# TISSUE CULTURE STUDIES ON

# *PEPEROMIA CLUSIFOLIA* HOOK. AND

# *STRONGYLODON MACROBOTRYS* A. GRAY

By

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# PREFACE

I hereby declare that this thesis, submitted for the degree of Master of Science in the Department of Botany, University of Natal, Pietermaritzburg, is the result of my own investigation, except where acknowledgement of other work is specifically indicated in the text.

The experimental work described in the thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg from January 1981 to December 1981, under the supervision of Dr N.A.C.Brown.

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## ABBREVIATIONS USED IN THE TEXT

| | |
|---|---|
| BA | $N^6$ - benzyladenine (=BAP) |
| BAP | 6 - benzylaminopurine (=BA) |
| IAA | Indole - 3 - acetic acid |
| IBA | Indole - 3 - butyric acid |
| K | Kinetin |
| NAA | $\alpha$ - naphthaleneacetic acid |
| 2iP | $N^6$ - isopentenyladenine |
| 2,4-D | 2,4 - dichlorophenoxyacetic acid |
| 2,4,5-T | 2,4,5 - trichlorophenoxyacetic acid |

## FRONTISPIECE - TOTIPOTENCY

Nothing is separate
Nothing's alone
Separation only makes it clear

Wondrous spark of light
Is made of many parts
These aren't apart at any height

Oh plant, I see you as you are
As myself, part of the whole
We together are complete

Oh leaf, I know you as you are
Cell upon cell, part of your whole
Rich green fabric woven so well

Each cell contains the secret within
Each one knows where the other has been

Separation will make the study complete
Which will you be - root, stem, or leaf?

You hold the power deep within
To always return to wherever you've been

Ever-present, power and might
Lulling grace, wondrous and quiet

Sharon Maraffa.

## ABSTRACT

Tissue culture studies were carried out on two ornamental plant species, *Strongylodon macrobotrys* A. Gray and *Peperomia clusifolia* Hook. Successful *in vitro* regeneration of plantlets was achieved in the latter species, using leaf and stem explants. The basal medium of Murashige and Skoog (1962), in combination with various levels of NAA and K, was utilised.

*Strongylodon* proved refractory to both establishment of a thriving callus culture and *in vitro* formation of roots and shoots. Several media were utilised, Miller's (1963) medium proving the most successful for the production of callus. Different combinations of the growth regulators NAA, IBA, BA and K were used to determine optimum levels of these substances for callus production. Root/shoot induction studies were carried out using the basal medium of Miller (1963) plus various concentrations of IBA in combination with K or BA. Alternatively, the basal medium was used without added growth regulators. Internodes, nodal segments, leaves, pulvini, flower parts and seeds were used in the study. No plantlets were obtained from *Strongylodon* explants.

CONTENTS

# GENERAL INTRODUCTION

The main intent of clonal propagation is to establish plants which are uniform and predictable of certain selected qualities (Murashige, 1976). In view of the substantially superior rate of plant increase, interest in the use of tissue cultures as an alternative to traditional asexual multiplication methods is currently spreading rapidly. Cell, organ and tissue culture techniques are today a valuable aid in many economically oriented activities, not the least of these being the commercial production of ornamental plants. Oglesby (1978) envisaged this method of propagation becoming a common procedure in nurseries in the U.S.A.and suggested that nurserymen view it as a helpful tool and not as a costly technique. The use of tissue culture for cloning ornamentals is, however, expensive and is presently limited to a certain number of species (Debergh and Maene, 1981).

Oglesby (1978) reported that, providing good starting plants and clean stock are utilized, plants derived from tissue culture were extremely uniform, grew faster and were of a superior quality when compared with those obtained using more conventional methods of propagation. The technique is not limited by factors such as seasonal availablility of seed and is particularly useful for the propagation of plants in which normal reproduction is slow. Variations in growth or habit due to propagation from seed may be eliminated and pathogen-free plants are recoverable from clones which are systemically infected. Tissue cultures are helpful during the initial establishment of large quantities of stock plants and also in

minimizing the number which must be retained as stock plants. Facilities and costs required to maintain stock plants are thus reduced and greenhouse space in the commercial nursery can be devoted to plants for sale (Oglesby, 1978). Rapid clonal increase also signifies faster and timely establishment of new cultivars and international exchange of genetic stocks is expedited and rendered safer (Murashige, 1976).

Tissue culture differs from the traditional methods of vegetative propagation primarily in the conditions under which the propagation is established, and not in the principles used (Murashige, 1978). Smaller propagules are utilised, an aseptic, artificially controlled environment is maintained and the propagules develop heterotrophically. Table 1 lists some ornamental genera which have, to date, shown potential for clonal multiplication through tissue culture. This list excludes the orchids,since at least 20 different orchid genera have been propagated successfully in this manner and the group could be dealt with separately. It is, however, interesting to note that general interest in the use of tissue cultures in plant propagation is attributable to the commercial success achieved with orchids (Murashige, 1974 ).

The production of extremely large numbers of plants from a single plant can be realized theoretically by the use of tissue culture techniques. Drew (1980) for example, detailed a procedure whereby 100 000 pineapple plants could be produced from a single shoot in 12 months and Oglesby (1978) cites the production of 20 000 *Hemerocallis* (daylily) plants from 30 explants in the space of 8 months. It is, however,

usually impractical to handle such numbers in a commercially acceptable manner and an economic method for commercial utilization needs to be formulated in most cases (Holdgate, 1977).

This project was concerned with the feasibility of the application of the technique of rapid clonal multiplication through tissue culture to the propagation of two dicotyledonous ornamental species, *Peperomia clusifolia* Hook. and *Strongylodon macrobotrys* A. Gray. The genus *Pepromia* has been the subject of previous tissue culture investigations (see Table 1) but, to date, *Strongylodon* has not been propagated using these methods.

Table 1 Some ornamental plants with demonstrated potential for clonal multiplication through tissue culture (adapted from Holdgate, 1977 and Murashige, 1974).

| Family | Genus | Reference |
|---|---|---|
| Agavaceae | *Dracaena* | Murashige (1974) |
| Amaryllidaceae | *Hippeastrum* | Mii, Mori & Iwase (1974) |
| | *Ipheion* | Hussey & Wyvill (1973) |
| | *Amaryllis* | Murashige (1974) |
| | *Narcissus* | Hussey & Wyvill (1973) |
| | *Alstroemeria* | Ziv, Kanterovitz & Halevy (1973) |
| Araceae | *Philodendron* | Murashige (1974) |
| | *Anthurium* | Pierik, Steegmans & van der Meys (1974) |
| Asclepiadaceae | *Hoya* | Marraffa, Sharpe, Tayama & Fretz (1981) |
| Asteraceae | *Chrysanthemum* | Hill (1968) |
| | | Ben-Jaacov & Langhans (1972) |
| | | Roest & Bokelmann (1973) |
| | *Gerbera* | Pierik, Steegmans & Marelis (1973) |
| Begoniaceae | *Begonia* | Ringe & Nitsch (1968) |
| | | Shigematsu & Matsubara (1972) |
| | | Fonnesbech (1974) |
| Blechnaceae | *Woodwardia* | Murashige (1974) |
| Bromeliaceae | *Aechmea* | Murashige (1974) |
| | *Ananas* | Murashige (1974) |
| | *Cryptanthus* | Murashige (1974) |
| | *Cryptbergia* | Murashige (1974) |
| | *Dyckia* | Murashige (1974) |
| | *Bilbergia* | Murashige (1974) |
| Caryophyllaceae | *Dianthus* | Hackett & Anderson (1967) |
| | *Phlox* | Konar & Konar (1966) |
| Cyathaceae | *Alsophila* | Murashige (1974) |

Table 1 continued

| Family | Genus | Reference |
|---|---|---|
| Daviliaceae | *Nephrolepis* | Murashige (1974) |
| Euphorbiaceae | *Euphorbia* | De Langhe, Debergh, & van Rijk (1974) |
| | *Kalanchoë* | Murashige (1974) |
| Geraniaceae | *Pelargonium* | Chen & Galston (1967)<br>Pillai & Hildebrandt (1968) |
| Gesneriaceae | *Gloxinia* | Haramaki & Murashige (1972) |
| | *Streptocarpus* | Appelgren & Heide (1972) |
| | *Saintpaulia* | Kukulczanka & Suszynska (1972) |
| Iridaceae | *Freesia* | Bajaj & Pierik (1974)<br>Davies (1971) |
| | *Gladiolus* | Ziv, Halevy & Shilo (1970) |
| | *Sparaxis* | Hussey & Wyvill (1973) |
| | *Schizostylis* | Hussey & Wyvill (1973) |
| Liliaceae | *Lilium* | Robb (1957)<br>Hackett (1969a;1969b) |
| | *Convalleria* | Sheridan (1968) |
| | *Hyacinthus* | Pierik & Woets (1971) |
| | *Tulipa* | Hussey & Wyvill (1973) |
| | *Scilla* | Hussey & Wyvill (1973) |
| Marantaceae | *Calathea* | Murashige (1974) |
| Peperomiaceae | *Peperomia* | Kukulczankska, Klimaszewska & Pluta (1977)<br>Henny (1978)<br>Berry (1978)<br>Hui & Zee (1981) |
| Primulaceae | *Cyclamen* | Stichel (1959) |
| Pteridaceae | *Adiantum* | Murashige (1974) |
| | *Microlepia* | Murashige (1974) |
| | *Pteris* | Bristow (1962) |
| Rosaceae | *Rosa* | Hill (1967) |
| Solanaceae | *Petunia* | Rao, Handro & Harada (1973) |

# 1. LITERATURE REVIEW

The literature covered in this review includes some generalized techniques (in order to obtain a basic understanding of the concept of tissue culture) as well as information pertaining solely to this project. The emphasis remains on *in vitro* propagation methods for ornamental plants and plants belonging to the same family or genus as *Peperomia clusifolia* (Peperomiaceae) and *Strongylodon macrobotrys* (Leguminosae).

## 1.1 Historical Perspective

Haberlandt's first attempts at plant cell culture in 1902 were aimed at developing a versatile tool to explore morphogenesis and to demonstrate the totipotency of plant cells. In explaining his failure to obtain indefinitely maintainable cultures, he proposed that certain hormones played critical roles in regulating cell division, enlargement and other morphogenetic phenomena. He predicted that totipotentiality of plant cells would be demonstrable through cultured cells. In the 80 years since Haberlandt formulated these ideas they have been verified and put into almost everyday use.

The first successful *in vitro* culture was organ culture. Robbins (1922) and Kotte (1922) (both cited by Gresshof,1978) developed media in which excised root tips continued to divide and grow to produce cultured roots. In 1934 White reported the successful tissue culture of tomato roots and Gautheret (1939) and Nobécourt (1939), working independently on *Daucus*, achieved the first successful callus cultures.

White (1939b) also reported the successful isolation of an indefinitely maintainable tobacco callus. Van Overbeek, Conklin and Blakeslee (1941) and Steward and Caplin (1952) made contributions to the development of a procedure which permitted establishment of callus cultures of diverse species, utilizing auxin and coconut milk as additives to the basal medium. Identification of the active principle in coconut milk followed after some years. Miller, Skoog, Okumura, von Saltza and Strong (1955) reported the isolation of kinetin from herring sperm DNA, and established its function as a substance promoting cell division. Kinetin-like substances were only positively identified in coconut milk in 1964 by Shaw and Srivastava.

The rooting of *Lupinus* and *Tropaeolum* shoot apices by Ball (1946) led to the demonstration by Morel and Martin (1952) that virus-free plants could be recovered from infected plants via shoot-tip cultures. In extending these observations to orchid shoot-tip culture, Georges Morel (1960) paved the way for the commercial application of tissue culture techniques. The current wave of tissue culture activity with other crops is an outgrowth of the success with orchids (Murashige, 1976). However, the identification of plant hormones and the disclosure of their roles in organogenesis was a prerequisite for the widespread application of tissue culture. In 1934 Kögl, Haagen-Smit and Erxleben identified the first plant hormone, the auxin IAA, and Skoog (1944) showed that auxin could stimulate root formation and inhibit shoot formation. Further studies by Skoog and his co-workers (Skoog and Tsui, 1948 and 1951; Miller and Skoog, 1953) culminated in the discovery of

the relationship of auxin : cytokinin balances to root and shoot initiation (Skoog and Miller, 1957). This classical finding has guided the development of *in vitro* propagation procedures of virtually all plants.

Observations of embryogenesis from somatic carrot cells *in vitro* (Reinert, 1958; Steward, Mapes and Mears, 1958) have also been influential in efforts to multiply plants clonally. An understanding of the concept of meristemoids (Torrey, 1966) is another fundamental step in achieving organized development in excised plant tissue.

Today a multitude of plants are cultured *in vitro* for a variety of different purposes. The use of tissue culture in the field of ornamental species is advancing rapidly and the following sections deal with the progress made in the families Peperomiaceae and Leguminosae.

### 1.1.1 Peperomiaceae

Literature available on the *in vitro* regeneration of members of the family Peperomiaceae is not extensive, information being limited to some species of the genus *Peperomia*.

Initial attempts at the *in vitro* culture of *P. sandersii* by Harris and Hart (1964) were a failure. 'Attempts to grow the (leaf) squares in sterile culture failed and of the many surface sterilising agents tried all either caused severe damage to the squares or failed to give sterility'. Subsequent investigations proved more successful. Kukulczanka, Klimaszewska and Pluta (1977) investigated the regenerative

potential of *P. scandens in vitro*. These workers utilised a basic Murashige and Skoog (1962) medium with NAA, K and 2,4-D (separately, or in combination) as growth regulators. The season of the year appeared to be an important factor contributing to regenerative potential and plantlets were obtained in all experiments. Explants from the basal part of the leaf and from the petiole produced the greatest number of plantlets. In 1978, Henny reported a method of propagating *Peperomia* 'Red Ripple' from leaf discs *in vitro*. The medium of Schenk and Hildebrandt (1972) was used in conjunction with various concentrations of NAA and K. Numerous shoots were produced in high K/ low NAA treatments and levels of NAA higher than 0,05 $mgl^{-1}$ resulted in callus development, root formation and media discolouration. Newly-formed shoots were rooted and the rooted plantlets could then be transplanted.

The investigations of Berry (1978) attempted to determine the influence of the explant source, chemical composition of the medium and light/dark incubation on callus development and root and shoot differentiation of *P. caperata*. The following conclusions were made (S. Berry - pers. comm.)* :

(1) Basal sections of leaves removed from the lower stem exhibited rapid development *in vitro*.

(2) Maximum callus formation occurred with high auxin ($3mgl^{-1}$ IAA, IBA, NAA or 2,4-D).

(3) Maximum root formation was observed on media with a low vitamin level (0,05 $mgl^{-1}$ nicotine, pyrodoxine, pentathion-

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-ate and riboflavin and 0,1 $mgl^{-1}$ thiamine), high auxin and low cytokinin (0,08 $mgl^{-1}$ K, BAP or 2iP).

(4) Medium vitamin levels (ten times the concentration of the 'low' levels given above), low auxin (0,03 $mgl^{-1}$) and low or high (one hundred times the 'low' level) cytokinin resulted in maximum shoot production.

(5) Dark incubation followed by light induction produced the best overall growth.

The basal medium used in all these treatments was that of Murashige and Skoog (1962).

Hui and Zee (1981) did not obtain consistently good yields of regenerated plants of *Peperomia* species using a defined medium supplemented with hormones. These workers thus found it necessary to propagate *P. viridis in vitro* from leaf discs on a medium supplemented with ginseng powder in addition to hormones. Korean ginseng (*Panax ginseng*) contains triterpenoid ginsenosides (Staba, 1977) and the powdered compound had been used previously to enhance plantlet regeneration in *Brassica* species (Hui and Zee, 1980). The basal medium used in these *Peperomia* experiments was that of Murashige and Skoog (1962). High regeneration of plantlets (up to 60%) was obtained on medium containing 2 $mgl^{-1}$ K and 1 $mgl^{-1}$ NAA but yields were not consistent. The addition of ginseng powder increased plantlet regeneration, number of shoots formed per leaf explant and increased the quality of the plantlets.

### 1.1.2 Leguminosae

Scowcroft and Adamson (1976) stated that despite the considerable use of legumes in tissue culture studies, cell cultures of legume species have proved refractory to plant regeneration. Boulter and Crocomo (1979) made a similar observation, namely that legumes in general have shown a poor ability of isolated tissues and cells to regenerate into whole plants.

Although *in vitro* legume cultures were first established in the 1950's by Nickell (1955; 1956), callus, suspension or regeneration cultures of several legume species have been achieved only recently (Phillips and Collins, 1979). Soybeans (*Glycine max*) have been cultured extensively since the 1960's (Miller, 1963) but to date no regeneration of plants from undifferentiated tissues has been achieved. Cell suspension and/or callus cultures have been described for several legumes, including garden, field, crop and forage species (Oswald, Smith and Phillips, 1977). Table 2 gives an up-to-date summary of these species.

With the exception of alfalfa (Saunders and Bingham, 1972; Bingham, Hurley, Kaatz and Saunders, 1975), regeneration of plantlets from callus cultures has not been extensive or reproducible (Bottino, Maire and Goff, 1979). In the majority of reports on regeneration of plantlets from legume callus, the primary explant has been excised from seedling and meristematic tissue (Gregory, Haq and Evans, 1980). These latter workers published the first report of plantlet regeneration

Table 2 List of some leguminous species for which suspension and/or callus cultures have been described.

| Species | Common name | Reference |
|---|---|---|
| *Canavalia ensiformis* | Jack bean | Collins, Vian & Phillips (1978) |
| *Glycine max* | Soybean | Philips (1974a;1974b) |
| *Medicago sativa* | Alfalfa | Bingham, Hurley, Kaatz & Saunders (1975) |
| *Melilotus alba* | Sweet clover | Taira, Haskins & Gorz (1977) |
| *Phaseolus vulgaris* | Bush bean | Mechta, Henshaw & Street (1967) |
| *Trifloium hybridum* | Alsike clover | Schenk & Hildebrandt (1972) |
| *Trifolium subterraneum* | Subterranean clover | Graham (1968) |
| *Vicia faba* | Broad bean | Grant & Fuller (1968) |

Table 3 Some legumes from which callus-derived plants have been regenerated.

| Species | Common name | Source of explant | Reference |
|---|---|---|---|
| *Acacia koa* | Koa | Seedling shoot tip | Skolmen & Mapes (1976) |
| *Indigophera enneaphylla* | | Cotyledon, hypocotyl | Bharal & Rashid (1979) |
| *Lotus corniculatis* | | Anther | Niizeki & Grant (1971) |
| *Medicago sativa* | Alfalfa | Anther | Saunders & Bingham (1972) |
| *Pisum sativum* | Garden pea | Shoot apices | Gamborg, Constabel & Shyluk (1974) |
| *Psophpcarpus tetragonolobus* | Winged bean | Mature leaf | Gregory, Haq & Evans (1980) |
| *Stylosanthes hamata* | | Cotyledon, radicle | Scowcroft & Adamson (1976) |
| *S. guianensis* | | Leaflets | Mroginski & Kartha (1981) |
| *Trifolium alexandrinum* | Berseem clover | Hypocotyl, anther | Mokhtarzedh & Constantin (1978) |
| *Trifolium incarnatum* | Crimson clover | Hypocotyl | Beach & Smith (1979) |
| *Trifolium pratense* | Red clover | Hypocotyl, epicotyl, cotyledons shoot and root apical meristems, young primary leaves | Phillips & Collins (1979) |
| *Trifolium repens* | Ladino clover | Whole germlings | Oswald, Smith & Phillips (1977) |

from callus derived from mature tissue - the leaf of the winged bean, *Psophocarpus tetragonolobus*. Mroginski and Kartha (1981) subsequently achieved plantlet regeneration from mature leaflets of *Stylosanthes guianensis*. Table 3 lists some legumes from which callus-derived plantlets have been regenerated.

Some workers report limited differentiation of legume callus i.e., either root or shoot production from callus cultures. Hildebrandt, Wilmar, Johns and Riker (1963) successfully induced formation of shoot primordia on callus of *Pisum sativum* stems. Root formation of both *P. sativum* (Torrey, 1967) and *Vigna unguiculata* (Davey, Bush and Power, 1974) has been observed in callus cultures.

Sources other than callus have provided some successful regeneration of legumes *in vitro*. Embryo cultures of red clover (Evans, 1962; Keim, 1953) resulted in the formation of plantlets, and anther cultures of the same species provided another plant regeneration system (Niizecki and Kita, 1973). Trinh, Lie-Schricke and Tran Tanh Van (1981) reported the direct *de novo* formation of plantlets from thin cell layers of different organs of the winged bean.

