IN VITRO PROPAGATION
OF
SCILLA NATALENSIS PLANCH.

by
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submitted in fulfilment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
in the
Department of Botany, Faculty of Science,
University of Natal, Pietermaritzburg

June 1999

PREFACE.

The experimental work described in this thesis was conducted in the Department of Botany, University of Natal, Pietermaritzburg, under the supervision of Professor J. van Staden. These studies were the result of my own investigation, except where the work of others is acknowledged.

Shelagh Alison McCartan
June 1999

I certify that the above statement is correct.

Professor J. van Staden
June 1999

WORK PRESENTED FROM THIS STUDY.

1. PAPERS PUBLISHED IN JOURNALS.

McCartan S.A. & van Staden J. 1998 Micropropagation of the medicinal plant, *Scilla natalensis* Planch. Plant Growth Reg. 25: 177-180

McCartan S.A. & van Staden J. 1999 Micropropagation of members of the Hyacinthaceae with medicinal & ornamental potential - A review. S. Afr. J. Bot. (In press).

2. PAPERS AND POSTERS PRESENTED AT CONFERENCES.

McCartan S.A. & van Staden J. 1995 *In vitro* propagation of *Scilla natalensis.* 21st Annual Congress of the South African Association of Botanists. (Paper)

McCartan S.A. & van Staden J. 1997 Rapid and economical propagation of the traditional medicinal plant, *Scilla natalensis* Planch. International Symposium for the Biotechnology of Tropical and Subtropical Species, Brisbane. Australia (Poster)

ACKNOWLEDGEMENTS.

I wish to thank:

Professor J. van Staden, Doctor J.F. Finnie and Doctor W.A. Cress for their invaluable input;

Professor R.P. Beckett and Mr G.D. Sharp (University of Port Elizabeth) for enduring endless statistical queries;

Ms J. L. S. Taylor for kindly providing photographs for Figures 3A, F, G and H;

Ms J. L. S. Taylor and Ms C. S. Ford for their help with the printing, photocopying and compiling of this thesis;

The Foundation for Research Development and the University of Natal Research Fund for financial assistance;

And lastly, but not least, my family and friends for their unwavering support.

ABBREVIATIONS.

2,4-D	2,4-dichlorophenoxyacetic acid
AC	activated charcoal
B5	*Gamborg et al* B5 medium
BA	benzyladenine
BDS	modified B5 medium
CH	casein hydrolysate
CM	coconut milk
EC	electrical conductivity
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
iP	6-[γ,γ]-dimethylallylamino purine
kinetin	N^6-furfuryladenine
MS	Murashige & Skoog medium
NAA	*α-napthyaleneacetic acid*
PAC	paclobutrazol
PGR's	plant growth regulators
TDZ	thidiazuron
YE	yeast extract

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ABSTRACT.

In South Africa, large quantities of *Scilla natalensis* are harvested from wild populations and sold as traditional medicine, which is reducing the density, distribution and genetic diversity of wild populations. The enforcement of existing legislation, however, has proved ineffective with plants being traded locally and internationally. It has therefore, been suggested that *ex situ* conservation through cultivation may alleviate pressures on natural resources. Conventional propagation of these plants, however, is usually fairly slow. *In vitro* propagation provides a rapid means of propagating selected chemotypes or cultivars, serving both conservation and commercial interests.

In the first part of the study, continuous culture systems were established for the three forms of *Scilla natalensis, S. natalensis sensu stricto* (Form A), *S. natalensis* syn. *S. kraussii* (Form B) and *S. natalensis* syn. *S. dracomontana* (Form C). The efficiency of the systems was strongly influenced by genetic factors, viz the form and epigenetic factors, viz the explant type, carbohydrate source, plant growth regulators and gelling agents. The form, Form A, Form B or Form C respectively, influenced shoot initiation with the larger forms generally producing more shoots than the smaller forms (Form A > Form B > Form C). The data confirmed that the three forms are significantly different in terms of their physiological response to carbohydrates, plant growth regulators and gelling agents *in vitro.* Since the effect of form could not be compensated for by the addition of either carbohydrates, plant growth regulators or gelling agents, this may provide some support for the reinstatement of these forms as three species, *Scilla natalensis* Planch., *S. kraussii* Bak. and *S. dracomontana* Hilliard & Burtt. The explant type, that is bulb or leaf explants respectively, significantly influenced shoot initiation. Leaf explants generally produced more shoots than bulb explants. The carbohydrate source significantly influenced shoot initiation. The explants generally produced more shoots when cultured on media containing glucose or sucrose than on media containing fructose, lactose, maltose and particularly mannitol. The combination of cytokinins and auxins significantly influenced shoot initiation. Shoot initiation was higher for combinations of kinetin: IAA than for combinations of kinetin: NAA or TDZ: NAA.

