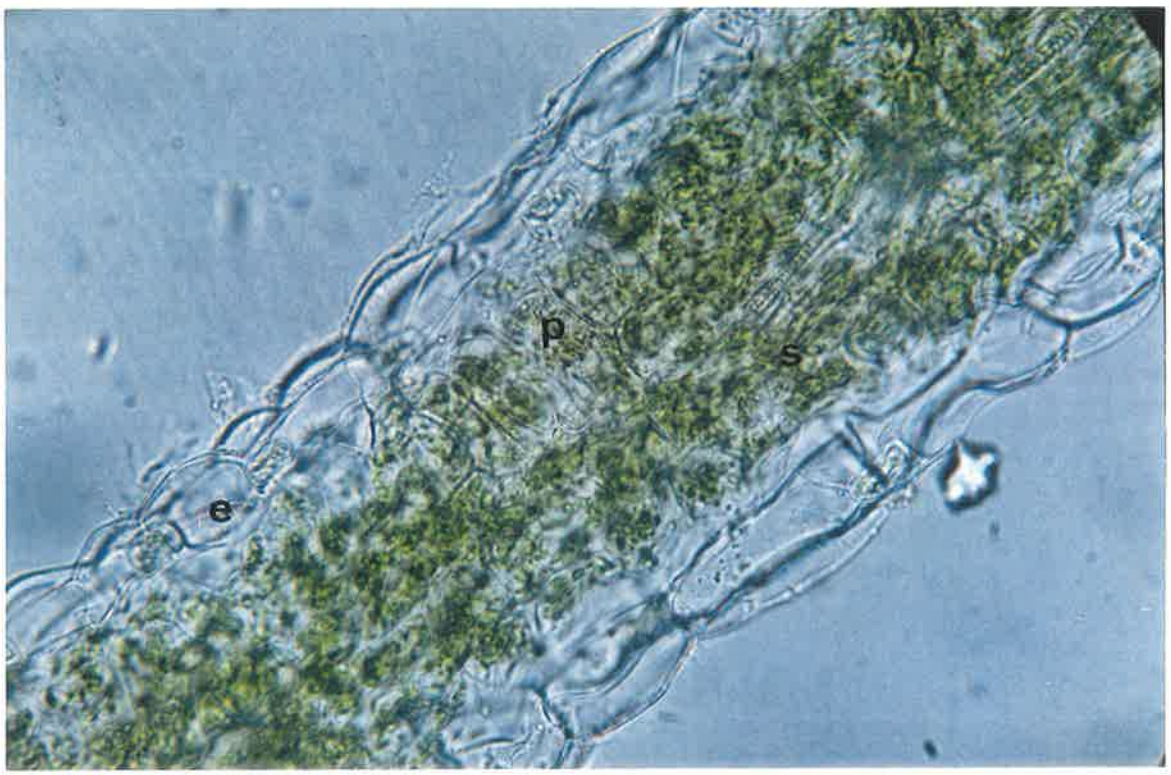
e. epidermis p. palisade parenchyma s.spongy parenchyma