'Embryoids' have been observed in alfalfa callus (Saunders and Bingham, 1972) and Mehta and Mohan Ram (1981) reported the presence of embryogenic structures in callus of *Psophocarpus tetragonolobus*. Previous studies on the winged bean by Bottino *et al*. (1979) and Venketeswaran and Huhtinen (1978) did not reveal the presence of these structures. The embryo-

-genic structures observed by Mehta and Mohan Ram (1981) were present only when plantlet regeneration was attempted on the same medium as that on which callus was initiated.

## 1.2 Stages in the Tissue Culture Method

In his 1974 review Murashige summarized the sequential steps to be considered in the establishment of a tissue culture programme. Debergh and Maene (1981) presented a modified version of the sequence aimed at reducing the cost of a commercial operation and broadening the range of plants which could be propagated economically *in vitro*.

The three stages in the tissue culture method should be, according to Murashige (1974), as follows:

Stage I Establishment of an aseptic culture
Stage II Propagule multiplication
Stage III Preparation for re-establishment of plants in soil.

Debergh and Maene (1981) proposed an additional stage prior to Stage I. Their scheme is outlined as follows:

Stage 0 Preparation of stock plants under hygienic conditions
Stage I Establishment of an aseptic culture
Stage II Induction of meristematic centres, their development into buds and their rapid multiplication
Stage IIIa Elongation of buds into shoots and preparation of uniform shoots for Stage IIIb
Stage IIIb Rooting and initial growth of the *in vitro* produced shoots under *in vivo* conditions.

The aim of Murashige's Stage I was to achieve prolonged survival *in vitro* of an infestation-free plant segment (Murashige, 1976). Debergh and Maene (1981) proposed that Stage I alone was insufficient for many ornamental plant species. Stock plants grown under controlled conditions prior to *in vitro* culture would, according to these authors, ensure healthier explants and a better, more uniform response to Stage I. They investigated the ability of numerous ornamental plant species to be propagated commercially by tissue culture and found no plants which would yield non-contaminated explants after the usual sterilisation procedures. They therefore devised Stage 0, during which period stock plants were grown in a greenhouse at a high temperature (25°C) and low humidity (70%). Watering was carried out directly into pots and never overhead. This regime increased the number of healthy, non-contaminated explants obtained and influenced their survival *in vitro*.

The classical Stage II (Murashige, 1974) aims for a rapid increase of organs and other structures which will ultimately give rise to plants. The culture supplies the shoots and materials necessary to maintain the stock. According to Murashige (1978) the increase expected during this stage can be achieved by inducing one of the following:

(a) Enhanced formation of axillary shoots, followed by rooting of individual shoots.

(b) Production of adventitious shoots followed by rooting of individual shoots.

(c) Somatic embryogenesis.

The scheme of Debergh and Maene (1981) provides a Stage II where tissue can remain for several generations without producing a shoot for rooting. In Stage IIIa the propagules formed in Stage II have to elongate to yield shoots usable as cuttings *in vivo*.The new Stage IIIb provides for the rooting of shoots *in vivo* instead of the classical *in vitro* Stage III. The disadvantages of rooting *in vitro* are, according to Debergh and Maene (1981), as follows:

(1) It is a labour-intensive step

(2) A delay in growth is usually manifest for *in vitro* rooted shoots after transfer to soil because it is difficult to induce a good functioning root system *in vitro*. *In vitro* formed roots usually die, growth ceases and then new roots start to develop.

(3) Rooting is generally not optimal when carried out in a constant regime for two to three weeks because exogenous auxins are required for root initiation but not for root elongation.

(4) Roots formed *in vitro* are usually damaged during planting and this increases the disease risk.

Debergh and Maene (1981) thus proposed a scheme to treat *in vitro* produced shoots as cuttings and root them under suitable conditions. Rooting of these shoots could be accomplished in the following ways:

(1) Plant shoots directly into normal planting material (substrate).

(2) Pre-treat the substrate with auxin.

(3) Pre-treat the planting material with auxin and plant into normal substrate.

The stages proposed by Debergh and Maene (1981) are not entirely new to the technique of tissue culture as applied to ornamental species. Knauss (1976) and Holdgate and Aynsley (1977) also advocated the use of a stage in which stock plants are prepared for *in vitro* propagation under hygienic conditions (Stage 0).

## 1.3 Constituents and Physical Qualities of the Nutrient Medium

The nutrient medium in each stage of the tissue culture procedure must be suitable in both composition and physical qualities. Both Murashige (1974) and de Fossard (1976) give excellent coverage to this complex topic and only the salient points will be mentioned here.

The assessment of requirements in nutrient medium composition can be simplified by considering the ingredients in three categories: inorganic salts, organic substances and natural complexes (Murashige, 1974). The inorganic salt requirement of a particular species (usually salts of chlorides, nitrates, sulphates, phosphates and iodides of calcium, magnesium, potassium, sodium, iron, manganese, zinc and boron) can be met by comparing various media used in work on similar species, genera or families. De Fossard (1976) suggests a broad spectrum experiment as an alternative to, or as an extension of, this method. Modifications can the

Table 4 Major inorganic salts present in various nutrient media (all values expressed as $mgl^{-1}$) (adapted from Narayanaswamy, 1977).

| Constituent | Gautheret (1942) | Murashige & Skoog (1962) | Blaydes (1966) | Miller (1963) |
|---|---|---|---|---|
| $MgSO_4.7H_2O$ | 125 | 370 | 35 | 71,5 |
| KCl | - | - | 65 | 65 |
| $CaCl_2.2H_2O$ | - | 440 | - | - |
| $KNO_3$ | 125 | 1 900 | 1 000 | 1 000 |
| $Ca(NO_3)_2.4H_2O$ | 500 | - | 347 | 1 000 |
| $NH_4NO_3$ | - | 1 650 | 1 000 | 1 000 |
| $KH_2PO_4$ | 125 | 170 | 300 | 300 |

be made to meet specific needs. The major inorganic salt constituents of some basic media are listed in Table 4. Extremely complex media are usually unnecessary for general plant propagation (Holdgate, 1977).

The organic constituents of a nutrient medium are usually as follows (Murashige, 1974): carbohydrates, certain vitamins, selected amino acids and amides, a nitrogen base such as adenine and various growth regulators (selected according to the objective to be achieved by the particular medium). The carbohydrate requirement is usually satisfied by the addition of sucrose (2% - 3%) and the most commonly used vitamins are thiamin, inositol, nicotinic acid and pyridoxin. Ascorbic acid (vitamin C) is sometimes used, particularly in combination with citric acid to prevent browning of freshly-excised tissues. Tocopherol (vitamin E) has also been used (in conjunction with high levels of iron) to enable control of the juvenile states in some callus cultures (Oswald *et al.*, 1977)

Only L - isomers of amino acids should be used, the D form usually being without effect, and amides are often more effective than their respective amino acids (Murashige, 1974). Adenine may be a desirable addition in certain cases as a stimulant for shoot production. Carefully regulated additions of growth regulating substances are made to the medium; auxins and cytokinins are the most critical organic constituents of the medium (Murashige, 1974). The order of effectiveness and stability of various auxins are:

IAA < IBA < NAA < 2,4-D < Tordon (Murashige, 1979).

IAA is the preferred auxin because it has the least side effects on growth but it is very unstable and cannot be autoclaved. The synthetic auxin 2,4-D is highly active. According to Murashige (1974), it acts as a stimulant to callus development but antagonizes organogenesis. Wilmar and Hellendoorn (1968) and Sunderland (1973) found 2,4-D to cause a rapid increase in ploidy in *Asparagus* and *Haplopappus* cultures, respectively. This auxin is, however, commonly used in the *in vitro* culture of legumes (Philips 1974a and 1974b; Arnison and Boll, 1974; Holsten, Burns, Hardy and Hebert, 1971). Of the cytokinins, BA and K are commonly used and are almost equivalent in effectiveness, BA being slightly stronger (Heide, 1965; Murashige, 1979). The cytokinin 2iP is more active but is very expensive. The choice of a particular cytokinin or auxin depends upon the effect required. The correct ratio of auxin:cytokinin must also be considered. Skoog and Miller (1957) discovered that a high auxin:low cytokinin ratio favours root initiation, whilst high cytokinin:low auxin induces shoot production and suppresses rooting (in tobacco). Callus development is enhanced by a high auxin concentration. Endogenous hormone levels may affect the response of the material to exogenously - supplied growth regulators (Heide, 1965; Kato and Kawahara, 1972). These interactions were also observed by Klimaszewska (1979) in his work on two *Peperomia* species.

Gibberellin added to the medium can interact with auxin and cytokinin in organogenesis (Pillai and Hildebrandt, 1968) or can inhibit this process (Schraudolf and Reinert, 1959). Gibberellins are not in common use in tissue culture but may

be useful in the stimulation of organ growth (Murashige, 1974).

Murashige (1974) suggests that natural complexes, such as coconut milk, should be used only when all attempts to use a chemically defined medium fail or when additional stimulation is required. Holdgate (1977) does not advocate the use of natural compounds at all.

It is sometimes necessary to include anti-oxidants to retard the browning and deterioration of the medium. In addition to vitamin C and citric acid, mentioned earlier, activated charcoal is often used as an adsorbant to remove phenolic compounds causing discolouration or other metabolites which inhibit morphogenesis (Wang and Huang, 1976; Fridborg, Pedersen, Landstrom and Erikkson, 1978; Weatherhead, Burdon and Henshaw, 1979). Activated charcoal may also have the disadvantageous effect of absorption of auxins and cytokinins from the medium (Murashige, 1979).

The pH of the medium is usually adjusted to the range between pH 5 and pH 6, for the following reasons (de Fossard, 1976):

(1) Most plant cells have pH readings between pH 5 and pH 8.

(2) Different elements become less available or unavailable to plant cells outside a certain pH range.

Drifts in pH do occur during culture and little is known of the influence of actual pH values on the development of a culture (Murashige, 1974). The pH of the medium is generally adjusted by the addition of dilute hydrochloric acid (HCl) or

sodium hydroxide (NaOH) to pH 5,5 or pH 5,8, prior to autoclaving.

Choice of a liquid or gel medium must be made according to the requirements of the plant under study. Aeration of liquid nutrient media is usually essential and is achieved by rotation or agitation of the culture vessels (Murashige, 1974). Solid media are usually gelled by means of purified agar, used at concentrations in the range 0,6% - 1% (w/v). The pH of the medium is important for successful gelling since the lower the pH, the softer the gel.

## 1.4 Decontamination Procedures

Adequate preparation and surface sterilisation of plant material is essential for successful aseptic technique. Naturally-occurring microflora on plant surfaces will proliferate rapidly on nutrient-rich tissue culture media. These organisms will, at least, retard growth and, at most, destroy the explant. Surface disinfestation can be accomplished at two levels (de Fossard, 1976). If the surface which is to be treated with disinfectant is to be part of the explant, damage to the surface by the sterilant must be avoided. If, however, the surface can be sterilised and removed aseptically, the sterilisation process can be more drastic.

A preliminary washing in water reduces the surface microbial population (de Fossard, 1976) and an alcohol swab or dip is often advantageous. The choice of the surface sterilant depends upon the sensitivity of the material to be sterilised.

The chemical should be easily removable because its retention will affect establishment of the culture (Yeoman, 1973). Chemicals commonly employed are sodium hypochlorite (often in the form of commercial bleach), calcium hypochlorite, mercuric chloride solution or a solution of bromine in water. Concentrations of these chemicals required for effective sterilisation differ according to the plant material used. The following sequence has been successfully employed for the sterilisation of bud material (Holdgate, 1977):

(1) Remove soil, dead and diseased tissue.

(2) Dip in 70% ethanol.

(3) Immerse for 3 - 15 minutes in 10% (v/v) solution of commercial bleach.

(4) Wash in sterile water - first a quick wash and then twice for five minutes.

(5) When further short-duration sterilisation is necessary during dissection it should be followed by copious washing.

Leaf material, stem sections and shoot tips can often be sterilised by washing in warm soapy water, followed by rinsing and surface sterilisation for 10 minutes in a 15% (v/v) solution of sodium hypochlorite plus two drops per 100 ml surfactant (e.g., Tween-20). Three rinses in sterile distilled water completes the treatment (Smith and Nightingale, 1979). Leaf material is especially difficult to sterilise and only very young leaves should be used (Yeoman, 1973). Leaf material

of *Peperomia* has been sterilised successfully using fairly dilute sodium hypochlorite solutions (0,5% - 1%) (Berry, 1978; Henny, 1978; Hui and Zee, 1981).

Sterile seedlings i.e., those grown from sterilised seed,are often the source of explants for tissue culture studies. In much of the work carried out on leguminous plants this is the case (Bottino *et al.* 1979; Mokhtarzedh and Constantin, 1978; Oswald *et al.* 1977; Phillips and Collins, 1979). Common sterilants used to treat these leguminous seeds were sodium hypochlorite (in concentrations ranging from 2% to 5%) and mercuric chloride (0,2%).

Complete flowers are usually sterilised in order to obtain sterile flower parts for anther and ovary culture (Mokhtarzedh and Constantin, 1978). Generally speaking, an adequate surface sterilisation technique for the particular plant tissue must be devised, using previous reports as a guideline. These reports, however, can be misleading as percentage contamination of cultures following a given sterilisation regime are seldom supplied by the authors.

## 1.5 Culture Environment

The major factors to be considered in the culture environment are light and temperature. The relative humidity is not very important because the relative humidity of the "microclimate" within the culture vessel is usually about 100% - depending on the temperature (Murashige, 1974).

### 1.5.1 Light Requirements

Light intensity, duration of the light exposure and quality of light should all be considered.

#### 1.5.1.1 Light intensity

Murashige (1974) recommends a light intensity of 1 000 lux for Stage I and Stage II cultures, and 3 000 lux - 10 000 lux for Stage III (suitable for several different plant species). The higher intensity is required to achieve maximum rates of transfer of propagula from tissue culture to soil. Under the scheme proposed by Debergh and Maene (1981), a moderate light intensity is required for Stage II cultures and those in Stage IIIa generally need a higher intensity. The requirements of the particular plant culture will usually lie within a fairly wide range of light intensities and will also depend on whether or not a callus culture is developed prior to initiation of morphogenesis. The literature available on legume culture and that of *Peperomia* indicates little standardisation in this sphere and little emphasis is placed on any aspect of illumination of the cultures.

#### 1.5.1.2 Duration of light exposure

The total radiant energy of specified quality to which the culture is exposed is the essential factor (Murashige, 1974). Thus, a combination of intensity and exposure period is involved. For a given species varying optima in the length of the daily exposure period will occur, depending on the light intensity used. Day-length does influence the development of

tissue cultures of plants which are normally responsive to photoperiod (Murashige, 1974). Legume cultures have been maintained, in general, under a 16-hour day regime (Trinh *et al*, 1981; Walker, Yu, Sato and Jaworski, 1978) or a continuous light regime (Cheyne and Dale, 1981). Henny (1978) and Hui and Zee (1981) grew *Peperomia in vitro* under a 16-hour day regime and Kukulczanka *et al*. (1977) utilised continuous light.

1.5.1.3 Light quality

This factor seems of little account in most published reports. However, the experiments of Weiss and Jaffe (1969) showed that the critical portion of the light spectrum for shoot induction is the blue region. Root induction is often stimulated by red light (Letouze and Beauchesne, 1969, cited by Murashige, 1974).

1.5.2 Temperature

The general practice has been to maintain cultures in an environment in which the temperature is constant - about 25°C (Murashige, 1974). This situation is adequate for the culture of many annual and tropical species, the life cycles of which are completed during a period of relatively uniform temperature conditions. The range of temperature over which *Peperomia* species have been cultivated is 25°C - 27°C. A wider temperature range exists in legume tissue culture: 20°C - 29°C.

# 2. GENERAL MATERIALS AND METHODS

The following basic procedures apply to the treatment of both *Peperomia* and *Strongylodon* material, and those techniques relating specifically to each genus are dealt with in 3.2 and 4.2, respectively.

## 2.1 Decontamination Procedures and Aseptic Techniques

All plant material brought into the laboratory from garden or greenhouse was sorted and washed under running water in order to remove dust and debris. It was then rinsed in distilled water and swabbed thoroughly with cotton wool soaked in 95% ethyl alcohol, the latter treatment being carried out with caution in the case of tender tissue to prevent complete breakdown of the cuticle and subsequent dehydration. All further treatment of the material was carried out on the laminar flow bench.

Prior to use, the surface of the laminar flow bench was swabbed down with 95% ethyl alcohol and the interior sprayed with the same. All instruments, glassware and media were steam-sterilised in an autoclave at a pressure of one bar and temperature of 120°C for 20 minutes. Instruments in use on the bench were placed in a beaker containing 95% ethyl alcohol and were flamed repeatedly, using a spirit burner, during the course of the work taking place on the bench. The worker's hands and forearms were washed thoroughly with soap and water and repeatedly swabbed with alcohol during the period of work. The mouths of all culture vessels were flamed prior to and after positioning of the explant on the medium.

The common sterilants NaOHCl and $HgCl_2$ were tested initially in order to establish effective surface-sterilisation procedures for use during subsequent experimentation on various types of plant material. The explants were steeped in sterilant solutions of different concentrations (1% - 5% NaOHCl and 0,1% - 0,5% $HgCl_2$) for varying lengths of time. Appendix Table A lists the results of these initial sterilisation procedures. The most successful sterilisation regimes for the different explants are detailed in the relevant sections dealing with specific materials and methods. A particular regime was counted as successful if 60% of the explants remained free from fungal or bacterial contamination after 10 days in culture. Tween-20 was used as a surfactant in the sterilant solutions (one or two drops Tween-20 per 100 ml solution). Continuous agitation of the solution during sterilisation ensured efficient distribution of the sterilant over the plant material. Three rinses in sterile distilled water completed the procedure, the explants remaining in the final rinse water for 20 - 30 minutes in order to remove all traces of the sterilant. Mercuric chloride is extremely difficult to remove by rinsing alone and thus a 40 milli-equivalent solution of sterile $CaCl_2$ was used to precipitate excess $HgCl_2$ prior to rinsing.

On completion of surface sterilisation, material was placed on a sterile Petri dish in order to remove any tissue visibly damaged by the sterilant. All further dissections took place on sterile Petri dishes and the explants thus prepared were transferred to the culture vessels containing the nutrient medium.

## 2.2 Preparation of Culture Media

Several different media were utilised during the course of the project and Tables 5 - 8 list the constituents of these media. All constituents were made up as stock solutions by dissolving the required amount of analytical grade chemical in glass-distilled water, the final volume being made up to 500 ml. Auxin and kinetin solutions were made up by dissolving the required amount of chemical in a few drops of dilute NaOH before making up to the desired volume with glass-distilled water and adjusting the pH to 7. Benzyl adenine stock solutions were made up by steaming the required amount of chemical in a few drops of dilute NaOH, making up to the desired volume with distilled water and adjusting the pH to 7. Auxin and cytokinin stock solutions were generally made up in smaller quantities than the other stocks in order to prevent age deterioration. All stock solutions were stored in glass reagent bottles at 5°C. Those solutions which contained light-sensitive constituents such as Fe EDTA or vitamin complexes were stored in bottles wrapped in aluminium foil to prevent exposure to light.

In order to obtain the final balanced culture medium, the correct quantity of each stock solution was combined with 30 $gl^{-1}$ sucrose and distilled water and the pH of the medium adjusted to 5,8. Eight grammes per litre Difco Bacto - agar was used to gel the medium. The medium containing the agar was steamed in the autoclave for 10 minutes to dissolve the agar, prior to dispensing into the culture vessels.