Optimal shoot initiation for Form A, Form B and Form C occurred on media containing 1 to 2 mg l^{-1} kinetin and 1 to 2 mg l^{-1} IAA. The gelling agent also influenced shoot initiation with media solidified with Gelrite® producing more shoots than media solidified with Oxoid or Unilab agar. Shoots were then rooted on media containing IAA, IBA or NAA and the plantlets were successfully acclimatised. These continuous culture systems can be used to produce large quantities of plantlets, which may alleviate pressures on natural resources and provide an alternative source of high quality plants for the burgeoning medicinal plant market.

In the second part of the study, the effect of carbohydrate source and concentration on growth and development of shoots of *S. natalensis* syn. *sensu stricto* (Form A) were determined. This has applications for the acclimatisation and germplasm storage of bulbous plants. The carbohydrate source and concentration significantly influenced the growth and development of shoots. In the absence of carbohydrates, the shoots were short with spindly leaves and short roots. When media were supplemented with high concentrations of fructose, the shoots were long with broad leaves, small bulbs, and few short to medium length roots at low concentrations. At higher fructose concentrations, however, the shoots were robust and short with narrow, sometimes deformed leaves, large bulbs, and few stunted, brown roots. When sucrose was substituted for fructose, the shoots were robust and long with narrow and often red-pigmented leaves, large bulbs, and many long, thick roots. When AC was used in combination with sucrose, however, the shoots were robust and short with few, and occasionally red-pigmented leaves, small to medium bulbs, and few, severely stunted roots. Optimal shoot growth and development in terms of shoot weight (FW) and quality occurred on media containing glucose or sucrose (40 to 60 g l^{-1}). The carbohydrate source and concentration also significantly influenced the physical properties of media particularly pH, electrical conductivity (EC) and gel-strength. The pH decreased slightly with increasing glucose concentration but decreased significantly with increasing fructose concentration when fructose was used alone or in combination with glucose. The pH also decreased significantly with increasing sucrose concentrations when sucrose was used in combination with Sigma AC. The EC decreased significantly with increasing fructose concentration when fructose was used alone but remained fairly

constant irrespective of glucose concentration when glucose was used alone or in combination with fructose. The EC also remained fairly constant irrespective of the sucrose concentration but decreased with increasing sucrose concentration when used in combination with AC. The gel-strength remained fairly constant irrespective of glucose. The gel-strength decreased with increasing fructose concentration when used alone or in combination with glucose. The gel-strength of media increased with increasing sucrose concentration although the addition of Sigma AC significantly decreased the gel-strength of media, which decreased with increasing sucrose concentration. The brand and concentration of AC also influenced gel-strength. The matrix plot suggested that the effect of carbohydrate source and concentration on the growth of shoots may be largely due to the indirect effects of these physical properties such as hydrolysis of carbohydrates, the spectrum and quantity of the breakdown products and the availability of nutrients, plant growth regulators and water rather than the direct effects of pH, EC and gel-strength *per se*.

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CHAPTER ONE

LITERATURE REVIEW OF THE HYACINTHACEAE.

1.1. INTRODUCTION.

The Asparagales forms a large and fairly homogenous complex of families. It is believed to be a monophyletic group, which evolved in parallel with the Liliales and Dioscorales. The Asparagales consists of 31 families including several new families. These families, which include the Alliaceae, Asparagaceae, Asphodelaceae, Draceanaceae, Eriospermaceae and Hyacinthaceae, comprise genera previously placed within the Liliaceae *sensu lato* (DAHLGREN, CLIFFORD & YEO, 1985; PERRY, 1985).