st. stoma

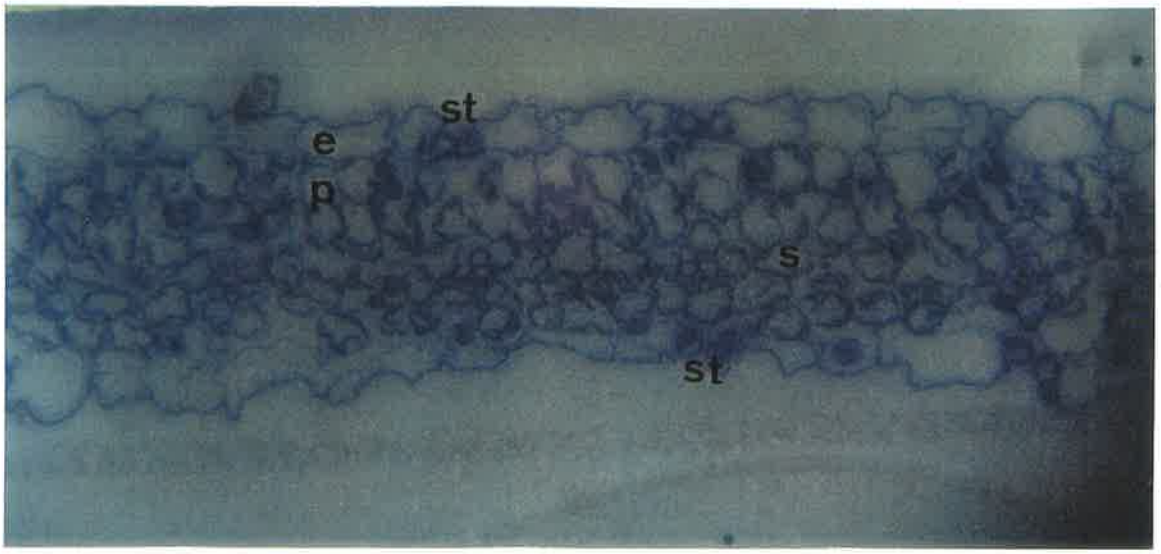
C



D



E



F

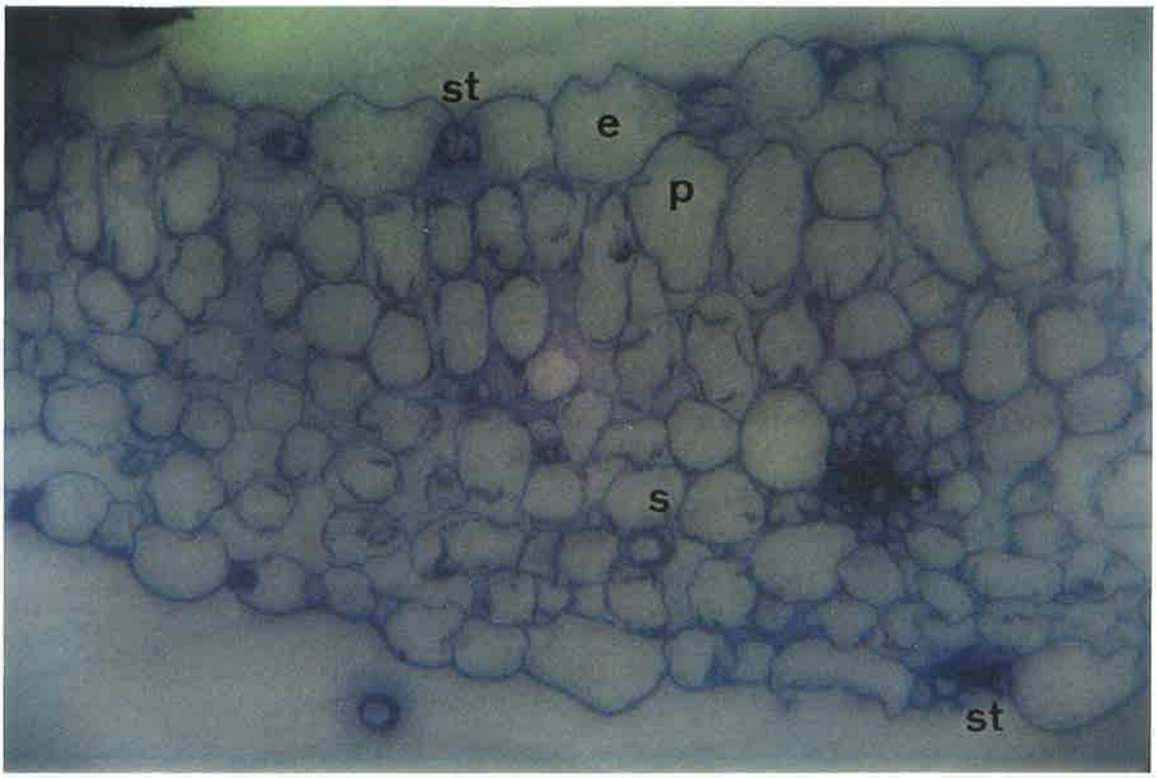


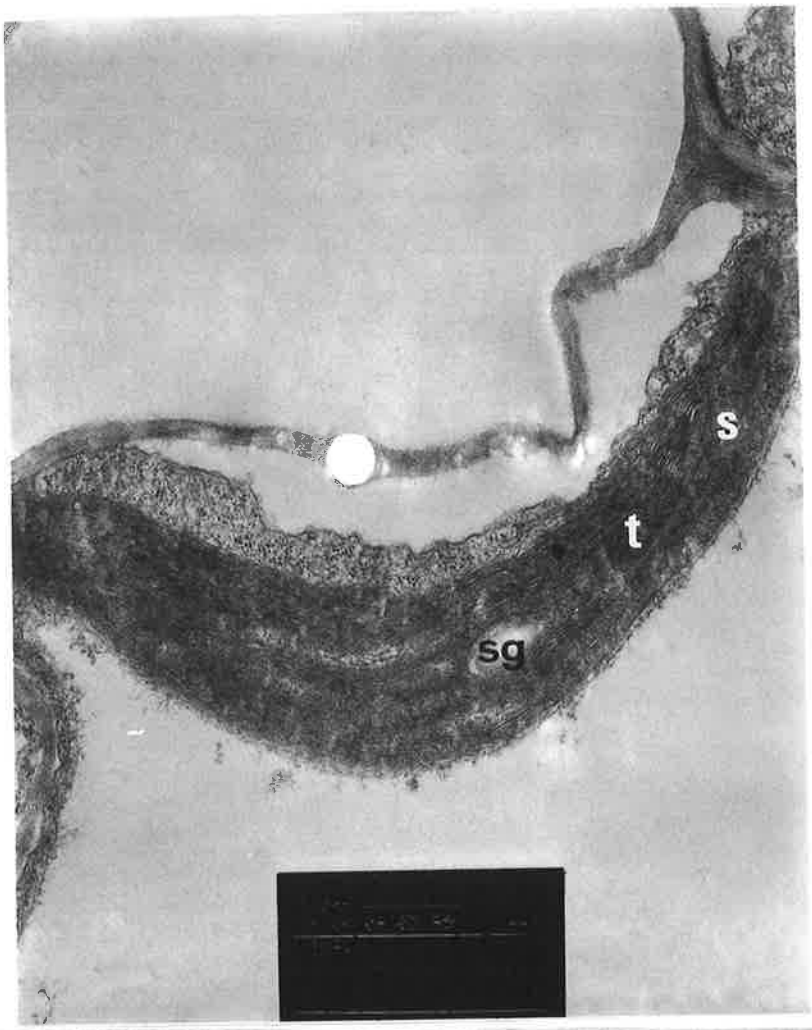
Figure 3.2.4 Transmission electron