The medium was dispensed in 15 ml aliquots into 80 mm x 25 mm glass tubes which were then sealed with Cap-O-Test caps, or in

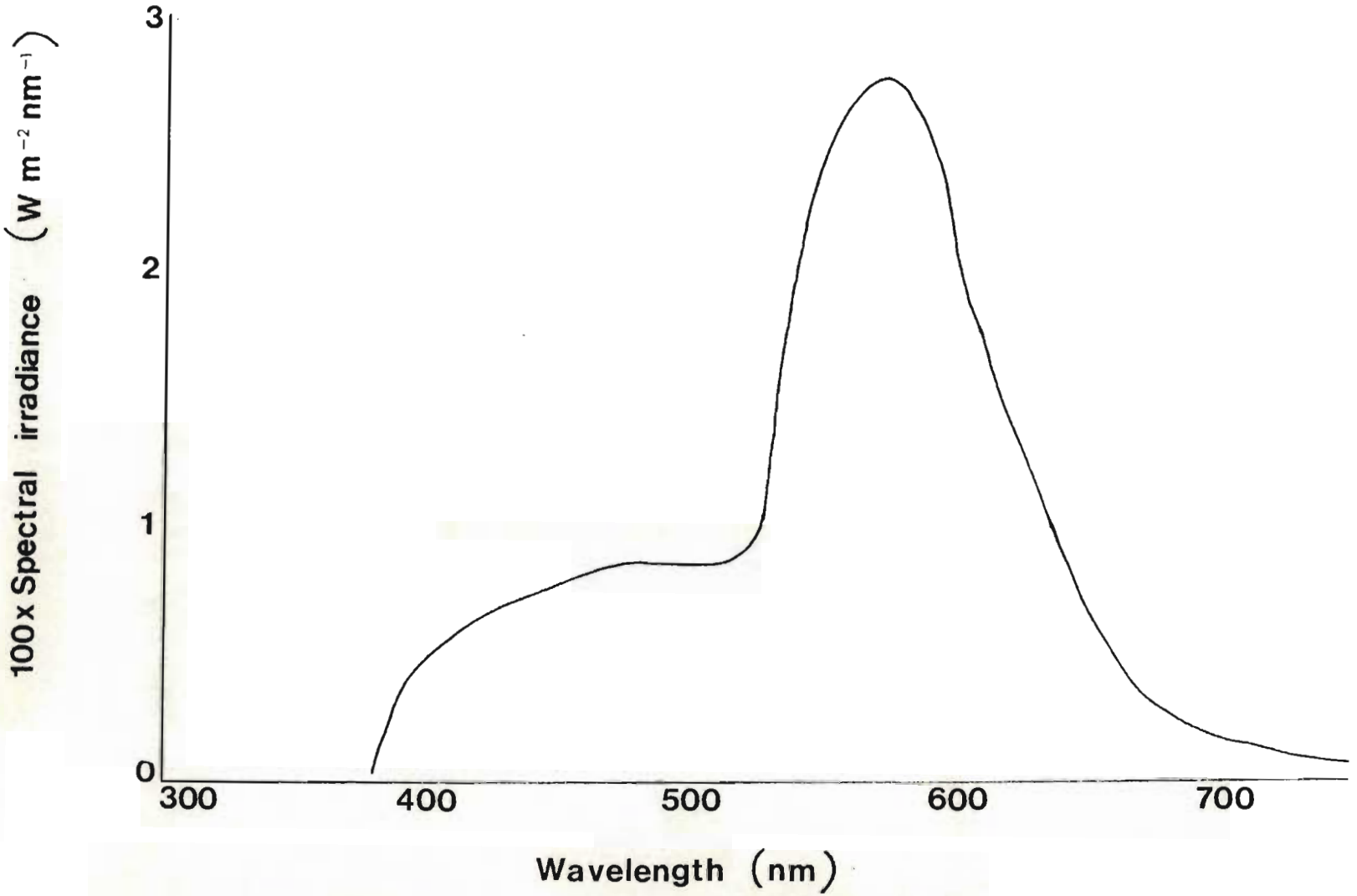



Fig. 1 Spectral irradiance in growth room where cultures of *Strongylodon macrobotrys* and *Peperomia clusifolia* were maintained.

30 ml aliquots into 50 ml conical flasks sealed with cotton wool bungs and aluminium foil caps. Occaisionally 50 ml aliquots were dispensed into 100 ml flasks and sealed as before. The culture vessels were then steam-sterilised in the autoclave.

## 2.3 Conditions for *in vitro* Culture

Cultures were maintained in a growth room where the total irradiance (measured over the range of wavelengths 380 nm - 750 nm using a spectroradiometer) was 3,63 $Wm^{-2}$. The spectral irradiance is illustrated in Fig.1 (See Appendix Table B for recorded irradiance readings). The cool white light tubes operated on a 16-hour light, 8-hour dark cycle. The room temperature varied between 23°C and 27°C.

## 2.4 Experimental Procedure

Variability among replicates receiving the same treatment has frequently been commented upon (Street, 1969: Vajrabhaya and Vajrabhaya, 1970) but its causes are ill-defined. This was borne in mind in the preparation of material for experiments and as many replicates were made as the availability of the plant material would allow. The exact number of replicates was different for different treatments. A further complicating factor was the loss of a certain number of replicates in every treatment owing to fungal contamination. Bacterial contamination was rarely observed.

Cultures were examined daily at the beginning of each experiment for a period of 10 days. Contaminated cultures were re-

moved and recorded. In the following period of culture a weekly examination of the treatments was made in order to observe callus and/or organ development.

Table 5 "Pea medium" of Gamborg, Constabel & Shyluk (1974) as prepared for the culture of *Strongylodon*.

| Stock solution | Constituents | Mass of constituents in stock solution ($gl^{-1}$) | Volume of stock solution in final medium ($ml l^{-1}$) | Mass of constituents in final medium ($mg l^{-1}$) |
|---|---|---|---|---|
| I | $NaH_2PO_4.H_2O$ | 15 | 10 | 150 |
| II | $KNO_3$ | 25 | 100 | 2 500 |
| III | $(NH_4)_2SO_4$ | 13,4 | 10 | 134 |
| IV | $CaCl_2$ | 15 | 10 | 150 |
| | $MgSO_4.7H_2O$ | 25 | | 250 |
| | $MnSO_4.H_2O$ | 1 | | 10 |
| | $H_3BO_3$ | 0,3 | | 3 |
| | $ZnSO_4.7H_2O$ | 0,2 | | 2 |
| V | $Na_2MoO_4.2H_2O$ | 0,025 | 10 | 0,25 |
| | $CuSO_4.5H_2O$ | 0,0025 | | 0,025 |
| | $CoCl_2.6H_2O$ | 0,0025 | | 0,025 |
| | KI | 0,075 | | 0,75 |
| | NaFeEDTA | 2,8 | | 28 |
| VI | Meso-inositol | 10 | 10 | 100 |
| | Nicotinic acid | 0,1 | | 1 |
| | Thiamine-HCl | 1 | | 10 |
| | Pyridoxine-HCl | 0,1 | | 1 |
| Additional | BAP | | | 2 $mg l^{-1}$ final medium |
| | NAA | | | 2 $mg l^{-1}$ final medium |
| | Sucrose | | | 30 $g l^{-1}$ final medium |

Table 6 "Alfalfa medium" of Saunders & Bingham (1972) as prepared for culture of *Strongylodon*.

| Stock solution | Constituents | Mass of constituents in stock solution ($gl^{-1}$) | Volume of stock solution in final medium ($ml l^{-1}$) | Mass of constituents in final medium ($mg l^{-1}$) |
|---|---|---|---|---|
| I | $Ca(NO_3)_2.4H_2O$ | 34,7 | 10 | 347 |
| | $NH_4NO_3$ | 100 | | 1 000 |
| II | $KNO_3$ | 10 | 100 | 1 000 |
| III | $KH_2PO_4$ | 30 | 10 | 300 |
| | $MgSO_4.7H_2O$ | 3,5 | | 35 |
| | KCl | 6,5 | | 65 |
| IV | KI | 0,08 | 10 | 0,8 |
| | $ZnSO_4$ | 0,15 | | 1,5 |
| | $H_3BO_3$ | 0,16 | | 1,6 |
| | $MnSO_4.H_2O$ | 0,44 | | 4,4 |
| | $Na_2EDTA$ | 0,22 | | 2,2 |
| | $FeSO_4.7H_2O$ | 1,6 | | 16 |
| V | Thiamine-HCl | 0,01 | 10 | 0,1 |
| | Nicotinic acid | 0,05 | | 0,5 |
| | Pyridoxine-HCl | 0,01 | | 0,1 |
| | Glycine | 0,20 | | 2 |
| Additional | NAA | | | 2 $mg l^{-1}$ final medium |
| | 2,4-D | | | 2 $mg l^{-1}$ final medium |
| | K | | | 2 $mg l^{-1}$ final medium |
| | Sucrose | | | 30 $g l^{-1}$ final medium |

Table 7 Miller's (1963) medium as prepared for the culture of *Strongylodon*.

| Stock solution | Constituents | Mass of constituents in stock solution ($gl^{-1}$) | Volume of stock solution in final medium ($ml l^{-1}$) | Mass of constituents in final medium ($mg l^{-1}$) |
|---|---|---|---|---|
| I | $KH_2PO_4$ | 3 | 100 | 300 |
| | $KNO_3$ | 10 | | 1 000 |
| | $NH_4NO_3$ | 10 | | 1 000 |
| | $Ca(NO_3)_2.4H_2O$ | 5 | | 500 |
| | $MgSO_4.7H_2O$ | 0,715 | | 71,5 |
| | KCl | 0,65 | | 65 |
| | $MnSO_4.4H_2O$ | 0,14 | | 14 |
| II | NaFe EDTA | 1,32 | 10 | 13,2 |
| | $ZnSO_4.7H_2O$ | 0,38 | | 3,8 |
| | $H_3BO_3$ | 0,16 | | 1,6 |
| | KI | 0,08 | | 0,8 |
| | $Ca(NO_3).3H_2O$ | 0,35 | | 3,5 |
| | $NH_4Mo_2O_2.4H_2O$ | 0,01 | | 0,1 |
| III | Myo-inositol | 10 | 10 | 100 |
| | Nicotinic acid | 0,2 | | 2 |
| | Pyridoxine-HCl | 0,08 | | 0,8 |
| | Thiamine-HCl | 0,08 | | 0,8 |
| Additional | Sucrose | | | 30 $gl^{-1}$ final medium |

Table 8 Medium of Murashige & Skoog (1962) as prepared for the culture of *Strongylodon* and *Peperomia*.

| Stock solution | Constituents | Mass of constituents in stock solution ($gl^{-1}$) | Volume of stock solution in final medium ($ml l^{-1}$) | Mass of constituents in final medium ($mg l^{-1}$) |
|---|---|---|---|---|
| I | $NH_4NO_3$ | 165 | 10 | 1 650 |
| | $CaCl_2.2H_2O$ | 44 | | 440 |
| | $MgSO_4.7H_2O$ | 37 | | 370 |
| | $KH_2PC_4$ | 17 | | 170 |
| | $H_3BO_3$ | 0,62 | | 6,2 |
| | $MnSO_4.4H_2O$ | 2,23 | | 22,3 |
| II | $KNO_3$ | 95 | 20 | 1 900 |
| III | $ZnSO_4.7H_2O$ | 0,86 | 10 | 8,6 |
| | KI | 0,084 | | 0,84 |
| | $Na_2MoO_4.2H_2O$ | 0,026 | | 0,26 |
| | $CuSO_4.5H_2O$ | 0,0026 | | 0,026 |
| | $CoCl_2.6H_2O$ | 0,0026 | | 0,026 |
| | $FeSO_4.7H_2O$ | 2,78 | | 27,8 |
| | $Na_2EDTA.2H_2O$ | 3,73 | | 37,3 |
| IV | Thiamine-HCl | 0,01 | 10 | 0,1 |
| | Pyridoxine-HCl | 0,05 | | 0,5 |
| | Niacin | 0,05 | | 0,5 |
| | Glycine | 0,02 | | 0,2 |
| Additional | Sucrose | | | 30 $gl^{-1}$ final medium |

Fig 2 *Peperomia clusifolia* Hook.

# 3. STUDIES ON *PEPEROMIA CLUSIFOLIA*

## 3.1 Introduction

*Peperomia clusifolia* Hook. is a member of the family Peperomiaceae, a small dicotyledonous family of succulent herbs often possessing attractive foliage (Heywood, 1978). The family is native to tropical and sub-tropical regions, especially tropical America and the West Indies. The genus *Peperomia* is a tropical American genus containing about 1 000 species. The leaves of the plant are alternate and the flowers are bisexual with no sepals or petals. A number of species are cultivated as ornamentals, *P. clusifolia* being grown as an indoor foliage plant. The genus is easy to propagate from stem and leaf cuttings by conventional methods. Previous tissue culture studies on members of the genus have resulted in the regeneration of plants from stem or leaf explants (as described in the literature review).

*P. clusifolia* has not been cultured previously *in vitro*, and was selected for this study as an ornamental plant which the author could use as a tool with which to familiarise herself with the application of the techniques of tissue culture propagation.

## 3.2 Materials and Methods

### 3.2.1 Source of Material

Well-established potted plants of *P. clusifolia* were provided by Tropical Plants and Gardens Nursery, Malvern, and were grown in a greenhouse until required. Young leaves (from

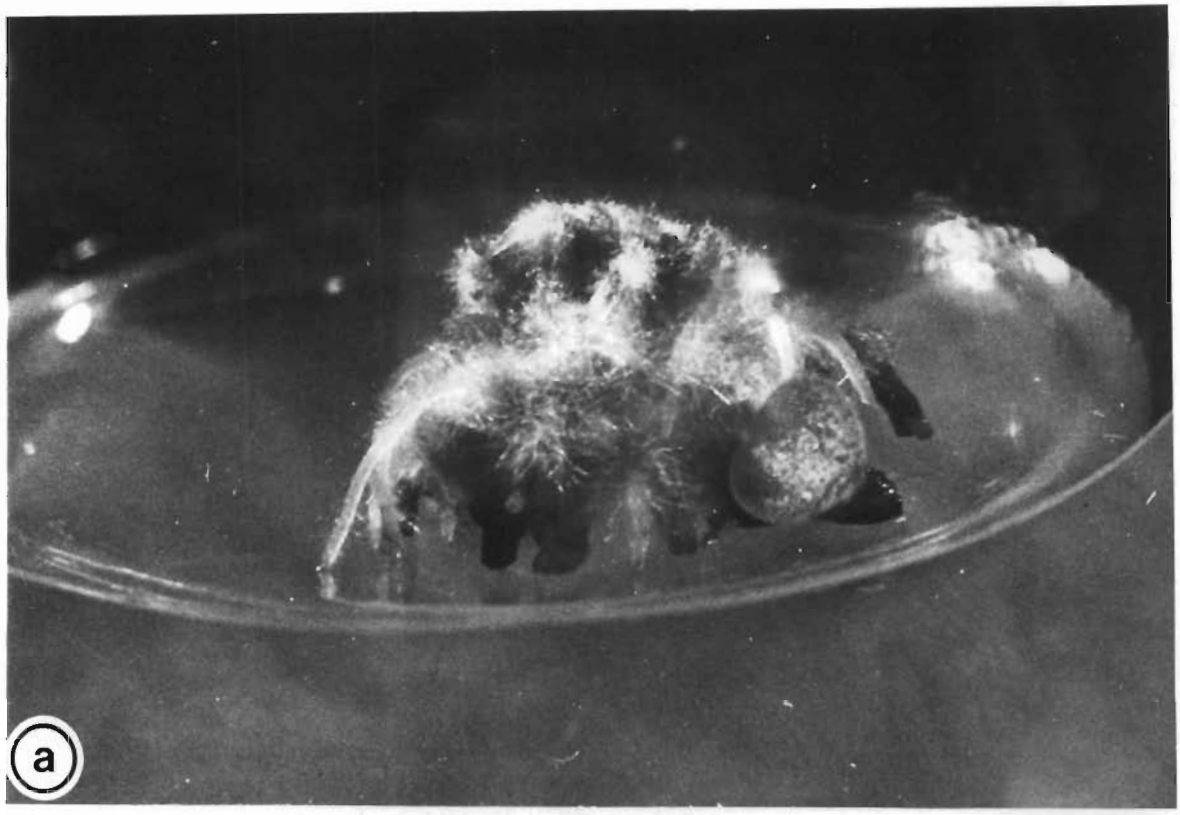
the third and fourth nodes), stems and floral shoots (pedicels) were removed from healthy plants and used immediately after picking.

### 3.2.2 Preparation of Plant Material

Leaves, stems and floral shoots were surface-sterilised in 2% NaOHCl for 15 minutes. Leaves were cut into pieces approximately 1 cm x 2 cm and stem and floral shoot material was cut into segments approximately 1 cm in length. These sterilised explants were then placed on to the medium. Leaf explants were placed with the abaxial surface in contact with the medium and stem and floral shoot explants were placed with their longitudinal axes parallel to the surface of the medium. Sufficient floral shoot material was available for two treatments only. Each tube of medium contained one explant.

## 3.3 Results and Discussion

Since the *in vitro* culture of *P. clusifolia* was used as an opportunity simply to become acquainted with the techniques of tissue culture, wide range experiments were not carried out. A single nutrient medium i.e. that of Murashige and Skoog (1962), which had been used previously for *Peperomia* tissue culture (Berry, 1978; Hui and Zee, 1981; Klimaszewska, 1979; Kukulczanka *et al.*, 1977), was selected. Several different hormone concentrations were utilised to test their effects on root and shoot induction. The use of 2% NaOHCl as surface sterilant resulted in a consistent survival rate *in vitro*, after 10 days in culture, of 10 - 15 explants per treatment


a


b

(55,5% - 83%).

Table 9 summarises the results obtained in the various treatments. Callus development was not extensive in any treatment and it was always closely followed by the formation of roots. Initial callus formation usually began after 14 - 16 days in culture and, in the case of leaf material, the roots generally developed on the upper surface of the callus and grew down into the medium. Where NAA was present at a level of 2 $mgl^{-1}$ in the medium, root elongation was retarded upon contact with the medium. This phenomenon probably occurred as a result of the detrimental influence of auxin on root elongation i.e., auxins are required for root initiation but high concentrations may impede further root development (Goodwin, 1978).

If no exogenous auxins or cytokinins were applied to the medium, the explants (of leaf, stem and floral shoot) exhibited no development whatsoever and rapid death of the explants followed. This would suggest that the level of endogenous hormones was insufficient to induce root, shoot or callus formation and thus morphogenesis *in vitro* was dependent upon externally-supplied hormones.

Equal concentrations of NAA and K at a relatively low level (1 $mgl^{-1}$) resulted in callus formation on all three explant types. Extensive root formation occurred on leaf explants in this treatment (Fig. 3a) and shoots were also formed. Both organ types were formed on stem explants. Floral shoot explants exhibited callus development only. In all treatments where callus formation preceeded organ formation, the first organ type to be formed was the root. Where no callus formation

Table 9 Response of *Peperomia clusifolia* explants grown on the medium of Murashige & Skoog (1962) supplemented with different amounts of the growth regulators, NAA and K.

| Treatment number | Growth regulator levels ($mgl^{-1}$) | | Response of explant | | |
|---|---|---|---|---|---|
| | NAA | K | Leaf | Stem | Floral shoot |
| 1 | 0 | 0 | No growth. Death after 5 days. | No growth. Death after 7 days. | No growth. Death after 5 days. |
| 2 | 1 | 1 | Callus formation after 14 days. Extensive root formation followed by shoot formation. | Callus formation after 16 days. Root and shoot formation. | Callus formation after 16 days. |
| 3 | 1 | 2 | Rapid root development followed by shoot development. | Polar development of shoots and roots. | - |
| 4 | 2 | 1 | Callus formation after 14 days. Rapid root development. Little shoot formation. | Callus formation after 14 days. Roots formed on one end of explant. Shoot formation on one explant only. | - |
| 5 | 2 | 2 | Callus formation after 14 days. Extensive root formation followed by shoot formation. | Minimal root/ shoot formation. | - |

occurred, root production followed shoot formation in all cases except the leaf explants of Treatment 3. The development of roots or shoots was, in all cases, more tardy on stem and floral shoot explants than on leaf explants. Callus only developed at the cut ends of the stem and floral shoot explants in contact with the medium. Callus development on leaf explants was initially peripheral. All roots formed in any treatment developed root hairs.

A high K: low NAA (2:1) ratio in the nutrient medium resulted in zero callus formation in all explant types. Although no callus was formed on the leaf material in this treatment, root development still occurred prior to shoot formation. Fig. 3b shows the acropetal development of shoots on stem explants of Treatment 3. Roots were subsequently formed on the basipetal end of the segment and are visible in the photograph. Some discolouration of the medium was noted in this treatment.

Callus development in the high NAA: low K (2:1) treatment involving equal quantities of NAA and K. Root formation on the explants of Treatment 4 was similar to that observed in Treatment 3. Shoot production did occur but was limited to one or two shoots per leaf explant. Only one stem explant produced a shoot in this treatment.

The 2 $mgl^{-1}$ NAA/ 2 $mgl^{-1}$ K treatment resulted in a little callus development on leaf explants followed by root and shoot formation comparable to that observed in Treatment 2. Very slow development occurred in the stem explants of this treatment. No callus was formed and root and shoot formation was minimal.