The Hyacinthaceae comprises several genera including *Bowiea, Eucomis, Ledebouria, Scilla* and *Urginea*. These genera are characterised by bulbs, thick and sometimes contractile roots, basal leaves and simple or more rarely branched racemes. The inflorescences comprise actinomorphic, bisexual flowers, which range from white, blue, violet, and more rarely yellow, red, brown to nearly black. The Hyacinthaceae are widely distributed, occurring in southern Africa and a region from the Mediterranean to SW Asia (DAHLGREN, CLIFFORD & YEO, 1985; DU PLESSIS & DUNCAN, 1989).

The genus *Scilla* contains several species, which occur in Africa, Europe and Asia (DAHLGREN, CLIFFORD & YEO, 1985). In recent years, many nomenclatural and taxonomic changes have been made at the generic and specific levels. These nomenclatural and taxonomic changes, however, are not universally accepted (BRYAN, 1988). In southern Africa, many species have been transferred to the genus *Ledebouria* (BRYAN, 1988), while others have been lumped to form fewer species (JESSOP, 1970: ARNOLD & DE WET, 1993). Consequently, *Scilla natalensis* Planch. is a polymorphic species, which comprises several forms.

Scilla natalensis Planch. *sensu stricto*, commonly known as Blue Squill, Blouberglelie or Inguduza (DU PLESSIS & DUNCAN, 1989; VAN WYK, VAN OUDTSHOORN, GERICKE, 1997), is characterised by large ovoid bulbs (100 mm diameter), long glabrous or pubescent leaves (300 to 450 mm long) and dense racemes of small blue flowers borne on long peduncles (300 to 400 mm long)(Figs. 1a & b). This form is widely distributed in Southern Africa occurring at altitudes of 760 to 1670 m in Lesotho, Swaziland, KwaZulu-Natal, eastern Free State and Gauteng (THISTLETON-DYER, 1896; ANONYMOUS, 1930; BRYAN, 1989; DU PLESSIS & DUNCAN, 1989; LE NARD & DE HERTOGH, 1993).

S. natalensis syn. *S. kraussii* Bak., an intermediate form, is characterised by medium globose bulbs (37.5 to 50 mm diameter), spreading densely pubescent leaves (50 to 75 mm long) and moderately dense racemes of small blue flowers borne on short peduncles (150 to 225 mm long)(Figs. 1c & d). This form occurs at altitudes of 1000 to 1670 m in Kwazulu-Natal and the Cape (THISTLETON-DYER, 1896; VAN DER MERWE, 1941).

S. natalensis syn. *S. dracomontana* Hilliard & Burtt, a dwarf form, is characterised by small globose bulbs (30 mm diameter), short pubescent leaves (10 to 35 mm long) and lax racemes of small blue flowers borne on very short peduncles (60 to 110 mm long) (Fig. 1e). This form occurs at altitudes of 1625 to 2100 m in the Drakensberg and surrounding foothills from Giant's Castle to the upper Umzimouti Valley, Kwazulu-Natal. This form occurs as extensive colonies on sandstone cliffs and in rock crevices (HILLIARD & BURTT, 1982).

Although the distributions of *S. natalensis sensu stricto* and syn. *S. dracomontana* are sympatric in the Drakensberg region, these forms can be distinguished from each other based on the size of the bulbs and the inflorescences. The leaves of *S. natalensis sensu stricto* and syn. *S. kraussii* also are young and erect at flowering, while the leaves of *S. natalensis* syn. *S. dracomontana* are mature and flat along the ground at flowering (HILLIARD & BURTT, 1982).