micrograph of the chloroplast of Sturt's desert pea

A. Chloroplast of normal leaf (37 000x)

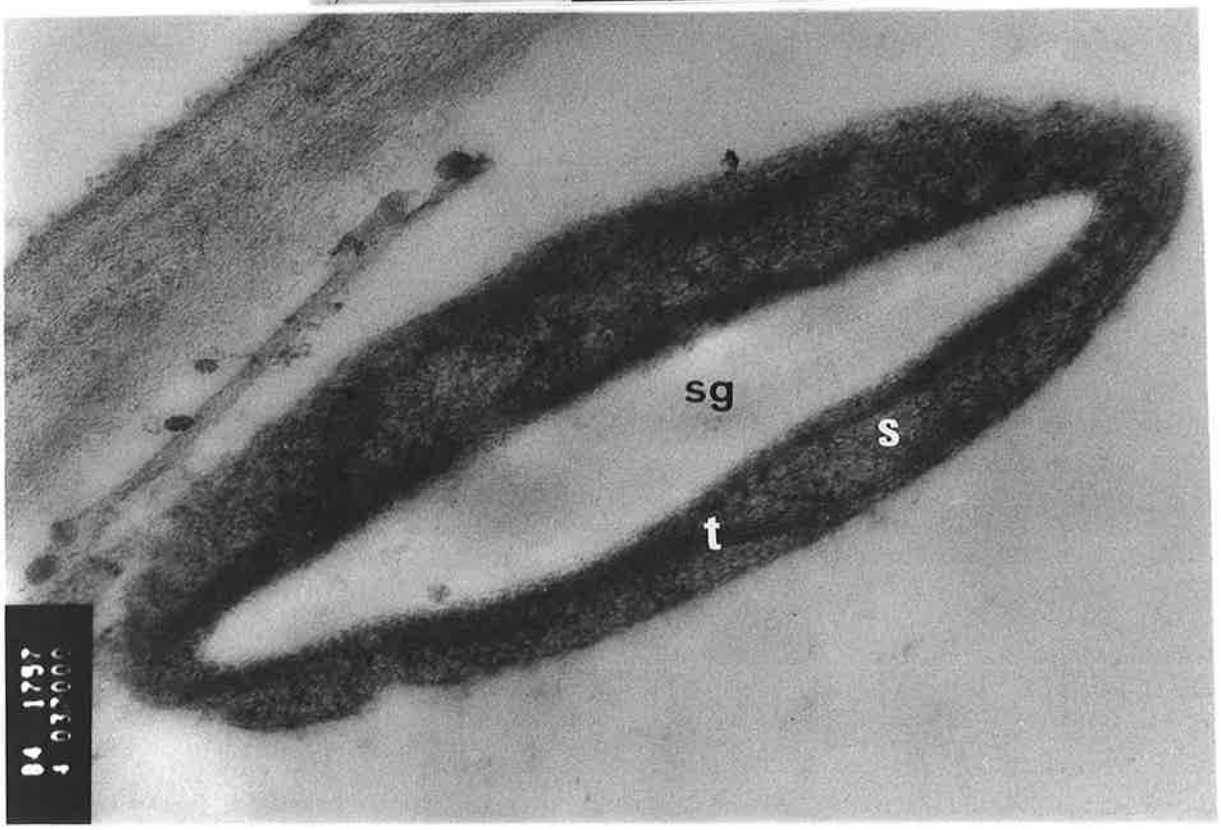
B. Chloroplast of hyperhydric leaf (37 000x)