Shoot formation on leaf and stem explants generally involved production of large numbers of shoots. Those explants exhibiting good shoot development were divided up into individual "plantlets". These were subcultured twice to fresh medium containing 2 $mgl^{-1}$ K and 1 $mgl^{-1}$ NAA, over a period of two months.

Rooted plantlets were transferred into pots containing an autoclaved mixture of equal parts soil, peat moss and sand. The pots were enclosed in polythene bags to maintain high humidity and grown on the laboratory window-sill in order to "harden-off" the plants. The bags were removed after a period of seven days and continued growth of the young plants occurred indoors. Seventy five per cent of these plants survived the transfer process.

## 3.4 Conclusion

The regenerative potential of *Pepromia clusifolia* was shown by these experiments to be high. The need for the presence of exogenously-applied hormones was evident but fairly low levels (1 $mgl^{-1}$) were required for organogenesis. The most successful treatments for shoot production were those containing 1 $mgl^{-1}$ NAA and 2 $mgl^{-1}$ K or 1 $mgl^{-1}$ NAA and 1 $mgl^{-1}$ K. These observations are similar to those made by Henny (1978) and Hui and Zee (1981). Maximum root production occurred in the treatments involving 2 $mgl^{-1}$ NAA and 1 $mgl^{-1}$ K or 1 $mgl^{-1}$ NAA and 1 $mgl^{-1}$ K. S.Berry (pers. comm.) obtained similar results for the *in vitro* root formation of *P. caperata*.

Leaf explants exhibited a higher regenerative potential than either stem or floral shoot explants. S. Berry (pers. comm.) found *Peperomia* easy to propagate *in vitro* from leaf explants. Floral shoot material was not induced to form organs in either of the two treatments in which it was included.

Further investigations could be aimed at optimising root and shoot development by the manipulation of hormone levels and changes in the constituents of the basal medium. The effect of light and dark on the development of cultures could be studied, and an in-depth analysis of the regenerative potential of the different plant parts carried out. The latter study could possibly be linked to an analysis of endogenous hormone levels in the various plant parts under study.

Fig. 4 The Jade vine, *Strongylodon macrobotrys* A. Gray.

# 4. STUDIES ON *STRONGYLODON MACROBOTRYS*

## 4.1 Introduction

The genus *Strongylodon* consists of about 20 species of high twining lianes (Herklots, 1976). The members of the genus are widespread from the Mascarenes to Melanesia, with two more or less distinct centres of distribution in the Malagasy Republic and the Philippine Islands. In each of these centres three or four endemic species occur. *S. macrobotrys* is a native of the Philippines and is an exquisite ornamental vine. The glossy green leaves are three-lobed and the petiole base is swollen into a distinct pulvinus. The green/turquoise flowers, which earned the plant its common name of "Jade Vine", occur in groups of two's, three's and four's. Each flower is borne on a slender violet pedicel, the whole raceme reaching lengths of up to one metre in good growing conditions.

The Jade vine is a member of the sub-family Papillionoideae, in the family Leguminosae (Willis, 1966). Previous tissue culture studies on legumes have been initiated for reasons such as improving forage and crop yields. Legumes are generally of interest as crop plants because of their capacity for nitrogen fixation, and tissue culture of legumes could be of specific value because of the possibility of somatic hybridisation between legumes and non-legumes (Scowcroft and Adamson, 1976). A leguminous tree, *Acacia koa* , which is endemic to the forests of Hawaii and is valued for its timber, has also been the subject of tissue culture propagation experiments since such trees are difficult to propagate by conventional

means (Skolmen and Mapes, 1976). The Jade vine is a legume prized neither for its nitrogen-fixing ability nor for its wood. It is a desirable ornamental plant and was chosen as the subject of this investigation for purely aesthetic reasons. The vine is able to be propagated fairly successfully from cuttings but is not a prolific seed-bearer.

## 4.2 Materials and Methods

An introductory account of the source of plant material, basic preparation of material after surface sterilisation and selection of nutrient media is presented here. For the sake of clarity, specific details relevant to particular explants and experiments are included with the Results and Discussion in the various sections of the next chapter.

### 4.2.1 Source of Material

Young plants of *Strongylodon macrobotrys* were obtained from the Tropical Plants and Gardens Nursery, Malvern and were maintained in a greenhouse. Conditions in the Pietermaritzburg greenhouse were, however, not ideal for optimum growth of this tropical vine. Greenhouse material was thus supplemented with cuttings taken from vines at the Botanical Gardens, Durban, and the gardens of Mr Richard Carte in Durban and Mrs Joan Wright in Pietermaritzburg. The Durban vines provided an early flower source. Stem and leaf material was collected from all the vines. It was observed that unless plant material was cut and used on the same day, the subsequent contamination rates in culture were exceedingly high. Thus, only fresh material was used throughout the course of the study.

4.2.2 Preparation of Plant Material

The surface sterilisation of the plant material complete, various operations were carried out to prepare the explants from different sources for transfer to the culture vessels. The most efficient sterilisation procedures for different explants are dealt with in 4.3.

4.2.2.1 Internodes

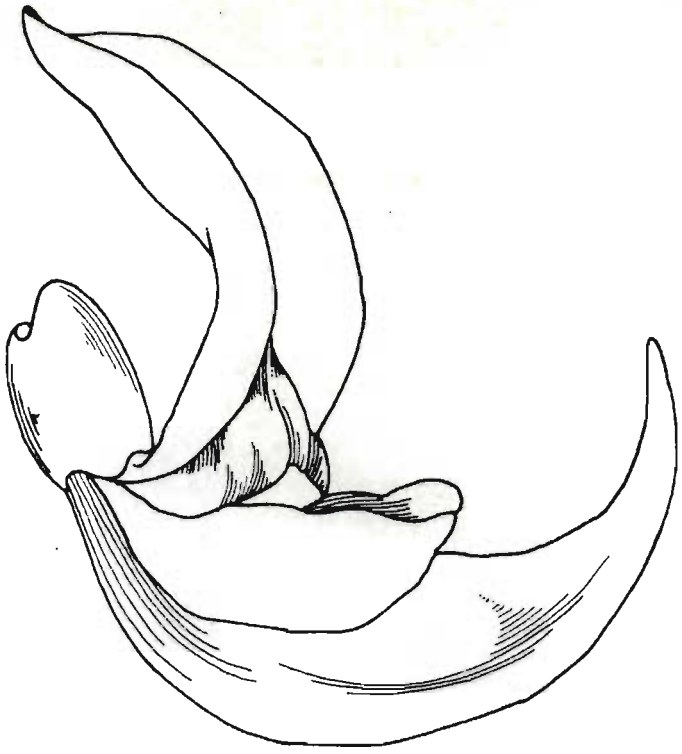
Sterilised stem pieces were cut into segments approximately 1,5 cm long and the outermost layers of bark aseptically removed with sterile forceps, in order to expose the meristematic tissue (see Fig. 16). The peeled explants were then placed horizontally on the medium in the culture vessel.

4.2.2.2 Leaf and pulvinus

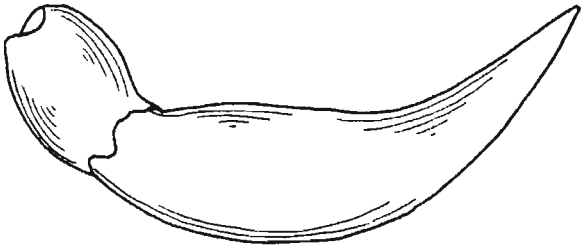
Leaf material was cut into pieces approximately 1 cm x 2 cm after surface sterilisation. In all experiments a comparison was made between those leaf pieces placed with the abaxial surface in contact with the medium and those pieces with this surface uppermost. The pulvinus was removed from the base of the petiole and dissected lengthways, the cut surface being placed in contact with the medium.

4.2.2.3 Flower

Recovery of anther and ovary material from immature flowers was carried out after the sterilisation of the entire intact flower. Three different stages in the development of the Jade vine flower are shown in Fig. 5(a-c). Only young and intermediate closed buds as shown in Fig. 5b and Fig. 5c



a



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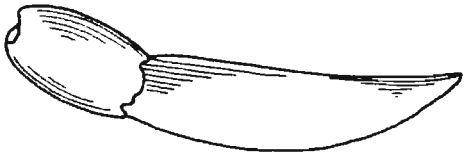

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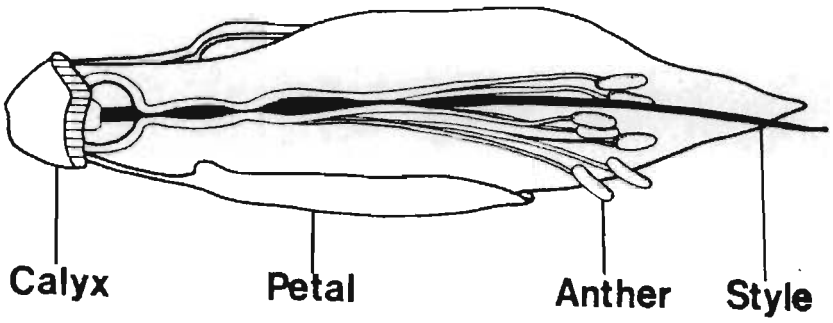



d

were used, since surface sterilisation of the large open flower (Fig. 5a) would have proved difficult. The dissection of the flower bud, as carried out on the laminar flow bench, for the removal of the parts required is shown in Fig. 5d. The anthers were removed from the filaments with care since contact between anthers and instruments should be avoided (Sunderland, 1973). Any damaged anthers were discarded and five anthers were placed into each tube containing the nutrient medium. Invariably a small piece of the filament remained attached to the anther. Since the exact position of the ovary was not easily visible in such immature flowers, a fairly large section of the style including the base of the perianth was excised (Fig. 5d). Pieces of petal and calyx were removed and the "ovary explant" placed on the medium.

4.2.2.4 Nodal material

These explants proved extremely difficult to render sterile by the means routinely employed for surface sterilisation. The decontamination procedures attempted and the degree of success of each regime are detailed in 4.3.4. It was noted that fungal contamination usually emanated from the bracts on either side of the bud. Removal of these bracts alone did not, however, facilitate clean culture. It was therefore necessary to use a dissecting microscope on the laminar flow bench to obtain bud explants free from the surrounding dirty material. The buds were also enclosed by tightly appressed bud scales which could be removed successfully while viewing under the microscope. The explants thus prepared were placed in an upright position on the medium.

4.2.2.5 Seed

Two immature seed pods were obtained and after a short period of dry storage in the laboratory became infected by an unidentified fungus. It was decided to open the pods and remove the seeds, although the pods were not ripe. Each pod contained two uncontaminated seeds. After sterilisation, the seed coat was removed and the cotyledons were cut into pieces which were then placed on nutrient medium. A great deal of spongy pod wall material was present and this too was surface sterilised and placed on nutrient medium.

4.2.3 Nutrient Medium

The initial choice of a nutrient medium suitable for the *in vitro* culture of a particular species is often influenced by the usefulness of certain media for tissue culture of members of the same genus or family as the plant under investigation. De Fossard (1976) suggests that the response of a hitherto untried species be examined on one or two published media or on several different combinations of medium ingredients, making up a broad spectrum experiment. The Jade vine belongs to the family Leguminosae and initial experiments were thus based on the media used previously in studies on the regeneration of legumes. The medium of Gamborg *et al.* (1974), which was initially developed for tissue culture studies of *Pisum sativum* (hereafter referred to as the "pea medium") and Saunders' and Bingham's (1975) medium for alfalfa (the "alfalfa medium") were used initially. Further experiments were carried out on a modified Murashige and Skoog (1962) medium ("M & S

medium") and on Miller's (1963) medium ("Miller's medium"). Details of the constituents of these media can be found in Tables 5 - 8. Various growth regulatory substances were added to the basal medium in different concentrations and combinations. Details of specific treatments are dealt with in 4.3. The auxins 2,4-D, NAA and IBA were used, as well as the cytokinins BA and K. In experiments dealing with the effects of growth regulators on callus formation (see4.3.1.1.), the natural growth regulatory substance coconut milk was added at a concentration of 10 ml per litre of medium.

### 4.2.4 Anatomical Studies

Segments of peeled and unpeeled stem material were cut free-hand (fresh material) or with the aid of a freeze microtome (material preserved for two days in 70% ethyl alcohol). A simple dehyrdation and staining schedule, as outlined in Appendix Table C, was followed and the sections viewed under the light microscope and photographed. The purpose of this procedure was to establish the regions of meristematic tissue within the stem and subsequently to use this knowledge to examine the site of callus formation. Several stem segments which produced callus were selected and dehydrated and embedded according to the schedule outlined in Appendix Table D. Monitor sections were cut using an ultramicrotome. These were stained with methyl blue and viewed and photographed under the light microscope.

## 4.3 Results and Discussion

The most efficient sterilisation regime for each type of explant is listed in Table 10. These were the regimes routinely employed in all treatments detailed in this section. Appendix Table A lists all sterilisation regimes attempted for decontamination of Jade vine explants.

Table 10 Decontamination of *Strongylodon macrobotrys* explants

| Explant type | Most efficient sterilant |
|---|---|
| Internodes | 5% NaOHCl for 10 mins |
| Leaf and pulvinus | 2% NaOHCl for 10 mins |
| Flower | 2% NaOHCl for 10 mins |
| Seed | 0,1% $HgCl_2$ for 5 mins |
| Pod wall material | 2% NaOHCl for 10 mins |
| Nodal material | No successful decontamination procedure |

### 4.3.1 Internodes

#### 4.3.1.1 Callus induction

In general, callus cultures show great genetic instability, so that callus-derived plants often cannot be used for clonal propagation (D'Amato, 1977). However, callus culture multiplication has proved successful with certain ornamentals (Holdgate, 1977). The initial aim in these experiments

was not to obtain callus but, if necessary, to use the production of callus as a transition step to obtaining plant regeneration.

Callus was produced on several media tested for their ability to support *in vitro* growth of the Jade vine. It was, however, obvious from the outset that the pea medium of Gamborg *et al.*(1974) was the least useful of the media tested,since callus produced on this medium was always brown and watery (Fig. 6). Explants on the pea medium produced callus after 10 days in culture. The alfalfa medium of Saunders and Bingham (1972) induced a thick mantle of callus on stem explants, callus formation beginning after seven days in culture. However, the callus produced was unhealthy and extremely friable (Fig. 7). Production of a callus suitable for root/ shoot induction studies was hampered by the browning of the callus. This discolouration occurred both in the initial stages of callus development and upon removal of callus from the mother explant for tranfer to fresh medium. Narayanaswamy (1977) states that calli exhibiting exuberant growth are least conducive to plantlet regeneration, and organ neoformation generally follows the cessation of unlimited proliferation. In the case of calli cultured on the alfalfa medium, this stage of development could not be reached as a result of the discolouration and unhealthy appearance of the callus.

Pigmentation of callus is influenced by a number of factors such as the level of dextrose, presence of soluble starch, nitrogen deficiency, temperature, light and exogenous auxin (Narayanaswamy, 1977). Many secondary products can be recovered

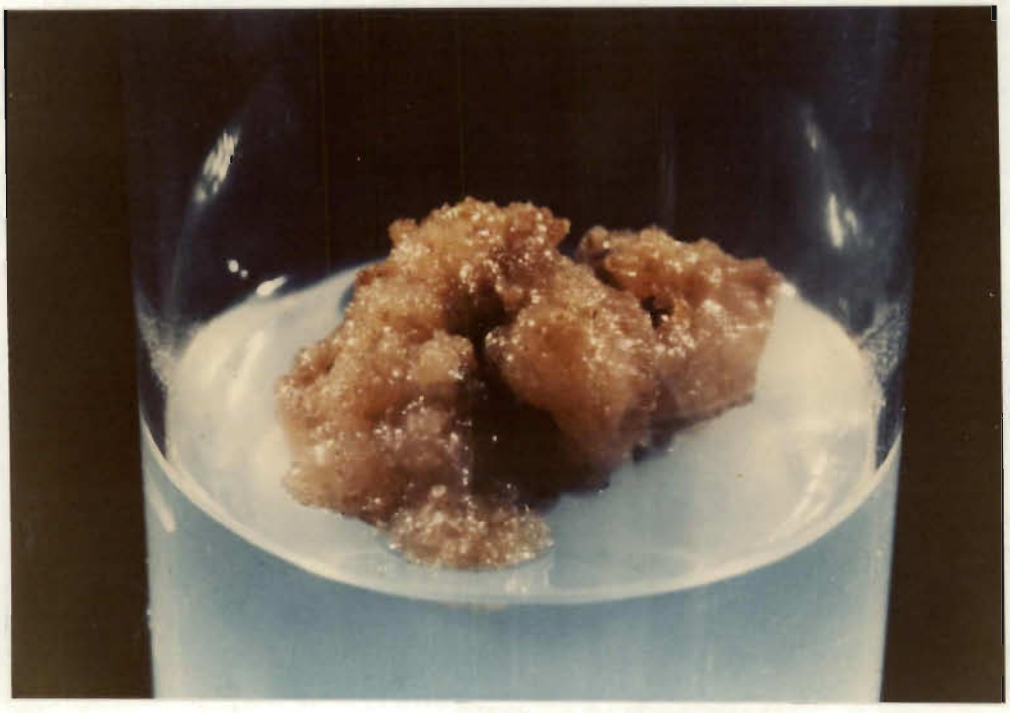


Fig. 6 Severely discoloured, watery callus produced from internode explants of *Strongylodon macrobotrys* on the medium of Gamborg, Constabel and Shyluk (1974) (x3)

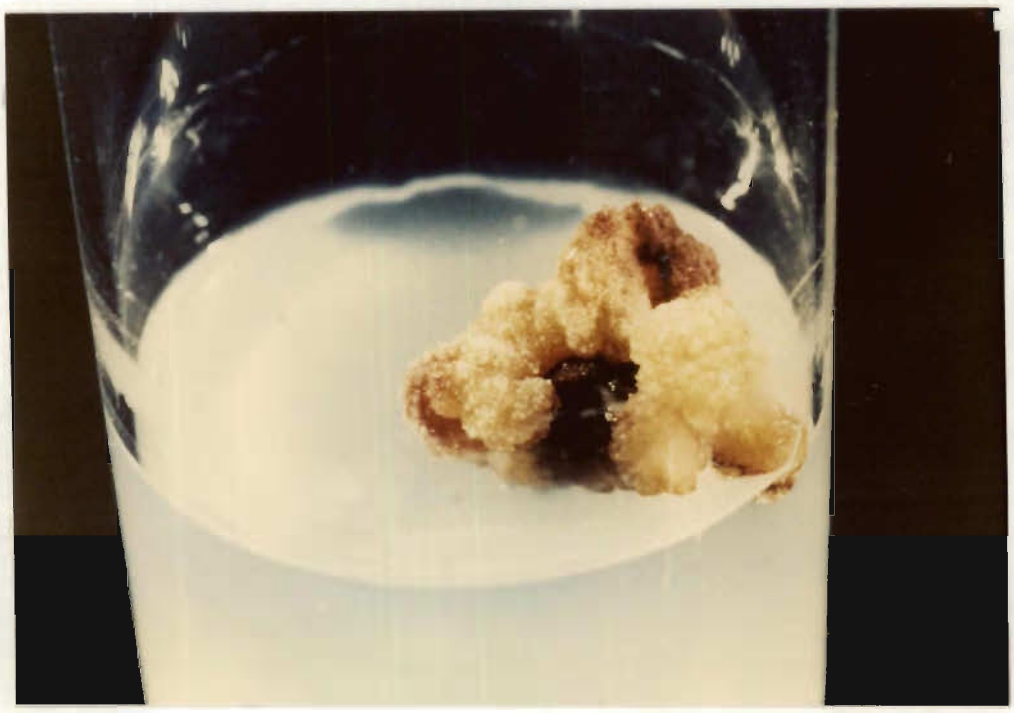


Fig. 7 Callus formation on internode explant of *Strongylodon macrobotrys* on the medium of Saunders and Bingham (1972) (x3)

from callus cultures (Butcher, 1977) and the production of phenolics is another common cause of discolouration (Murashige, 1979).

The natural pigmentation and other characteristics, such as friability, are species-dependent. The discolouration as observed in this callus is usually indicative of declining viability (Oswald *et al*, 1977). The following factors were investigated in an attempt to overcome this detrimental discolouration and ultimate death of the callus cultures:

(1) Production of phenolic compounds

(2) Oxidation of phenolic compounds to quinones

(3) Presence of 2,4-D as the exogenous auxin

Activated charcoal has been used, with varying degrees of success, in the absorption of phenolic compounds from tissue culture media (e.g., Fridborg and Erikkson, 1975; Wang and Huang, 1976). The addition of activated charcoal (1% w/v) to the alfalfa medium used initially for callus induction had an adverse effect on callus development. Stem explants on the modified medium exhibited a slight swelling after eight days in culture but were dead after 14 days. The experiment was repeated twice with younger plant material and the results were the same as those initially observed. Activated charcoal is known to absorb auxins from certain media (Murashige, 1979) and this could possibly account for the lack of callus development observed. The results of the charcoal treatment were essentially the same as the results of those treatments in which

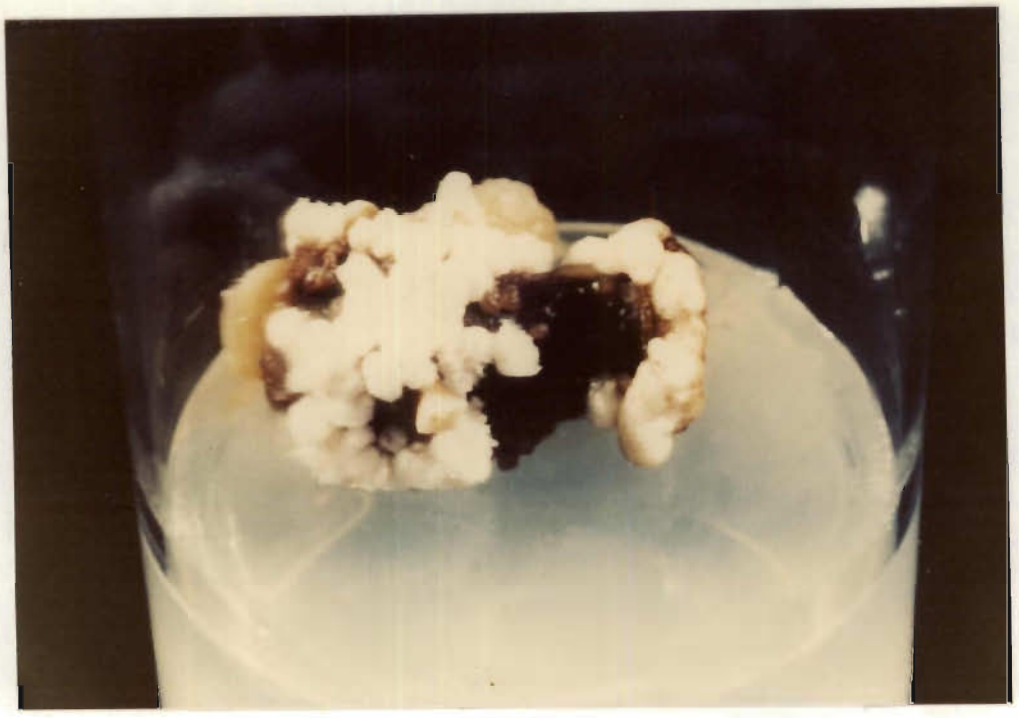
growth regulators were omitted from the basal medium.

Citric acid is a chemical compound commonly used in the control of oxidation processes causing discolouration (Murashige, 1974). The oxidation of phenols to quinones, which can then be further oxidised to produce complex coloured substances, occurs via the action of the group of enzymes known as the phenol or polyphenol oxidases (Salisbury and Ross, 1969). Citric acid acts as a reducing agent to prevent the occurrence of the browning reaction. Addition of citric acid to the medium on which the original explant was placed did not, however, have a noticeable effect on the colour of the callus. Similarly, transfer of the discoloured callus to medium containing citric acid had no beneficial effects.

Phillips and Collins (1979) found that use of 2,4-D as the auxin in legume tissue culture media enhanced the watery response of the callus and Oswald *et al*. (1977) resolved the problem of discolouration in clover and soybean cultures by using 2,4,5-T instead of 2,4-D as the auxin. Jade vine callus established on the complete alfalfa medium (containing NAA as well as 2,4-D) was transferred to the same medium lacking 2,4-D. A slightly healthier callus did develop but was still not satisfactory. Later experiments involving Miller's (1963) medium provided more conclusive evidence against the inclusion of the synthetic auxin 2,4-D in the nutrient medium. The discolouration phenomenon was most pronounced upon transfer of the callus from the mother explant to fresh medium. It is possible that there is a degree of dependence of the callus on the mother explant for various nutrients, growth regulators and enzyme complexes. Removal from the source of these sub-

-stances could result in a decline of viability of the callus (manifest by the discolouration and ultimate death of the callus). This effect would presumably be most noticeable in the situation where the nutrient medium did not provide the essential growth requirements of the callus. Yeoman and Aitchison (1973) state that excision of the explant and the various factors required for growth and maintenance of tissue cultures presumably act by altering the accessibility of genetic information. The response of a piece of excised tissue in culture thus depends on the endogenous growth substances present at the time of excision (Yeoman, 1973). The effect of excision from the mother explant on callus may be governed by similar factors. Excision (of an explant) will produce a wound response, accompanied by induction of cell division, and callus may result. However, maintenance of callus, once established, requires exogenous nutrients and growth regulators (Yeoman and Aitchison, 1973). These latter authors also stress that the exact biochemical expression of cells in tissue culture may be considerably modified by conditions imposed by the medium.

The browning problem was largely overcome by the ultimate use of Miller's medium as the basal medium (Fig. 8). A comparison of the constituents of Miller's medium and the alfalfa medium (which is a modified Blaydes' (1966) medium) shows few major differences. The form in which nitrogen is made available to plant tissue in culture has received some attention. Yatazawa and Furuhashi (1968) and Yatazawa, Furuhashi, Kurihara and Ohnishi (1968) noticed the effects of the nitrogen source on growth and metabolism of rice callus. Singh (1978), in his



a

b

Fig. 8 Callus development on internode explants of *Strongylodon macrobotrys* on Miller's (1963) medium

a Early stages of development (x3)

b Slight discolouration on older callus (x3)

investigations on shoot bud differentiation of *Dioscorea deltoidea*, observed root and shoot differentiation only when ammonium nitrate was the nitrogen source. Similar effects were noted by Halperin and Wetherell (1965) in a study on *in vitro* embryogenesis of carrot. In their work on organogenesis in callus of *Pisum sativum*, Gamborg *et al*.(1974) noted larger and more vigorous shoots on medium with a high total nitrogen content (in both the ammonia ($NH_4$) and the nitrate ($NO_3$) forms). Both Miller's medium and the alfalfa medium supply nitrogen in the nitrate form.

The levels of inorganic constituents are similar in both Miller's and the alfalfa medium. The presence of a greater quantity of magnesium (in the form of $MgSO_4.7H_2O$) in Miller's medium could possibly have an effect on colouration of the callus, since this element is an essential constituent of the chlorophyll molecule. A larger proportion of green-coloured callus was obtained using this medium. Calcium is present in a higher quantity in Miller's than in the alfalfa medium and this is also the case for manganese and zinc. The iron content is less in Miller's than in the alfalfa medium and the former medium contains molybdenum and copper which are not present in the latter. Copper and iron are involved in reactions in the mitochondria (de Fossard, 1976) but it is difficult to assess the extent to which these micro-nutrients would affect the formation of healthy, light-coloured callus.

Miller's medium was initially used in conjunction with the growth regulators K and NAA, since these are the compounds used routinely in the Plant Physiology Laboratory of the

Botany Department, University of Natal, Pietermaritzburg, for the initiation and continued culture of soybean callus for the cytokinin bioassay. Although 2,4-D has been regularly employed as the auxin for *in vitro* cultures of legumes (Bingham 1975; Philips 1974a and 1974b; Arnison and Boll, 1974: Holsten *et al.*, 1971), a comparison of the alfalfa medium and Miller's medium, with and without 2,4-D, confirmed earlier suspicions that 2,4-D did exert an effect on the discolouration of the callus. Experiments involving the removal of 2,4-D from the alfalfa medium did not show an exaggerated effect (as stated on Page 59). However, addition of 2,4-D to Miller's medium resulted in a pronounced browning effect when compared with Miller's medium without 2,4-D (K and NAA were present in the Miller's medium in the same concentrations as in the alfalfa medium i.e., 2 $mgl^{-1}$. 2,4-D was added at a concentration of 2 $mgl^{-1}$).

Certain researchers have tended to avoid using the synthetic auxin 2,4-D because it is thought to cause chromosomal aberrations, polyploidy and severe suppression of organ differentiation (Gresshof, 1978). The consequences of its incorporation into tissue culture media do, however, appear to be a source of some controversy. Singh and Harvey (1975, cited by Gresshof, 1978) found that high levels of 2,4-D in *Vicia* cultures resulted in highly stable diploid cultures, while low 2,4-D levels increased chromosomal abnormality. Murashige (1974) stated that 2,4-D stimulated callus production *in vitro* but antagonized organogenesis. This was substantiated by Crocomo *et al.* (unpublished observations cited by Boulter and Crocomo, 1979) who showed that media containing NAA, IAA, K and 2,4-D produced rapid callus development in legumes but little or no root formation. In contrast, Liau and Boll (1970) and Cambra (1966)

found 2,4-D to be more effective in organ initiation than IAA or NAA at equivalent concentrations. Witham (1968) stated that when, in soybean and tobacco callus cultures, the level of 2,4-D was high, K became inhibitory to growth. Further conflicting evidence was put forward by Oswald *et al.* (1977) and Walker *et al.*(1978). The latter group (working on *Medicago sativa* ) found 2,4-D to be a suppressor of organogenesis whilst the former observed the opposite effect in their investigation into callus and plant regeneration of *Trifolium repens* and *Glycine max*.

In view of the conflicting evidence related here and the author's observations on discolouration, 2,4-D was omitted from further experimentation in *Strongylodon* callus cultures. Miller's medium in conjunction with hormones other than 2,4-D did induce callus which exhibited a marked improvement in colour and friability. Some discolouration was still observed and could possibly be attributed to the presence of phenolic compounds in the mother explant i.e., as distinct from phenolic compounds produced as metabolites in culture. Scattered pockets of phenolic compounds were observed in stem sections stained with the saffranin/ fast green stain and viewed under the compound microscope. However, this would not account for discolouration of callus on removal from the mother explant.

An initial comparison of the efficacy of certain hormones in callus production (using Miller's medium as the basal medium) suggested that a combination of NAA and BA produced a healthier (greener) callus than did a similar combination of NAA and K. In order to ascertain optimum levels of these growth regulators for callus production, concentration grids were set

up to test the effects of NAA, IBA, BA and K. Each treatment was replicated 10 times. After 20 days in culture, photographs were taken to represent the type and quality of callus growth obtained (Figs. 9 - 12). Within the concentration range investigated a combination of the growth regulators NAA and BA produced the healthiest callus. A thick mantle of callus was induced at a concentration of 1 $mgl^{-1}$ BA within the range 0 - 2 $mgl^{-1}$ NAA. The overall greatest volume of healthy callus was produced at a concentration of 1 $mgl^{-1}$ BA in combination with 1 $mgl^{-1}$ NAA. Callus production in the other treatments was not as prolific, neither was survival in culture of these calli consistent enough for further organogenesis experiments. The exception to this was the callus produced in the treatments involving a combination of 1 $mgl^{-1}$ BA and 1 $mgl^{-1}$ IBA, or 1 $mgl^{-1}$ K and 1 $mgl^{-1}$ IBA.

The controls i.e., basal medium alone - no growth regulators added, in each grid did not produce callus and died within 14 days of the initiation of the experiment. This result was similar to that obtained when activated charcoal was added to a medium containing growth regulators. A coconut milk treatment was included in order to observe the effect of a natural growth regulatory complex. The coconut milk was added to the basal medium alone and the amount of callus induced was only slightly greater than that observed where no growth regulators were present.

In all the treatments except those involving NAA/ BA combinations, the least callus development was observed where no auxin was present. Those treatments comprising auxin alone were generally only marginally more productive than the "no auxin" treatments.

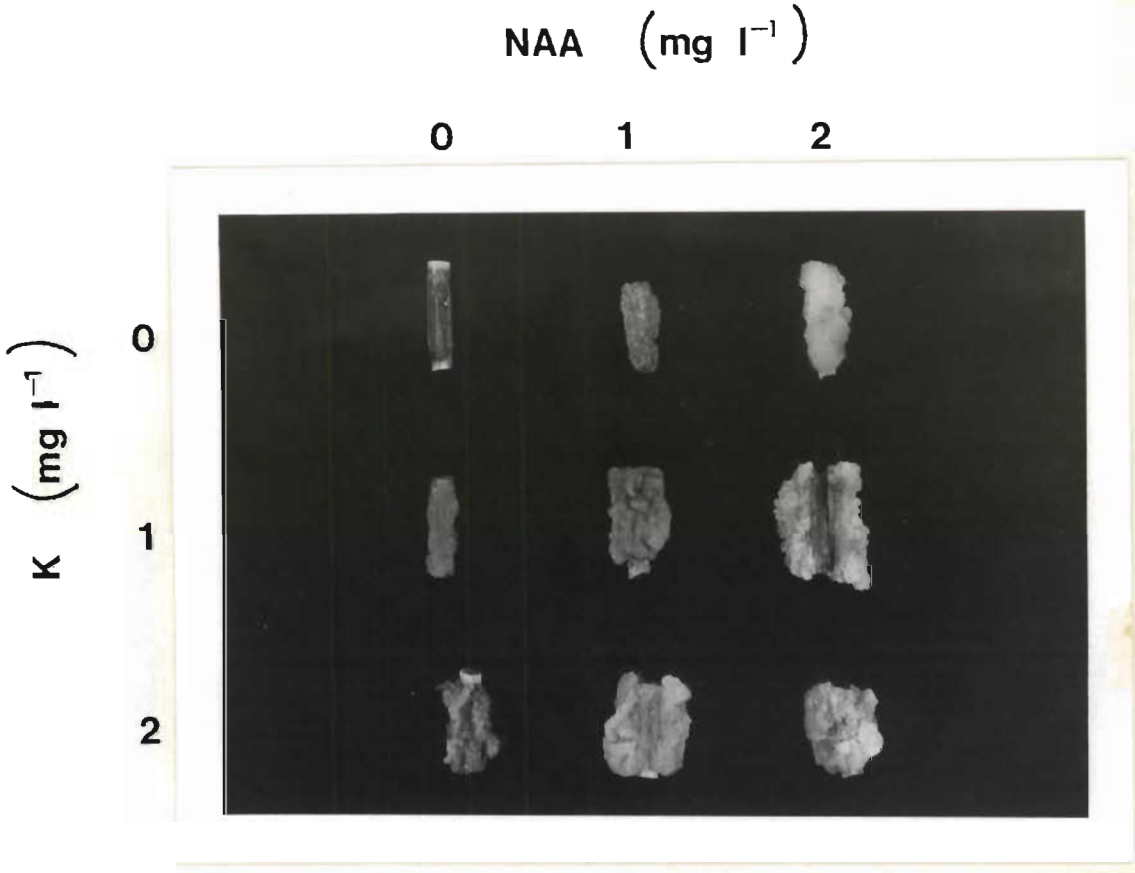



Fig. 9 Effect of different concentrations of NAA and K on callus proliferation on internode explants of *Strongylodon macrobotrys*.

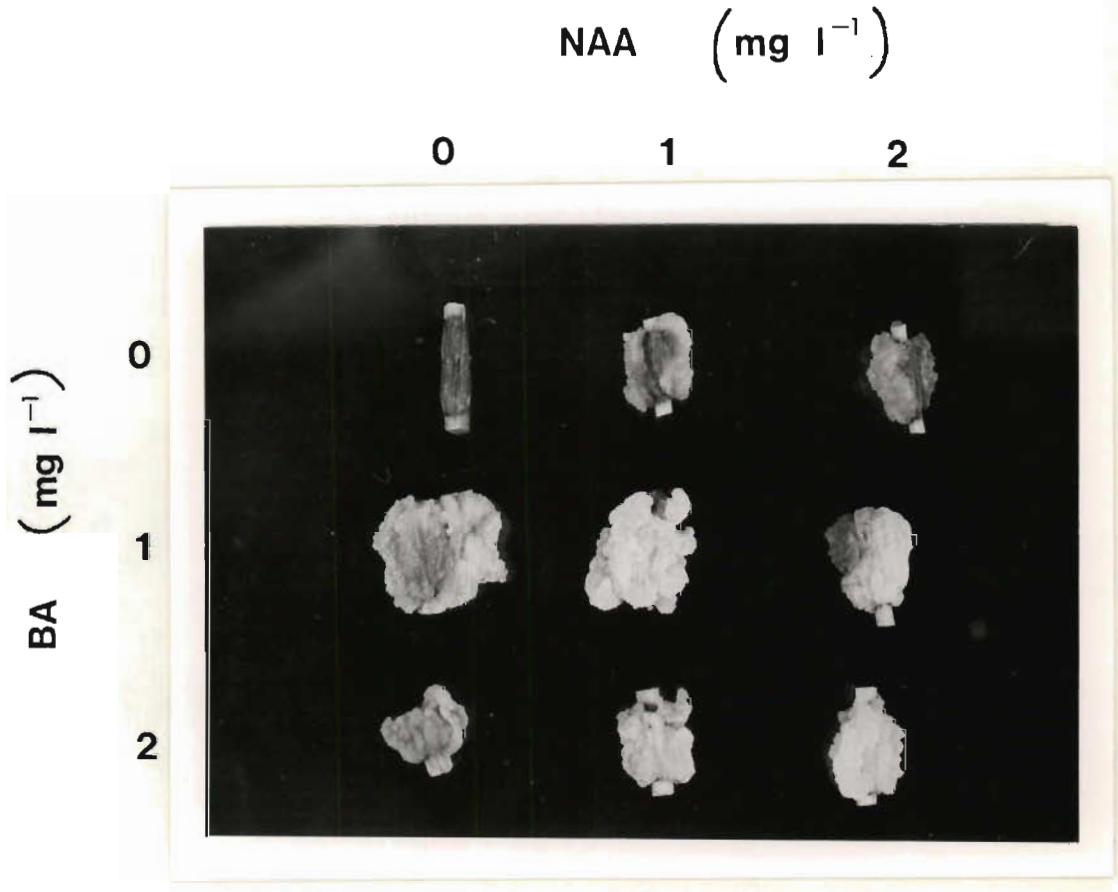



Fig. 10 Effect of different concentrations of NAA and BA on callus proliferation on internode explants of *Strongylodon macrobotrys*

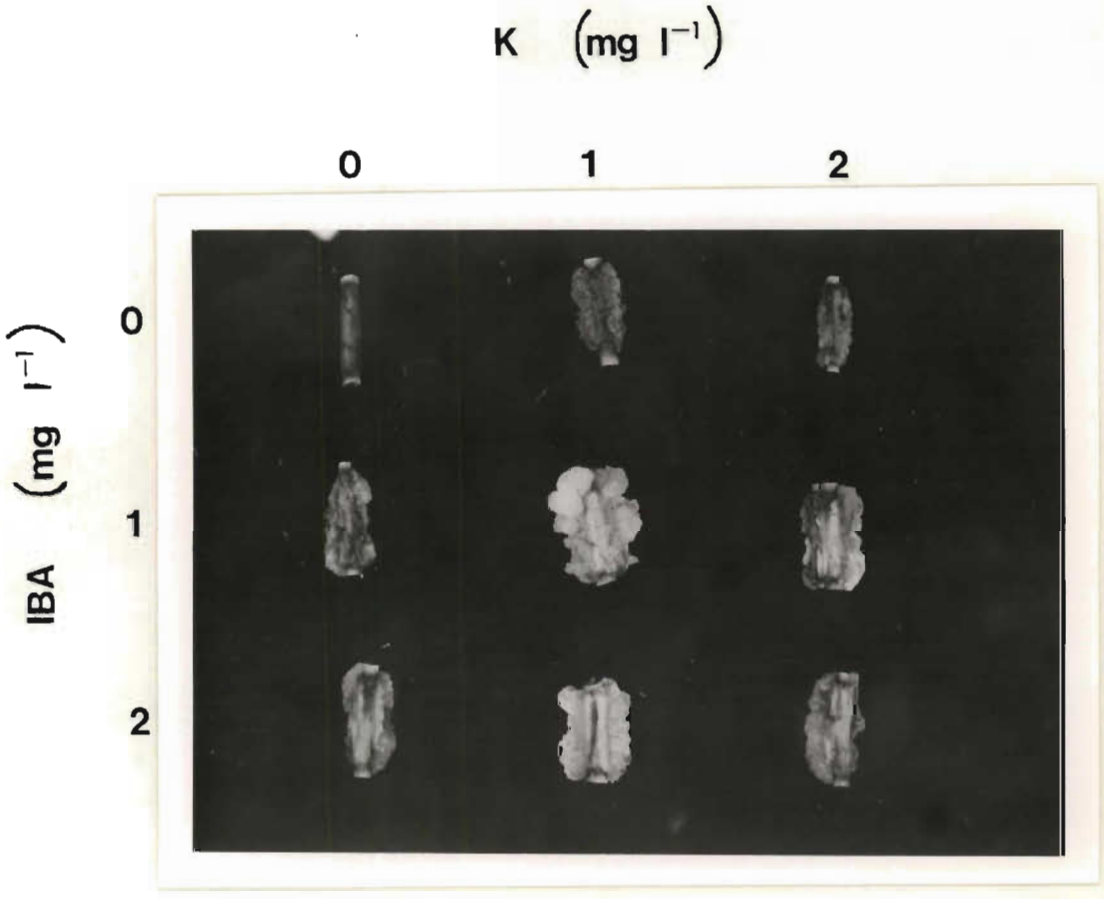



Fig. 11 Effect of different concentrations of IBA and K on callus proliferation on internode explants of *Strongylodon macrobotrys*.