s. stroma t. thylakoid layers sg. starch granule

A



B



Chapter 4. Discussion

The results of all tissue culture experiments clearly indicated that it was possible to reduce the incidence of hyperhydricity while optimising other desirable growth parameters.

Factors which influenced hyperhydricity in these experiments included the type of support matrix and tube closure, which played a role in controlling medium water content and vial humidity (including gas accumulation) respectively, and cytokinin regime, which played a role in regulation of cell division. In these experiments, various support matrices with different water evaporation rates and various tube closures with different water transmission rates were tested to determine their effect on hyperhydricity in Sturt's desert pea cultures. In the present experiments, increases in agar concentration decreased the incidence of hyperhydricity. Similar results were observed for *Cynara scolymus* (Debergh, 1983), *Dianthus caryophyllus* (Ziv *et al.*, 1983), *Malus pumila* (Pasqualetto *et al.*, 1988), *Gypsophila paniculata* (Han *et al.*, 1991) and *Digitalis obcura* (Lapena *et al.*, 1992). Moreover, the use of highly permeable closures (cotton wool, holed cap) produced less hyperhydricity than did more air-tight closures such as screw caps. Cotton wool, which showed the highest water transmission rate, produced the lowest hyperhydricity and screw caps, which showed the lowest water transmission rate, produced the highest. These results for *Swainsona formosa* are in agreement with those of Rossetto *et al.* (1992), who found that aeration using a cap with a hole covered by double filter paper layers successfully minimised hyperhydricity in *Conostylis wanganensis, Diplolaena andrewsii, Drummondita ericoides, Eremophila resinosa, Eucalyptus 'graniticola', Lechenaultia pulvinaris* and *Sowerbaea multicaulis*. Hakkaart and Versluijs (1983) also found that looser types of closures (such as cotton wool) minimised hyperhydricity in *Dianthus cariophyllus*. The use of perforated lids during the whole culture period also depressed hyperhydricity in *Gypsophila paniculata* (Dillen and Buysens, 1989; Han *et al.*, 1991).

Increases in agar strength and permeability of tube closures also reduced shoot number (to be discussed later). To address this problem, experiments 3 and 4 were design to evaluate various combinations of agar concentration, tube closure type and cytokinin (BAP) regime. The results showed that increases in BAP concentration and BAP exposure time increased hyperhydricity. However, hyperhydricity was also affected by agar concentration and closure type. Thus, high agar strength and high permeability closures reduced hyperhydricity at all levels of BAP concentration and exposure time. The tendency for hyperhydricity to increase with increases in BAP concentration was also reported by other authors, who demonstrated the significance of exogenous cytokinin in the induction and promotion of hyperhydricity (Debergh 1983; Pasqualetto *et al.* 1988; Leshem *et al.* 1988b; McLaughlin and Karnosky 1989; Ma *et al.*, 1990; and Bouza *et al.* 1992).

Hyperhydricity increased in cultures where agar was replaced by alternative support matrices. Agar (12 g/l) was the supperior support matrix at most parameters studied. This may have been at least partly due to the higher water evaporation rate from alternative support matrices, which presumably increased the humidity in the culture vessel, although this was not tested. Agar differed significantly from the other support matrices in having significantly lower water loss from its surface. In these experiments, the combination of agar (lowest water evaporation rate) and cotton wool closures (highest water transmission rate) produced negligible hyperhydricity. The use of support matrices and tube closures also affected other culture attributes. These results showed similarities to those of Matsubara *et al.* (1991), who found that agar produced more normal shoots and greater dry weight than did gelrite and paper bridges in carnation cultures. Higher incidences of hyperhydricity were found in *Malus pumila* (Fausto and Alvaro, 1987) and in *Nicotiana tabacum* (Gorinova *et al.,* 1993) cultured in liquid media than were found on agar media. Moreover, bananas cultured in media gelled with phytagel (no specification) had higher multiplication rates than those in liquid media or on cellulose substrate (Alvard *et al.,* 1993).

The possible process by which stress conditions induce hyperhydricity may be as follows. Poorly aerated tissue and high content of cytokinin concentration induced the emission of large amounts of ethylene in studies of Jackson *et al.* (1991) on *Ficus lyrata* and *Gerbera jasmesonii* and Phan (1991) on *Malus sylvestris* respectively. Excess atmospheric ethylene would tend to retroinhibit its own biosynthesis and consequently decrease the activity of phenylalanine ammonia-lyase (PAL) and acidic peroxidase, thus hindering the lignification processes (Kevers *et al.*, 1984). A deficiency of lignin would reduce wall pressure allowing more water uptake by cells and initiating the hyperhydric malformation. Moreover, the presence of a high level of cytokinin maintains the meristematic cells in a state of active cellular division. Newly-divided cells which are not adequately protected by cell walls because of deficiencies of lignin and cellulose (Kevers and Gaspar, 1985), allow water to diffuse into the cell from the highly humid environment. Thus, this condition results in the enlargement of newly-divided cells as seen in the microscopic study of leaf and meristem tissues. Furthermore, tissue water content of hyperhydric shoots was shown to be significantly higher than that of normal shoots. The condition of cell enlargement in hyperhydric shoots was also observed in epidermal cells of hyperhydric leaves, producing lower stomata densities on hyperhydric leaves than on normal leaves. This was in agreement with the results of Yassen *et al.* (1992), who found that hyperhydric leaves of soybean, chicory and carnation had fewer stomata per unit area than non-hyperhydric leaves.

Abnormal stomata on hyperhydric leaves may be a result of plant responses in reducing water or gas uptake from the culture vessel due to poor ventilation. Although no data about gas accumulation inside tubes was generated in this study, the research of Jackson *et al.* (1991) on *Ficus lyrata* and *Gerbera jasmesonii* found that poorly ventilated tubes had higher accumulated gas than ventilated tubes. This was similar to results of Miguens (1993) and he suggested that deformations of the guard cell of hyperhydric *Datura insignis* may be due to erroneous deposition of cell wall constituents or deformation of the cells.

Hyperhydric leaves showed larger intercellular spaces than normal leaves. Moreover, the palisade parenchyma and the spongy parenchyma of hyperhydric leaves were loosely connected compared with those of normal leaves. These cell layers were not dissimilar in structure to those of many hydrophytic and aquatic plants which have undifferentiated mesophyll and aerenchyma, respectively (Mauseth, 1988). Therefore, it is suggested that the structure of hyperhydric leaves was influenced by environmental factors. These results are in agreement with those of Rossetto *et al.* (1992) on *Conostylis wonganensis*, Von Arnold and Erriksson (1984) on *Picea abies* and Williams and Taji (1991) on *Olearia microdisca.*

Callus width and shoot proliferation increased with increases in BAP concentration or exposure time to BAP. However, 10 days exposure to 5 μM BAP was the optimal treatment for producing shoots, since shoot numbers declined with BAP exposures of greater than 10 days. Humidity and gas accumulation in the culture vessel, and water content in the medium (as varied by tube closure and agar strength, respectively) also seemed to play important roles in callus growth and propagation ratio. It was shown that increases in agar concentration or the use of more permeable tube closures tended to reduce the width of callus and the number of shoots. Debergh *et al.* (1981) stated that increases in agar concentration might reduce the availability of cytokinin. This was stated from the fact that the propagation ratio of globe artichoke cultures was reduced to one on the medium without cytokinin, as well as the medium with a high gel concentration. Similar results were achieved in *Dianthus caryophyllus* and in *Gypsophila paniculata* cultured in different gel concentrations (Hakkaart and Versluijs, 1983; and Han *et al.,* 1991, respectively). Other similarities were found in the research of Leshem *et al.* (1988), who found that BAP strongly increased shoot number and encouraged the growth of friable callus on melon plantlets. In addition, reducing cytokinin concentration in European larch cultures reduced adventitious bud production (McLaughlin and Karnosky, 1989).