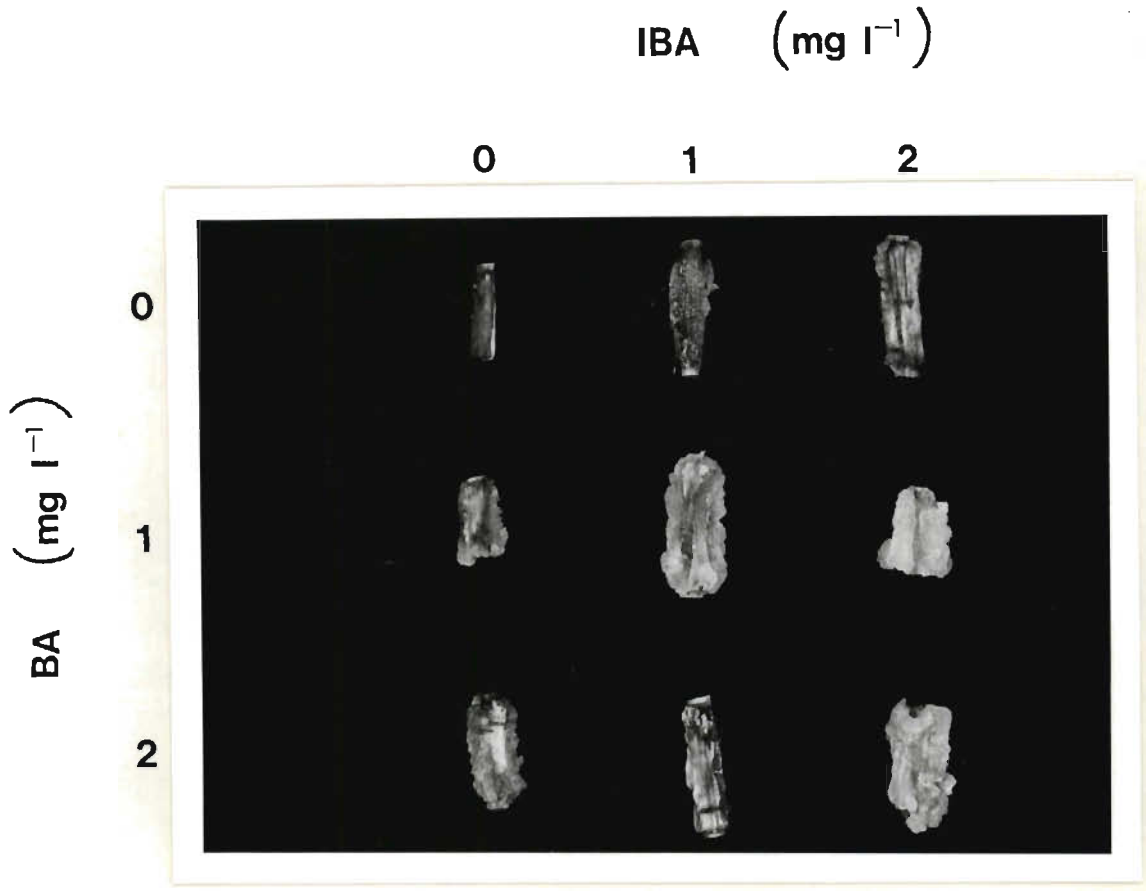



Fig. 12 Effect of different concentrations of IBA and BA on callus proliferation on internode explants of *Strongylodon macrobotrys*.

In the case of the NAA/ BA treatments, a slight drop in callus production could be observed in the "no cytokinin" treatments and in the treatment where 2 $mgl^{-1}$ BA was used in the absence of NAA. Treatments involving "high" concentrations of cytokinin i.e., 2 $mgl^{-1}$ BA or K (again with the exception of the NAA/ BA combinations) also exhibited impaired callus development. However, in the case of 2 $mgl^{-1}$ K in combination with 2 $mgl^{-1}$ NAA (Fig. 9) and 2 $mgl^{-1}$ BA with 2 $mgl^{-1}$ IBA (Fig. 12) callus production was not as low as expected for that concentration of cytokinin. In all four combination series the callus produced on media containing 1 $mgl^{-1}$ BA or K was the healthiest and most prolific.

Miller's medium plus 1 $mgl^{-1}$ NAA and 1 $mgl^{-1}$ BA was utilised subsequently as the medium for the induction of callus to be used in root and shoot regeneration experiments.

4.3.1.2 Root and shoot induction

According to Gamborg *et al* .(1974), regeneration of plants from cells cultured *in vitro* occurs either by embryogenesis or chemically induced organogenesis. Since embryogenesis is limited to cell lines of a few species (Halperin, 1970; Gamborg, Constabel and Miller, 1970), the common procedure is to induce organogenesis and plant regeneration by supplementing the media with combinations of cytokinins and auxins (Hildebrandt, 1970; Winton, 1971).

The view of Kohlenbach (1976) is that there are no generally applicable formulae for regeneration, but there are several important factors to be considered in the induction of organs:

(1) Removal of auxin - after a preculture with auxin, its ommission may lead to root formation.

(2) Ratio of cytokinin:auxin i.e.,high cytokinin:auxin may induce shoot formation and low cytokinin:auxin may induce root formation.

(3) The absolute concentration of the phytohormones.

(4) The nature of the auxins and cytokinins e.g.,BA in combination with IAA or NAA.

(5) Phytohormones other than auxins and cytokinins e.g., abscisic acid or gibberellins.

(6) Reduced nitrogen (N) i.e.,the presence of reduced N could result in embryo production and its absence result in root production.

In 1951, Skoog and Tsui proposed that other media constituents can alter the effectiveness of the phytohormones. Quantitative interactions between growth factors, rather than specific morphogenetic substances, may provide a common mechanism for the regulation of all types of morphogenetic phenomena. Skoog and Miller (1957) elaborated this theory but the mechanism cannot be universally demonstrated (Thorpe, 1978). Murashige (1978) suggests the addition of organic addenda such as adenine and tyrosine to enhance shoot multiplication. Further emphasis is placed on the constituents of the media by Thorpe (1978). He acknowledges the fact that some plants require a media change for organ formation. Tran Thanh Van (1981), in an excellent review on the control of morphogenesis in *in vitro* cultures, states that changes in the macro-micronutrient ratio and the

addition of various substances such as charcoal or organic compounds (vitamins, amino acids, polyamines, phenolamines, polypeptides, steroids or diverse plant extracts) can affect morphogenesis. Steward, Kent and Mapes (1967) suggest that various growth regulatory stimuli may need to be applied to cells in the correct amounts and right sequence and under the correct culture conditions for organ formation.

This stage of the experimentation was plagued initially by the lack of suitable callus (because of the browning problem) for transfer to regeneration media. The initiation of meaningful experiments to investigate the regenerative potential of the Jade vine callus was thus delayed until the establishment of Miller's medium as a satisfactory medium for callus production. Inconsistent results still occurred, however, and could possibly be attributed to differing levels of endogenous growth regulators in the mother explant or callus itself.

Callus cultured on Miller's medium plus 1 $mgl^{-1}$ BA and 1 $mgl^{-1}$ NAA was subcultured after four weeks on to fresh medium containing the same levels of growth regulators. After a further three-week period an increase in the quantity of callus permitted transfer to the treatments as outlined in the grid diagram (Fig. 13). Studies on *Zea mays* (Ku, Cheng, Kuo, Kuan, An and Huang, 1978) and *Trifolium pratense* (Phillips and Collins, 1979) have shown that a change in the type of growth regulators or inhibitors can be used to induce morphogenesis in those species considered reluctant to form roots and shoots. Since, in the case of the Jade vine, insufficient healthy callus was available for experiments incorporating all the factors involved in the control of morphogenesis, it was decided to attempt this

method of induction. Hence NAA was replaced by IBA and treatments investigating the influence of BA and K at different levels of IBA were set up. Miller's medium was used throughout.

| IBA ($mgl^{-1}$) \ K / BA ($mgl^{-1}$) | 0 | 1 | 2 | 5 | 10 |
|---|---|---|---|---|---|
| 0 | | | | | |
| 1 | | | | | |
| 2 | | | | | |
| 5 | | | | | |
| 10 | | | | | |

Fig. 13 Diagram to illustrate treatments involving combinations of K or BA with IBA.

Care was taken to transfer callus pieces of similar size to each 50 ml flask containing 30 ml medium. Each treatment was replicated five times. The cultures were carefully observed over a period of 20 days. After only 10 days in culture callus in some of the treatments began to turn brown and healthy callus was present in very few of the treatments. Absolutely no signs of any root or shoot development were observed in any of the treatments. At the end of the observation period the decline in viability of the callus began to accelerate. The callus

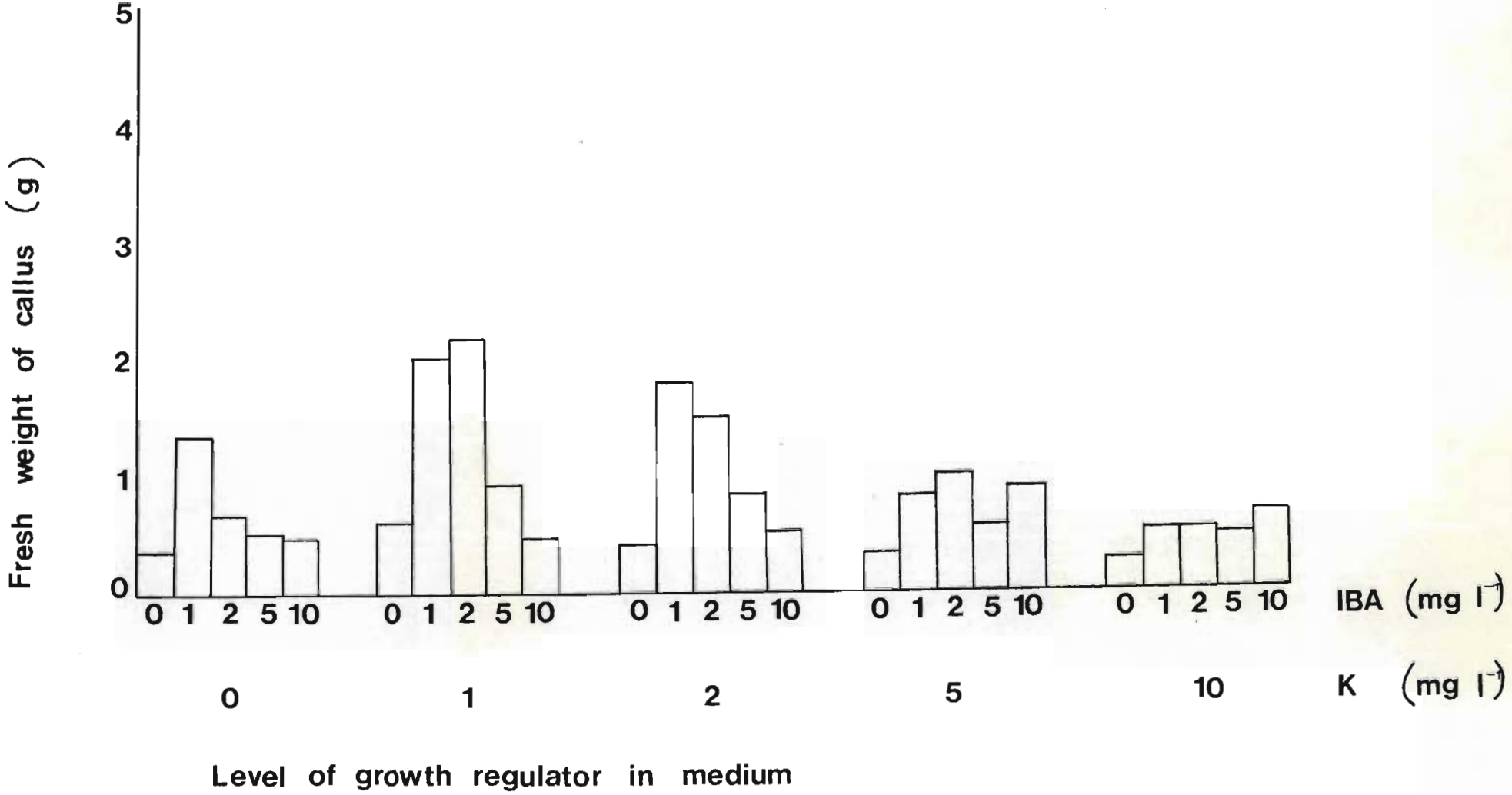



Fig. 14 Influence of K, at various levels of IBA, on proliferation of *Strongylodon macrobotrys* callus.

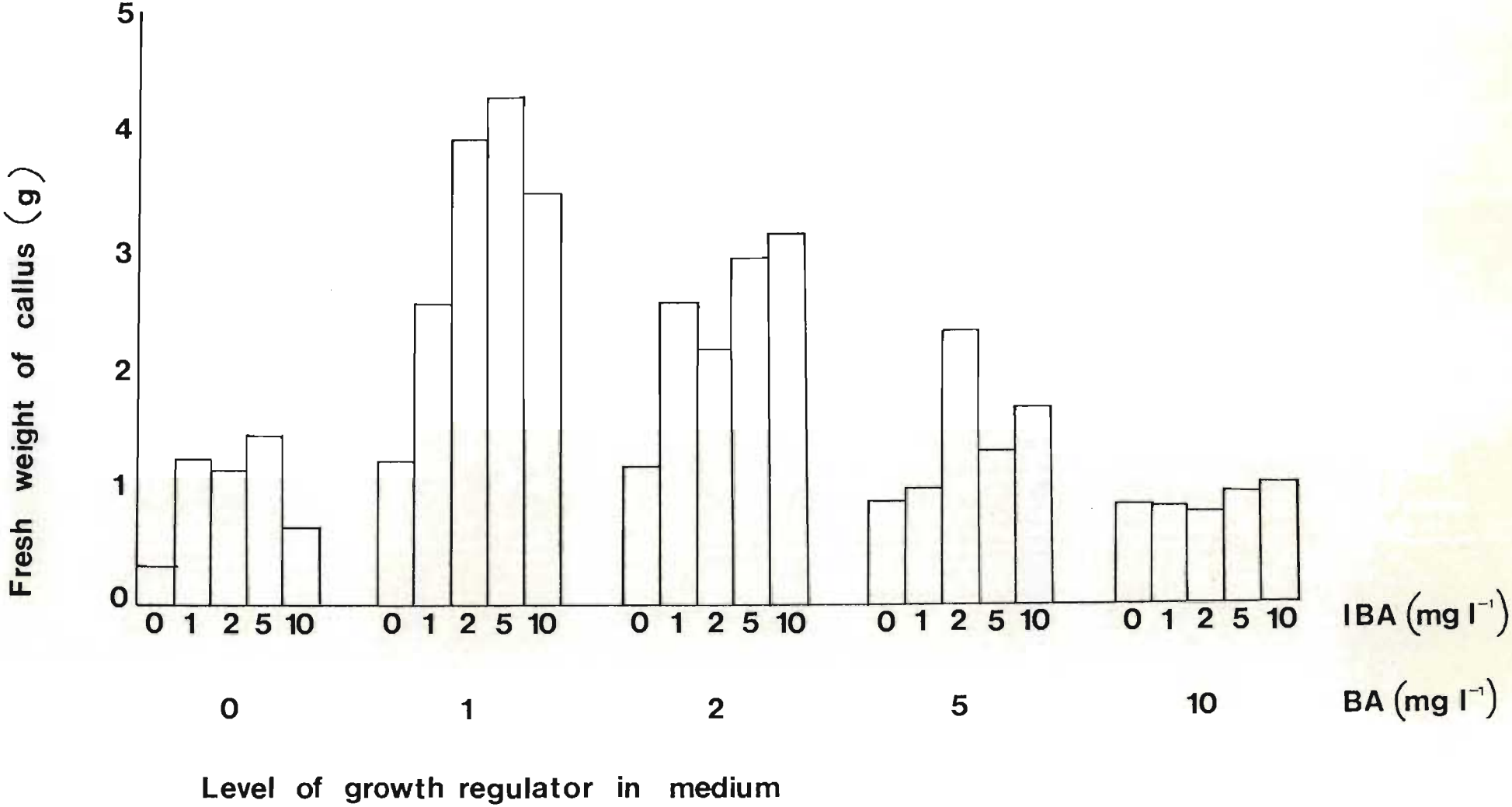



Fig. 15 Influence of BA, at various levels of IBA, on proliferation of *Strongylodon macrobotrys* callus.

from each treatment was therefore weighed in order to establish trends in callus production under the various growth regulator regimes. Appendix Tables E and F list the mass of callus obtained from the different treatments. The missing values in the K/ IBA treatments are due to contamination of three flasks of callus. The results are presented graphically in Figs. 14 and 15.

A statistical analysis of the results was carried out to determine the significance of the effects of the growth regulators on callus production. The results of these analyses are presented in the "analysis of variance" tables (Tables 11, 14 and 17). Standard errors (S.E.'s) of the means and values of least significant differences (L.S.D.'s) are presented and the results of the different treatments discussed in the light of these computations.

Table 11 Analysis of variance of experiment to investigate the effect of IBA and BA on proliferation of *Strongylodon macrobotrys* callus.

| Source | DF | SS | MS | F |
|---|---|---|---|---|
| IBA treatment | 4 | 27,263 | 6,816 | 12,171 ** |
| BA treatment | 4 | 91,777 | 22,944 | 40,971 ** |
| Interaction (IBA/BA) | 16 | 26,067 | 1,629 | 2,909 * |
| Error | 100 | 56,035 | 0,560 | |
| Total | 124 | | | |

DF = degrees of freedom

SS = sum of squares

MS = mean squares

F = F-test of significance

** = significance at 1% level

Table 12 Treatment means of experiment to investigate the effect of IBA and BA on proliferation of *Strongylodon macrobotrys* callus.

| | Treatment means (g) | | | | | |
|---|---|---|---|---|---|---|
| IBA ($mgl^{-1}$) | BA ($mgl^{-1}$) | | | | | IBA marginal means (g) |
| | 0 | 1 | 2 | 5 | 10 | |
| 0 | 0,346 | 1,216 | 1,168 | 0,870 | 0,826 | 0,885 |
| 1 | 1,254 | 2,562 | 2,570 | 0,978 | 0,822 | 1,637 |
| 2 | 1,154 | 3,910 | 2,142 | 2,300 | 0,762 | 2,053 |
| 5 | 1,452 | 4,286 | 2,914 | 1,282 | 0,922 | 2,171 |
| 10 | 0,650 | 3,482 | 3,108 | 1,656 | 1,066 | 1,992 |
| BA marginal means (g) | 0,971 | 3,091 | 2,380 | 1,417 | 0,880 | 1,748 |

Table 13 Standard Errors (S.E.'s) and Least Significant Differences (L.S.D.'s) for means of IBA/ BA treatments in experiments to investigate the effect of these growth regulators on proliferation of *Strongylodon macrobotrys* callus.

| Source | S.E. | L.S.D.(5%) | L.S.D.(1%) |
|---|---|---|---|
| Single yield | 0,150 | 0,419 | 0,554 |
| Means in body of table of means | 0,067 | 0,187 | 0,248 |
| Marginal means (IBA or BA) | 0,150 | 0,419 | 0,554 |

Coefficient of variance = 42,91%

From Table 11 it can be seen that although both factors (IBA and BA) had a significant effect on callus yield, the interaction between the two factors is also significant. Thus, neither factor can be held responsible for a particular response and Tables 12 and 13 must be examined bearing this in mind.