In contrast to shoot number, length of shoots decreased with increases in BAP concentration or exposure time. However, agar concentration did not affect this parameter. Furthermore, the length of shoots decreased with increasing permeability of closures, although this effect was not observed in experiment one due to the different support matrices used. These results are in agreement with those of Dillen and Buysens (1989) and Han *et al.*(1991), who found that permeable lids produced shorter plantlets of *Gypsophyla paniculata* than did normal plastic lids. The effects of cytokinin on shoot number and growth were similar to those found in the research of Lauzer and Vieth (1990) on *Cyanara scolymus*, where the number of shoots was increased in the presence of BAP although the size of the shoots was reduced. Moreover, Williams and Taji (1991) found that cytokinin stimulated bud production in *Olearia microdisca* cultures.

Anatomical studies of hyperhydric shoots showed them to have shorter internodes and wider stems than normal shoots, lack of clear differentiation in the developing parts of the shoot apex, and meristems composed predominantly of parenchyma. This suggested reduced meristematic activity and resultant poor shoot growth. Similar results were found by Werker and Leshem (1987) for *Dianthus caryophyllus*. They suggested that the earlier cessation of meristematic activity in the stem and leaves may well be the cause of various structural abnormalities.

Shoot vigour also declined with increases in BAP concentration and exposure. In contrast, vigour tended to increase with increases in agar strength. Tube closures seemed to have less effect, and the results were not consistent between the experiments. In these experiments, hyperhydric shoots were low in vigour (leaves showed light green colour, some of them were chlorotic, plantets were broad and stems thick). Light green colour or chlorosis may be due to a reduction in chloroplast numbers in the mesophyll cells of hyperhydric leaves compared with normal leaves. The ultrastructure of chloroplasts showed that those from hyperhydric leaves had larger starch granules and fewer thylakoid stacks than did the chloroplasts of normal leaves. These abnormalities in the ultrastructure of chloroplasts suggested that the chloroplasts were not functioning

normally, and may be affecting the vigour of cultures. However, further research is required to elucidate the physiological affects of hyperhydricity on chloroplast function.

From results discussed above, it can be seen that increases in agar concentration, permeability of tube closure and decreases in BAP concentration reduced the incidence of hyperhydricity, shoot number, callus width but increased shoot vigour. Therefore, it can be recommended that 10 g/l agar, screw cap with a hole and 1μM BAP are the best condition in minimising the incidence of hyperhydricity on Sturt's desert pea while optimising other desirable growth parameters.

Chapter 5. Conclusions

Results obtained during this study showed techniques for reducing hyperhydricity in the micropropagation of Sturt's desert pea. The effects of support matrix, tube closure and cytokinin regime were examined in relation to hyperhydricity and various parameters of culture growth. Solid agar media were found to be optimal for minimising hyperhydricity. Increasing agar concentration reduced hyperhydricity. Moreover, hyperhydricity was reduced by fitting vials with of high permeability closures. However, increasing closure permeability also reduced the culture proliferation rate. The combination of 1 μM BAP, 10 g/l agar and screw cap with a hole produced the best result in minimising hyperhydricity while maximising the performance of other growth parameters.

To improve our understanding of hyperhydricity, the anatomy of hyperhydric shoots was investigated. Abnormal tissue was found in between the two guard cells in stomata. Furthermore, the density of stomata was higher in normal leaves than in hyperhydric leaves, the mesophyll cells in hyperhydric leaves contained fewer chloroplasts than in normal leaves, and the chloroplasts of hyperhydric leaves had larger starch granules and fewer thylakoid stacks than did normal leaves. Moreover, the structural components of the hyperhydric apical meristem were found to be inadequately differentiated, suggesting reduced meristematic activity in these cells.

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