All treatments involving addition of either IBA or BA or both to the basal medium resulted in a significantly higher callus yield than the yield produced by the control (0 $mgl^{-1}$ IBA/ 0 $mgl^{-1}$ BA). In the case of the IBA treatments a level of 5 $mgl^{-1}$ IBA produced the highest callus yield (averaged over the range of K concentrations). However, the yield from this treatment was not significantly higher than the yields from either the 2 $mgl^{-1}$ IBA or the 10 $mgl^{-1}$ IBA treatments.

The BA treatment which produced the highest yield of callus (averaged over all IBA levels) was the 1 $mgl^{-1}$ BA treatment. This was significantly higher than the yield produced by any other BA treatment.

In combination with different levels of BA, the IBA treatments resulted in a distinct trend in callus production. Low yields were obtained when IBA (at all levels) was used in combination with 0 $mgl^{-1}$ or 10 $mgl^{-1}$ BA. The highest yields were obtained from treatments consisting of IBA plus BA levels of 1 $mgl^{-1}$ or 2 $mgl^{-1}$. This type of trend was not well marked in the case of the BA treatments. The lowest callus yields were not necessarily obtained from those BA treatments involving a combination of BA with either high or low levels of IBA. No consistent pattern of maximum callus production at each level of BA could be observed. The combination of IBA and BA which produced the

highest callus yield overall was 5 $mgl^{-1}$ IBA/ 1 $mgl^{-1}$ BA. This yield was significantly higher than the yield from any other combination of these growth regulators.

Before an analysis of the IBA/ K treatments could be carried out, substitutions had to be made for the values lost through contamination of the callus. Substituted values for missing data must be incorporated in such a way that the error sum of squares of the analysis of variance remains at a minimum (Rayner, 1967). In this analysis, the mean of the existing four values for each treatment involved was substituted as the fifth missing value. This fulfilled the "minimum error sum of squares" requirement (D.O. Chalton - pers. comm.*). The analysis is continued as for the IBA/ BA treatments. Specific comparisons can be made, if required, between those means with and without missing values. The differences are, however, slight enough to be ignored and the results are thus discussed without special reference to those treatments in which one replicate was lost by contamination.

Unlike the situation which existed in the IBA/ BA treatments, addition of various levels of IBA and K to the medium did not always result in callus yields which were significantly greater than the yield obtained from the control (no added hormones). However, only two of the treatments, viz. 0 $mgl^{-1}$ IBA/ 5 $mgl^{-1}$ K and 0 $mgl^{-1}$ IBA/ 10 $mgl^{-1}$ K resulted in yields lower than that of the controls.

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*D.O. Chalton, Department of Biometry, University of Natal, Pietermaritzburg.

Table 14 Analysis of variance of experiment to investigate the effect of IBA and K on proliferation of *Strongylodon macrobotrys* callus.

| Source | DF | SS | MS | F |
|---|---|---|---|---|
| IBA treatment | 4 | 16,864 | 4,216 | 25,707** |
| K treatment | 4 | 9,581 | 2,395 | 14,604** |
| Interaction (IBA/K) | 16 | 9,307 | 0,582 | 3,549* |
| Error | 100 | 16,446 | 0,164 | |
| Total | 124 | 52,198 | | |

Table 15 Treatment means of experiment to investigate the effect of IBA and K on proliferation of *Strongylodon macrobotrys* callus.

| | Treatment means (g) | | | | | |
|---|---|---|---|---|---|---|
| IBA ($mgl^{-1}$) | K ($mgl^{-1}$) | | | | | IBA marginal means (g) |
| | 0 | 1 | 2 | 5 | 10 | |
| 0 | 0,378 | 0,608 | 0,412 | 0,338 | 0,274 | 0,402 |
| 1 | 1,382 | 2,040 | 1,820 | 0,820 | 0,528 | 1,318 |
| 2 | 0,680 | 2,152 | 1,514 | 1,008 | 0,528 | 1,176 |
| 5 | 0,512 | 0,908 | 0,770 | 0,568 | 0,474 | 0,646 |
| 10 | 0,388 | 0,458 | 0,518 | 0,900 | 0,676 | 0,588 |
| K marginal means (g) | 0,668 | 1,233 | 1,007 | 0,727 | 0,496 | 0,826 |

Table 16 Standard Errors (S.E.'s) and Least Significant Differences (L.S.D's) for means of IBA/K treatments in experiment to investigate the effect of these growth regulators on proliferation of *Strongylodon macrobotrys* callus.

| Source | S.E. | L.S.D.(5%) | L.S.D.(1%) |
|---|---|---|---|
| Single yield | 0,081 | 0,797 | 1,053 |
| Mean in body of table (without missing value) | 0,181 | 0,507 | 0,670 |
| Mean in body of table (with missing value) | 0,202 | 0,400 | 0,529 |
| IBA/K marginal means (without missing values) | 0,083 | 0,164 | 0,217 |
| IBA/K marginal means (with missing values) | 0,081 | 0,160 | 0,212 |

Coefficient of variance = 23,17%

Once again an analysis of variance illustrates the significance of the interaction effect on yield of the two growth regulators. The IBA treatment which resulted in the highest callus yield was the 1 $mgl^{-1}$ IBA treatment (averaged over all levels of K). This yield was almost significantly higher than the second-highest yielding IBA treatment i.e., 2 $mgl^{-1}$ IBA.

The callus yield produced by the 1 $mgl^{-1}$ K treatment (averaged over all IBA levels) was the highest yield of all the K treatments, the difference between this yield and that obtained from the 2 $mgl^{-1}$ K treatment being significant at the 5% level.

Trends in callus yields from these treatments were as follows:

(a) For the IBA treatments, low levles of callus production generally occurred at the different levels of IBA in

combination with concentrations of K of 0 $mgl^{-1}$ and 10 $mgl^{-1}$. A peak in callus production usually occurred at the various levels of IBA in combination with 1 $mgl^{-1}$ K. The exception to this was observed in the treatment involving 10 $mgl^{-1}$ IBA, the peak occurring at a concentration of 5 $mgl^{-1}$ K.

(b) Treatment means for K treatments once again showed a trend of low callus production for K in combination with levels of IBA of 0 $mgl^{-1}$ and 10 $mgl^{-1}$. Exceptions to this trend occurred in the 5 $mgl^{-1}$ and 10 $mgl^{-1}$ treatments. Maximum callus production occurred in the K treatments in combination with levels of IBA of 1 $mgl^{-1}$ or 2 $mgl^{-1}$. This was true for all treatments except the 10 $mgl^{-1}$ K treatment, where the peak occurred at a level of IBA of 10 $mgl^{-1}$.

The combination of IBA and K which produced the highest yield of callus was 2 $mgl^{-1}$ IBA/ 1 $mgl^{-1}$ K.

Combined analysis of the two sets of data i.e., IBA/ BA and IBA/ K results, was carried out to compare the efficacy of the two different cytokinins in combination with the auxin. This combined analysis is justified because experimental conditions for both experiments were identical, the original source of callus was the same and the same experimental design was used in both cases. Tables 17 - 19 are used in the discussion of the differences of the effects of the auxin/ cytokinin combinations.

Table 17 Combined analysis of variance of data from IBA/K and IBA/BA treatments of *Strongylodon macrobotrys* callus.

| Source | DF | SS | MS | F |
|---|---|---|---|---|
| Difference between experiments | 1 | 53,103 | 53,103 | 144,302** |
| Variation in IBA factor between experiments | 4 | 14,425 | 3,606 | 9,799** |
| Difference between BA and K | 4 | 22,580 | 5,645 | 15,340** |
| Difference between interactions IBA/BA and IBA/K | 16 | 15,325 | 0,958 | 2,603** |
| Pooled error | 197 | 72,481 | 0,368 | |
| Total | 222 | 177,914 | | |

Table 18 Differences of means of IBA/K and IBA/BA treatments of *Strongylodon macrobotrys* callus.

| | Difference of means BA - K (g) | | | | | |
|---|---|---|---|---|---|---|
| IBA ($mgl^{-1}$) | BA/K ($mgl^{-1}$) | | | | | IBA marginal means (g) |
| | 0 | 1 | 2 | 5 | 10 | |
| 0 | -0,032 | 0,608 | 0,756 | 0,532 | 0,552 | 0,483 |
| 1 | -0,128 | 0,522 | 0,750 | 0,158 | 0,294 | 0,319 |
| 2 | 0,474 | 1,758 | 0,628 | 1,292 | 0,234 | 0,877 |
| 5 | 0,940 | 3,378 | 2,144 | 0,714 | 0,448 | 1,525 |
| 10 | 0,262 | 3,024 | 2,590 | 0,756 | 0,390 | 1,404 |
| BA/K marginal means (g) | 0,303 | 1,858 | 1,374 | 0,690 | 0,384 | 0,922 |

Table 19 Standard Errors (S.E.'s) and Least Significant Differences (L.S.D.'s) for differences of means of IBA/K and IBA/BA treatments of *Strongylodon macrobotrys* callus.

| Source | S.E. | L.S.D.(5%) | L.S.D.(1%) |
|---|---|---|---|
| Difference of means in body of table (without missing value) | 0,384 | 1,064 | 1,399 |
| Difference of means in body of table (one with a missing value) | 0,407 | 1,096 | 1,440 |
| Difference of marginal means (without missing values) | 0,575 | 0,476 | 0,626 |
| Difference of marginal means (one with a missing value) | 0,174 | 0,478 | 0,629 |

Once again, inclusion of missing values makes little difference to the analysis but L.S.D's for differences involving more than one missing value can be computed from the data given, if required.

The statistical significance of the difference between the interactions IBA/BA and IBA/K (from Table 17) indicates that the common factor (IBA) interacted differently with each of the factors BA and K. The table of differences of means of the two experiments shows that the magnitude of the interaction of IBA with BA or K, varies according to the level of hormone involved in a particular treatment. The trends in the different interactions are thus different for each level of IBA. Individual comparisons can be made from the table of differences of means, but this would serve little purpose here.

The healthiest callus in both sets of treatments was produced at levels of BA or K of 1,2 or 5 $mgl^{-1}$. In the BA treatment containing 10 $mgl^{-1}$ BA and 10 $mgl^{-1}$ IBA, severe discolouration

of the medium occurred. In both sets of treatments, levels of K and BA of 1 $mgl^{-1}$ resulted in the greatest callus production. This treatment also resulted in production of callus which maintained its viability for the longest period of time. All callus grown on medium wihtout growth regulators died within 10 days of the initiation of the experiment.

Failure of these treatments to induce organogenesis could be attributed to the lack of adequate investigation into factors other than hormone concentrations. Although initiation of organised development involves a shift in metabolism in which phytohormones play an important role (Thorpe, 1978), changes in media composition, light and temperature could have been investigated. The production of a stable, healthy callus was, however, a limiting factor in these trials. In a "reluctant" legume species such as *Psophocarpus tetragonolobus*, thin cell layers have been found to be more morphogenetically competent than the corresponding internodes (Trinh,*et al.*, 1981). These latter workers utilised thin cell layers from the epicotyl of two-week old plants and from the internodes of four-week old plants. Direct neoformation of buds occurred on these explants and the buds were capable of adventitious root formation. If seeds of the Jade vine had been more readily available and seedling establishment been successful, this mode of organogenetic induction would have been attempted.

In addition to the classical approach whereby organ formation is induced via manipulation of hormone levels, experiments without exogenously applied hormones have met with success in the culture of some legume species. Walker *et al.*(1978), working on the regeneration of alfalfa (*Medicago sativa* L. cv.

"Regen S") omitted all growth regulators from the regeneration medium, after callus had been initiated on the same basal medium containing hormones. Conditions promoting formation of either shoots or roots were thus identified by the transfer of tissues from an induction medium containing growth regulators to a regeneration medium lacking growth regulators. High concentrations of 2,4-D and low concentrations of kinetin in the induction medium promoted optimal shoot formation in tissue subsequently transferred to regeneration medium. Conversely, low levels of 2,4-D and high levels of kinetin promoted the subsequent formation of roots on the regeneration medium.

Venketeswaran and Huhtinen (1978) observed shoot regeneration of the winged bean *Psophocarpus tetragonolobus* after two or three sub-cultures of callus in the absence of hormones. However, Bottino *et al.* (1979) failed to achieve similar results in their study of the regenerative potential of the callus of the winged bean. Cells were plated on to the Murashige and Skoog (1962) medium containing no hormones and the subsequent callus growth was subcultured monthly over a 3-month period. No shoot or root formation was observed.

Meijer and Broughton (1981), in their investigations on *in vitro* culture of the legume *Stylosanthes guianensis*, observed similar effects to those cited by Walker *et al.* (1978) and Walker, Wendeln and Jaworski (1979). Continued differentiation of organs of *Stylosanthes* was, however, observed only on calli which already showed development of shoot buds before transfer to medium lacking hormones.

The callus of the Jade vine did not respond favourably to this "no-hormone" treatment. Callus was initiated on Miller's medium with the addition of BA and NAA in different concentrations to result in four different treatments as follows:

2 $mgl^{-1}$ BA + 1 $mgl^{-1}$ NAA

4 $mgl^{-1}$ BA + 0,1 $mgl^{-1}$ NAA

2 $mgl^{-1}$ NAA + 1 $mgl^{-1}$ BA

4 $mgl^{-1}$ NAA + 0,1 $mgl^{-1}$ BA

The object of the treatments was thus to repeat, in principal, the experiments of Walker *et al.* (1978) by sub-culturing callus formed on the above media to media without added growth regulators. Callus was grown on the initial media for a period of 2 weeks before transfer to the "no-hormone" medium - Miller's medium with no added hormones. Callus pieces of approximately equal size were transferred to 50 ml flasks containing the medium. After a period of 15 days on this medium,callus in all four treatments began to show signs of declining viability and was transferred to fresh Miller's (1963) medium. The rate of decline was not arrested by this subculture and after a further 15 days in culture most of the callus was dead. At no stage was the formation of roots or shoots observed.

Bottino *et al.* (1979) cited varietal differences as a possible explanation for the discrepancy between their results and those of Venketeswaran and Huhtinen (1978),in their respective investigations of the *in vitro* culture of the winged bean. A great variation in response to different treatments within a family, between genera and between species is likely to be observed. Hence, the Jade vine would not necessarily react in the same

way as the winged bean or alfalfa to this method of organ induction. The same can obviously be said of any treatment applied to different plants in tissue culture studies. Differences in basal medium composition should also be taken into account, since it is possible that the nutritional composition of the induction and regeneration media and not the hormones *per se* plays a dominant role in root and shoot induction. Walker *et al.* (1978) stated that the effect of the regeneration medium on the pattern of organ formation provides a complex and somewhat confusing picture. They stated further that the use of certain media as regeneration media obscured patterns of organogenesis which had previously been observed.

### 4.3.2 Leaf and Pulvinus

Jade vine leaves, although plentiful and easy to obtain, were not a useful source of explant material since they were extremely difficult to surface-sterilise. An effective sterilant invariably resulted in necrosis of the leaf explants before the effect of the medium on *in vitro* development could be assessed. The most effective sterilant was 2% NaOHCl (10 minute soak) which resulted in 50% contamination after 10 days in culture. The surviving explants exhibited necrotic patches and minute quantities of callus were produced only where the cut surfaces of the leaf veins, particularly the mid-rib, came into contact with the medium. This reaction in culture occurred on each of the following media, to a greater or lesser degree:

(1) Pea medium + 2 $mgl^{-1}$ NAA + 2 $mgl^{-1}$ BA

(2) Alfalfa medium + 2 $mgl^{-1}$ K + 2 $mgl^{-1}$ NAA + 2 $mgl^{-1}$ 2,4-D

(3) Miller's medium + 1 $mgl^{-1}$ BA + 1 $mgl^{-1}$ NAA

(4) M & S medium + 2 $mgl^{-1}$ BA + 1 $mgl^{-1}$ IBA.

Owing to the high degree of contamination and/or death of explants it was difficult to assess the effect of contact of the adaxial or abaxial surface of the leaf with the medium. There appeared to be no difference in response between these treatments. Well-developed pulvini proved to be excellent yielders of callus and this material was included in the experimental treatments of the stem material. The response of the pulvinus explants to these treatments was identical to that observed in the case of the stem explants, except that the former produced much greater quantities of callus from explants of a comparable size. No root/shoot induction studies were carried out on this callus.

4.3.3 Flower

Table 20 shows the effect of different media and hormone concentrations on anther and ovary explants excised from young and intermediate buds. The desired response of direct plantlet formation from anthers and ovary material was not realised and the formation of callus was the chief response. The stage of development of anthers selected is critical, with respect to the response elicited. Reinert and Bajaj (1977) stated that, in general, closed flower buds which had anthers containing uninucleate microspores were most suitable for the induction of androgenesis. It is possible that the regenerative potential of buds at an earlier stage of development than those used in these experiments should have been investigated. Although

Table 20 Response of anther and ovary explants from *Strongylodon macrobotrys* to different media.

| Medium | Hormones (mgl ) | Response | | | |
|---|---|---|---|---|---|
| | | Young bud | | Intermediate bud | |
| | | Anther explant | Ovary explant | Anther explant | Ovary explant |
| M&S ($\frac{1}{2}$)[a] | 2BA 1IBA | Blackening and death of anthers. | Callus (+)[b] | Blackening and death of anthers. | Callus (+) |
| M&S ($\frac{1}{2}$) | 5BA 1IBA | Slight swelling of anthers. Callus (++++) | Callus (+++++) | Blackening and death of anthers. | Callus (+) |
| Miller's | 2BA 1IBA | Blackening and death of anthers. Callus (+++) | Callus (+++++) | Blackening and death of anthers. | Callus (+) |
| Miller's | 5BA 1IBA | Blackening and death of anthers. Callus (+) | Callus (+++++) | Blackening and death of anthers. | Callus (+) |

a: M&S medium made up with half-strength macro- and micro- nutrients i.e. Stocks I-IV (see Table 8) and full strength Stocks V and VI.

b: Callus development after 14 days in culture on a scale of 1 - 5 (+ - +++++). Callus developed on filaments in the case of the anther explants.

Table 21 Results of decontamination procedures carried out on nodal material of *Strongylodon macrobotrys*.

| Procedure | Result |
|---|---|
| 5% NaOHCl for periods up to 20 mins. | *Total contamination after 7 days. |
| Explants soaked in 1% Benomyl for 30 mins prior to sterilisation in 5% NaOHCl for 10 mins. | Total contamination after 5 days. |
| 10% NaOHCl for 10 mins. | Total contamination after 7 days. |
| 0,1% $HgCl_2$ for 1 min. | Total contamination after 7 days. |
| 0,5% $HgCl_2$ for 1 min. | Fungal contamination of 40/50 explants. Death of all remaining nodes. |
| 1% $HgCl_2$ for 1 min. | Fungal contamination of 50/99 explants. Death of 32 remaining nodes. |

*"Total" contamination is the term used although one or two explants did survive each treatment.

death of the anthers did occur in all treatments, with one exception as indicated in Table 20, those anthers excised from the younger flowers remained healthier for longer than their older counterparts. The selection of the media used was fairly arbitrary. The basal media of White (1943), Murashige and Skoog (1962) and Nitsch and Nitsch (1969) have been used for anther culture (Reinert and Bajaj, 1977). Mokhtarzedh and Constantin (1978) used M & S medium to regenerate plants from anther callus of the legume berseem clover (*Trifolium alexandrinum*). A comparison was thus made between M & S and Miller's medium, which had proved successful in establishment of callus on Jade vine stem explants. The results of this comparison were inconclusive because of the scale of the experimentation.

### 4.3.4 Nodal Material

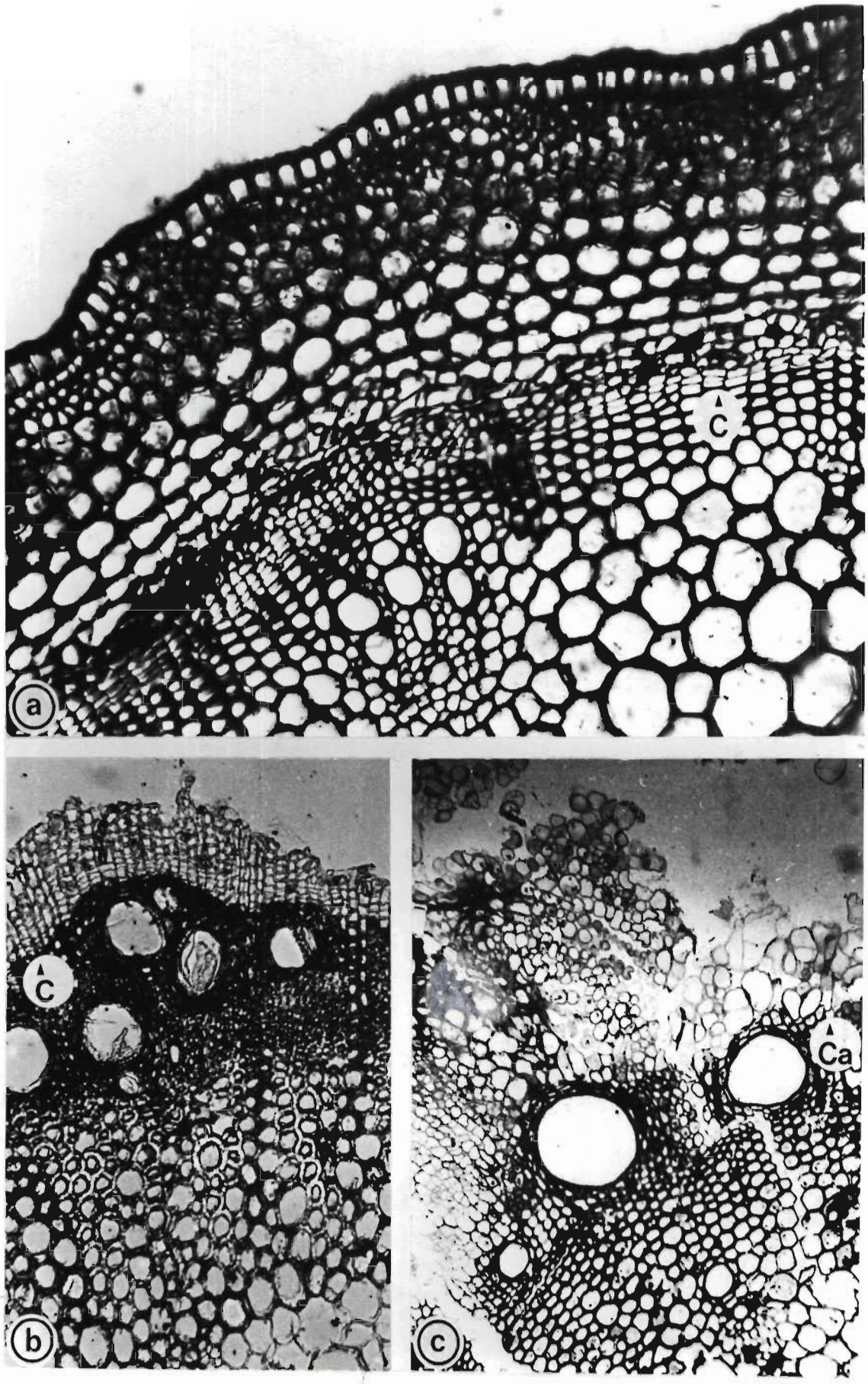
This phase of the investigations was plagued by overwhelming difficulties in decontamination of the nodal material. The nodes where buds develop in the axils of the leaves are ideal "settling grounds" for debris and fungal spores and when viewed, using the scanning electron microscope, were observed to be covered in foreign material. Table 21 shows the sterilisation procedures attempted and the degree of success of each regime. The removal of bracts and bud scale leaves,as described in 4.2.2.4,did result in a slightly improved efficacy of the NaOHCl treatments,but the $HgCl_2$ treatments were not repeated in view of the death of the explants (see Table 21 ).

It was hoped to initiate a "seasonal" study to examine the possible effects of bud dormancy on subsequent growth *in vitro* of those few explants which did survive uncontaminated. The

initial aim was to use nodal material collected in August, September, October and November in order to assess the effects of different hormone treatments on bud development (using Miller's as the basal medium). However, in view of the serious decontamination problems experienced these effects could not be investigated. As an alternative strategy a standard medium, employing set levels of hormones selected for their possible ability to induce shoot elongation of the buds, was used for each set of material collected. Thus Miller's medium with a relatively high level of cytokinin ($4mgl^{-1}$ K) and low level of auxin ($1mgl^{-1}$ NAA) was used as the medium for the material collected on a monthly basis.

Throughout the course of the study no shoot development (or root growth) was observed in any of the buds which survived in each monthly treatment. Callus developed consistently at the cut base of the explants where contact was made with the medium.

Several factors could possibly be responsible for the failure of the nodal explants to develop *in vitro*. Firstly, the decontamination problem itself prevented an adequate investigation into the requirements for growth. The medium selected as the standard growth medium did not necessarily supply the essential nutrients and growth regulators for shoot development, although it did support callus growth. Secondly, although the technique for removing all extraneous matter was useful in improving the surface sterilisation of the explants, it may have caused damage to the young buds. Thirdly, a dormancy factor could have influenced bud elongation *in vitro*. Vigorous growth of the Jade vine is observed under Durban's climatic conditions during December and January. During this period up to four metres of new growth may be observed. Growth declines during


a
C
b
C
c
Ca

the cooler months until July/August when the vine begins to flower. Cuttings to be rooted are normally taken from the vine once flowering is complete (B. Blaydes - pers. comm.)*. It would therefore appear likely that bud material collected from the end of the flowering period (November) and in December and January would have the greatest chance of survival and development *in vitro*.

### 4.3.5 Seed

The cotyledon explants produced roots on Miller's medium containing 0,5 $mgl^{-1}$ K and 1 $mgl^{-1}$ NAA after 15 days in culture. Callus began to develop on these roots and on the cotyledon tissue after a further 10 days in culture. Tranfer of this material to Miller's medium plus 4 $mgl^{-1}$ BA and 1 $mgl^{-1}$ NAA (in an attempt to induce shoot production and slow callus development) did not result in a change in response. The roots continued to elongate and the callus proliferated.

Pod wall material produced a fairly vigorous callus on Miller's medium plus 1 $mgl^{-1}$ BA and 1 $mgl^{-1}$ NAA. However, this callus declined rapidly and no root/shoot induction tests were carried out on it.

### 4.3.6 Anatomical studies

Fig. 16 shows sections of unpeeled and peeled stem segments as well as the origin of callus on an unpeeled stem segment. Peeling of the stem resulted in exposure of the cambium (C) to the medium and Fig. 16c shows callus cells (Ca) developing from this meristematic region. It can be observed

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*B. Blaydes, Production and Display Officer, Durban Parks, Recreation and Beaches Department

that the peeling treatment removed a fair amount of meristematic tissue and this could account for poor callus development in some cases.

## 4.4 Conclusion

Plant regeneration is a requirement for a well-defined tissue culture system. This requirement was not met in the study of the *in vitro* development of *Strongylodon macrobotrys*. Legumes are similar to most species in that the ability for plant regeneration seems to be lost in cultures of callus as the sole tissue (Jelaska, 1974). In the case of the Jade vine, the production of a healthy callus was itself a difficult task. Maintenance of a callus and its subsequent use in regeneration experiments was hampered by a rapid decline in callus viability. Experiments were conducted to ascertain optimum growth regulator concentrations for callus production. Subsequent subculture of calli to media containing different levels of growth regulators (or no growth regulators at all) was carried out in an attempt to induce root or shoot regeneration. No organs developed as a result of these treatments and the trends observed in callus proliferation at different growth regulator levels were inconclusive.

The first line of research to be followed in a continuation of this work could be the establishment of a suitable medium for culture of the vine via callus. However, in accordance with the views expressed by Hussey (1981a and 1981b), who illuminated the serious drawbacks to the use of callus for *in vitro* propagation, it is this author's contention that a method without resort to callus should be sought. With this in mind,

a more in-depth study of bud material could be undertaken. The formidable problem of the decontamination of the nodal explants would have to be overcome before satisfactory results could be achieved. Use of seeds to produce sterile seedlings for further *in vitro* investigations could be another avenue to follow.

Although the *in vitro* regeneration of other legume species has been achieved, the degree of regeneration of whole plants from cultured tissue does vary from species to species (Narayanaswamy, 1977). Intervarietal differences in plant regeneration have also been observed amongst members of the family Leguminosae (Bingham *et al.*, 1975; Malmberg, 1979; Phillips and Collins, 1979). It is not, therefore, altogether surprising to discover difficulties in the *in vitro* regeneration of the Jade vine.

Different morphogenetic potentials have been illustrated in different plant parts (Bharal and Rashid, 1979) and a further line of research could, therefore, involve a detailed study of the regenerative potential of, for example, apical meristems and immature embryos of *S. macrobotrys*. In this project leaf material proved unsuitable for culture, but a more detailed investigation of anthers and ovaries, both of which demonstrated an ability to be cultured *in vitro*, could be carried out. Evans, Sharp and Flick (1981) state that few generalisations can be made with regard to the role of plant hormones in the regeneration of legume species *in vitro*. In addition, regeneration of organs often occurs more readily on simpler media than those required for actual tissue or cell culture (Narayanaswamy, 1977).

It would thus appear that manipulation of the medium composition as a whole could have more beneficial effects on plantlet formation than simply changing the levels of the growth regulators present in the medium.

Conditions of light and temperature may have been less than ideal during the course of the experimentation, although they complied, in magnitude, with the generally accepted "norms" for similar studies. The peak in spectral irradiance in the culture room (Fig. 1) occurred in the region of the yellow and yellow-green wavelengths i.e., 550nm - 600 nm (Kondratyev, 1969). It is possible that the spectral irradiance of the blue region which, according to Weiss and Jaffe (1969), is critical for shoot development, was too low. The use of Gro-Lux lighting tubes could overcome this problem. Although it has not been possible to induce differentiation at will in legume tissue culture (Bharal and Rashid, 1979), the lack of a suitable medium and/or ideal culture environment constituted a major barrier to this investigation. The failure to produce a viable callus was an overall limitation to the scale and depth of the study.

# 5. GENERAL CONCLUSION

Plant tissue culture or aseptic culture of cells and organs has gained considerable prominence in research and commercial applications (Murashige, 1980). The propagation of ornamentals via tissue culture, as investigated in this study, should be viewed in the light of its commercial acceptability. Evans *et al.* (1981) advise that the rate of tissue culture propagation must be faster than conventional means of propagation in order to be economically viable. Virtually all marketable orchids are clonable through tissue culture and about 100 laboratories in the U.S.A. can propagate 300 other plants by the method (Murashige, 1980). Bearing the economic factors in mind, even the relatively successful *Peperomia* culture would have to be improved upon before it could be considered as an alternative to conventional means of propagation.

Any tissue culture study presents an almost infinite number of parameters for investigation and the scale of this project imposed limitations on those factors which could be investigated. However, the establishment of the effects of those factors tested does provide a pointer for the direction of further studies.

"Our usual method of reporting research leaves out all the fun and adventure. It leaves in only the bare bones of orderly fact, as we can arrange them best after prolonged and sober study. This process may engender an image of the scholarly Scientist who operates according to a coldly logical Scientific Method ..... . But what keeps you and me plugging away at research is not some lofty sense of scholarly achievement, it is an intensely human reward of fun and adventure."

John Decker.

Appendix Table A Results of decontamination procedures carried out on explants of *Peperomia clusifolia* and *Strongylodon macrobotrys*.

| Explant source | Sterilant | Duration of sterilisation | Percentage contamination after ten days |
|---|---|---|---|
| 1. *P. clusifolia* | | | |
| Leaf, stem and floral shoot | 2% NaOHCl<br>0,1% $HgCl_2$ | 10 mins<br>30 secs | 33% (32/98 explants)<br>Death of 55% explants (54/98) owing to strength of sterilant |
| 2. *S. macrobotrys* | | | |
| Internodes | 0,5% NaOHCl | 7 mins | 58% (42/72 explants) |
| | 1% NaOHCl | 20 mins | 51% (37/72 explants) |
| | 5% NaOHCl | 10 mins | 38% (27/72 explants) |
| | 0,1% $HgCl_2$ | 30 secs | 46% (33/72 explants) |
| | 0,5% $HgCl_2$ | 30 secs | 42% (30/72 explants) |
| Leaf and pulvinus | 0,5% NaOHCl | 7 mins | 100% (72/72 explants) |
| | 2% NaOHCl | 10 mins | 50% (36/72 explants) |
| Flower | 2% NaOHCl | 5 mins | 8% (2/25 anther explants<br>No ovary explants contaminated. |
| Nodal material | See Table 21 for details | | |
| Seed | 0,1% $HgCl_2$ | 5 mins | 0% (0/8 explants) |
| Pod wall | 2% NaOHCl | 10 mins | 10% (2/20 explants) |

Appendix Table B Spectral irradiance in the growth room where cultures of *Peperomia clusifolia* and *Strongylodon macrobotrys* were maintained.

| Wavelength (nm) | Spectral irradiance ($x10^{-2}Wm^{-2}nm^{-1}$) |
|---|---|
| 380 | 0,041 |
| 400 | 0,473 |
| 425 | 0,688 |
| 450 | 0,779 |
| 475 | 0,858 |
| 500 | 0,847 |
| 525 | 0,948 |
| 550 | 2,352 |
| 575 | 2,760 |
| 600 | 2,260 |
| 625 | 1,330 |
| 650 | 0,720 |
| 675 | 0,320 |
| 700 | 0,160 |
| 725 | 0,110 |
| 750 | 0,080 |

Appendix Table C Dehydration and staining schedule for sections of *Strongylodon macrobotrys* stem (adapted from Johansen, 1940).

| Treatment number | Treatment | Time |
|---|---|---|
| 1 | 70% alcohol | 1 min |
| 2 | Saffranin made up in 70% alcohol | 15 min |
| 3 | 95% alcohol | 1 min |
| 4 | 100% alcohol | 1 min |
| 5 | 100% alcohol | 1 min |
| 6 | Xylene/alcohol | 1 min |
| 7 | Fast green stain | 15 sec |
| 8 | Xylene/alcohol | 30 sec |
| 9 | Xylene | 1 min |
| 10 | Mount in D.P.X. | |

Appendix Table D Fixation, dehydration and embedding schedule for sections of *Strongylodon macrobotrys* stem sections and callus (embedding technique adapted from Luft, 1961).

| Treatment number | Treatment | Time |
|---|---|---|
| 1 | Tissue trimmed. Primary fixation in 5% gluteraldehyde made up in 0,05M Na-cacodylate buffer (pH 7,2) | 20 hours |
| 2 | Washed in 0,05M Na-cacodylate buffer (pH 7,2) | Two washes, each of 30 mins duration |
| 3 | Post fixation in 2% sodium tetroxide made up in 0,05M Na-cacodylate buffer (pH 7,2) | Four hours in refrigerator |
| 4 | Dehydration in graded alcohol series | Elapsed time of 60 mins |
| 5 | Rinsed in absolute alcohol | Two rinses, each of 15 mins duration |
| 6 | Washed in propylene oxide | Two washes, each of 30 mins duration |
| 7 Embedding: | | |
| (i) | Three parts propylene oxide: one part Epon-Araldite | Two hours |
| (ii) | Two parts propylene oxide: two parts Epon-Araldite | One hour |
| (iii) | One part propylene oxide: three parts Epon-Araldite | Eight hours |
| (iv) | Pure Epon-Araldite | 24 hours |
| 8 | Tissue mounted in moulds and polymerized at 70°C | 48 hours |

Appendix Table E Final mass of *Strongylodon macrobotrys* callus after treatment with growth regulators IBA and BA (5 replicates per treatment).

| | Mass of callus (g) | | | | |
|---|---|---|---|---|---|
| IBA ($mgl^{-1}$) | BA ($mgl^{-1}$) | | | | |
| | 0 | 1 | 2 | 5 | 10 |
| 0 | 0,24 | 1,16 | 2,00 | 1,47 | 0,71 |
| | 0,39 | 1,56 | 0,52 | 0,27 | 0,59 |
| | 0,51 | 1,36 | 1,38 | 0,86 | 0,26 |
| | 0,27 | 0,92 | 0,64 | 1,40 | 0,97 |
| | 0,32 | 1,08 | 1,30 | 0,35 | 1,60 |
| Total | 1,73 | 6,08 | 5,84 | 4,35 | 4,13 |
| 1 | 1,62 | 1,47 | 2,10 | 1,19 | 0,46 |
| | 1,27 | 4,88 | 1,28 | 0,83 | 0,95 |
| | 0,76 | 2,34 | 3,34 | 1,45 | 0,52 |
| | 2,06 | 2,48 | 2,89 | 1,08 | 1,51 |
| | 0,56 | 1,64 | 3,24 | 0,34 | 0,67 |
| Total | 6,27 | 12,81 | 12,85 | 4,89 | 4,11 |
| 2 | 1,58 | 3,19 | 2,42 | 2,15 | 1,21 |
| | 0,80 | 3,27 | 2,23 | 1,32 | 0,49 |
| | 1,96 | 4,17 | 1,56 | 2,91 | 0,51 |
| | 0,55 | 5,54 | 2,40 | 2,18 | 0,64 |
| | 0,88 | 3,38 | 2,10 | 2,94 | 0,96 |
| Total | 5,77 | 19,55 | 10,71 | 11,50 | 3,81 |
| 5 | 1,72 | 4,01 | 2,21 | 1,05 | 0,93 |
| | 0,79 | 5,82 | 3,37 | 1,49 | 0,70 |
| | 1,45 | 2,58 | 1,65 | 1,80 | 0,74 |
| | 2,40 | 5,45 | 3,99 | 0,63 | 1,18 |
| | 0,90 | 3,57 | 3,35 | 1,44 | 1,06 |
| Total | 7,26 | 21,43 | 14,57 | 6,41 | 4,61 |
| 10 | 0,49 | 3,29 | 1,93 | 1,22 | 0,96 |
| | 0,49 | 3,81 | 1,77 | 1,50 | 1,80 |
| | 0,99 | 4,54 | 2,63 | 1,94 | 1,05 |
| | 0,78 | 4,29 | 3,93 | 1,15 | 0,51 |
| | 0,58 | 1,48 | 5,28 | 2,47 | 1,01 |
| Total | 3,25 | 17,41 | 15,54 | 8,28 | 5,33 |

Appendix Table F Final mass of *Strongylodon macrobotrys* callus after treatment with growth regulators IBA and K (5 replicates per treatment).

| | Mass of callus (g) | | | | |
|---|---|---|---|---|---|
| IBA ($mgl^{-1}$) | K ($mgl^{-1}$) | | | | |
| | 0 | 1 | 2 | 5 | 10 |
| 0 | 0,60 | 0,77 | 0,61 | 0,24 | 0,21 |
| | 0,33 | 0,53 | 0,30 | 0,34 | 0,27 |
| | 0,44 | 0,65 | 0,32 | 0,33 | 0,28 |
| | 0,30 | 0,22 | 0,50 | 0,42 | 0,38 |
| | 0,22 | 0,87 | 0,33 | 0,36 | 0,23 |
| Total | 1,89 | 3,04 | 2,06 | 1,69 | 1,37 |
| 1 | 1,22 | 0,19 | 1,63 | 0,97 | 0,65 |
| | 0,84 | 2,68 | 2,78 | 0,81 | 0,44 |
| | 1,74 | 3,26 | 1,27 | 0,72 | 0,52 |
| | 1,61 | 1,91 | 2,36 | 0,82 | 0,73 |
| | 1,50 | 2,16 | 1,06 | 0,78 | 0,30 |
| Total | 6,91 | 10,20 | 9,10 | 4,10 | 2,64 |
| 2 | 0,70 | 1,40 | 0,50 | 0,45 | 0,37 |
| | 0,92 | 1,58 | 1,61 | 0,50 | 0,81 |
| | 0,69 | 3,07 | 1,88 | 1,29 | 0,54 |
| | 0,41 | 1,86 | 1,41 | 1,14 | 0,52 |
| | | 2,85 | 2,17 | 1,66 | 0,40 |
| Total | 2,72 | 10,76 | 7,57 | 5,04 | 2,64 |
| 5 | 0,40 | 0,45 | 0,64 | 0,77 | 0,45 |
| | 0,40 | 0,80 | 1,23 | 0,36 | 0,94 |
| | 0,30 | 1,05 | 0,50 | 0,78 | 0,16 |
| | 0,93 | 1,44 | 0,70 | 0,65 | 0,31 |
| | 0,53 | 0,80 | | 0,28 | 0,51 |
| Total | 2,56 | 4,54 | 3,07 | 2,84 | 2,37 |
| 10 | 0,24 | 0,47 | 0,52 | 0,85 | 1,29 |
| | 0,33 | 0,57 | 0,74 | 1,05 | 0,55 |
| | 0,58 | 0,31 | 0,35 | 1,09 | 0,60 |
| | 0,42 | 0,54 | 0,46 | 0,95 | 0,26 |
| | 0,37 | 0,41 | 0,52 | 0,56 | |
| Total | 1,94 | 2,29 | 2,07 | 4,50 | 2,70 |

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