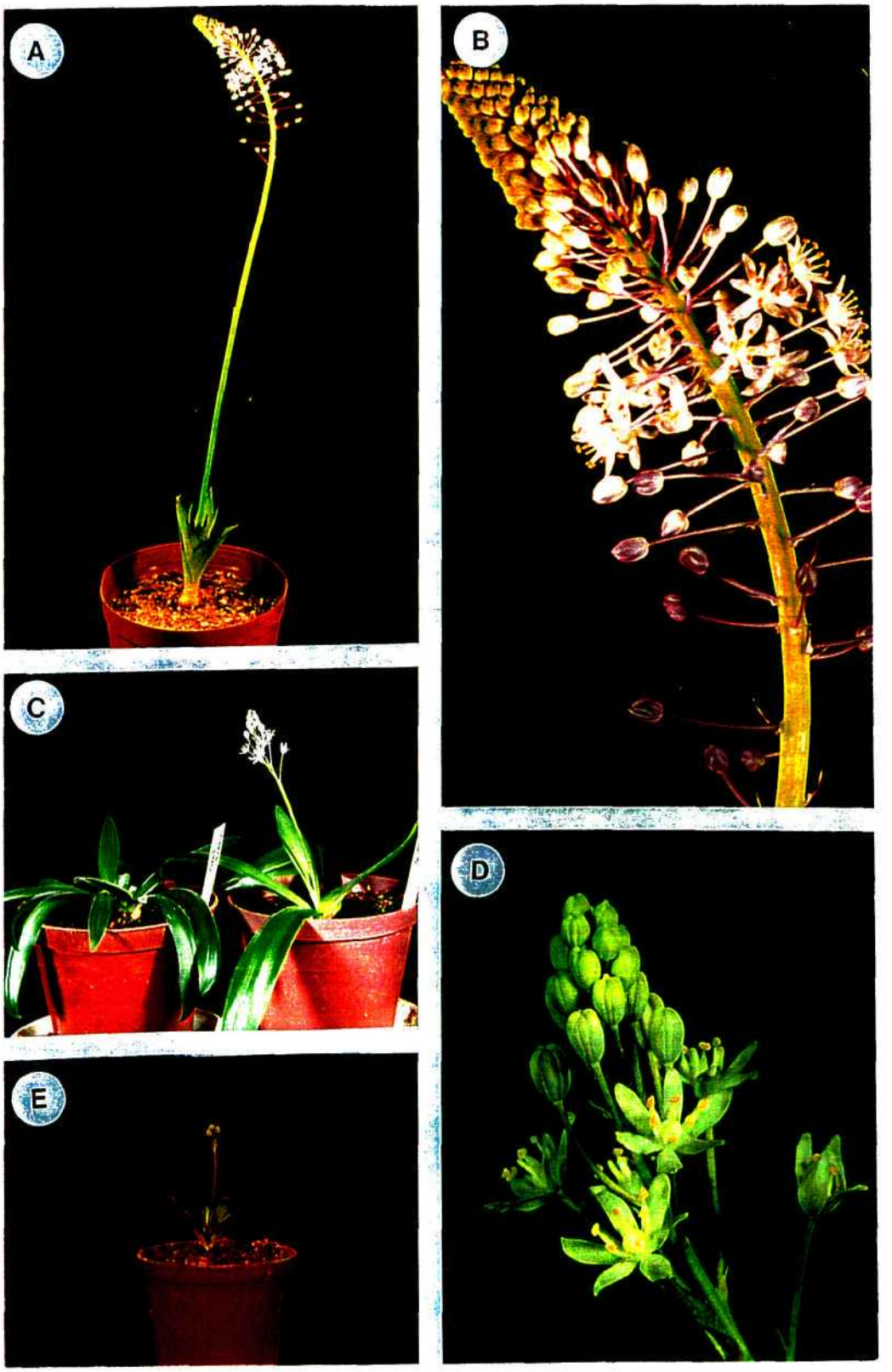


Fig. 1A: Whole plant of *S. natalensis sensu stricto* (Form A); B: Inflorescence of *S. natalensis sensu stricto*; C: Whole plant of *S. natalensis* syn. *S. kraussii* (Form B); D: Inflorescence of *S. natalensis* syn. *S. kraussii*; E Whole plant of *S. natalensis* syn. *S. dracomontana.*

1.2. ECONOMIC IMPORTANCE OF THE HYACINTHACEAE.

1.2.1. MEDICINAL AND PHARMACEUTICAL POTENTIAL.

In South Africa, approximately 20000 tons of plant material, which is worth about R270 million, is harvested, processed and sold as traditional medicine annually (GOSLING, 1998). Approximately 14 % of this plant material comprises bulbs (MANDER, 1997), which are destructively harvested, processed and sold for the treatment of various ailments (Table 1). These bulbs, which are sold whole, sliced or chopped, are usually administered as decoctions, emetics and enemas (WATT & BREYER-BRANDWIJK, 1962; JACOT GUILLAMOD, 1971; HUTCHINGS, 1989; MANDER, MANDER, CROUCH, McKEAN & NICHOLS, 1995; VAN WYK, VAN OUDTSHOORN & GERICKE, 1997).

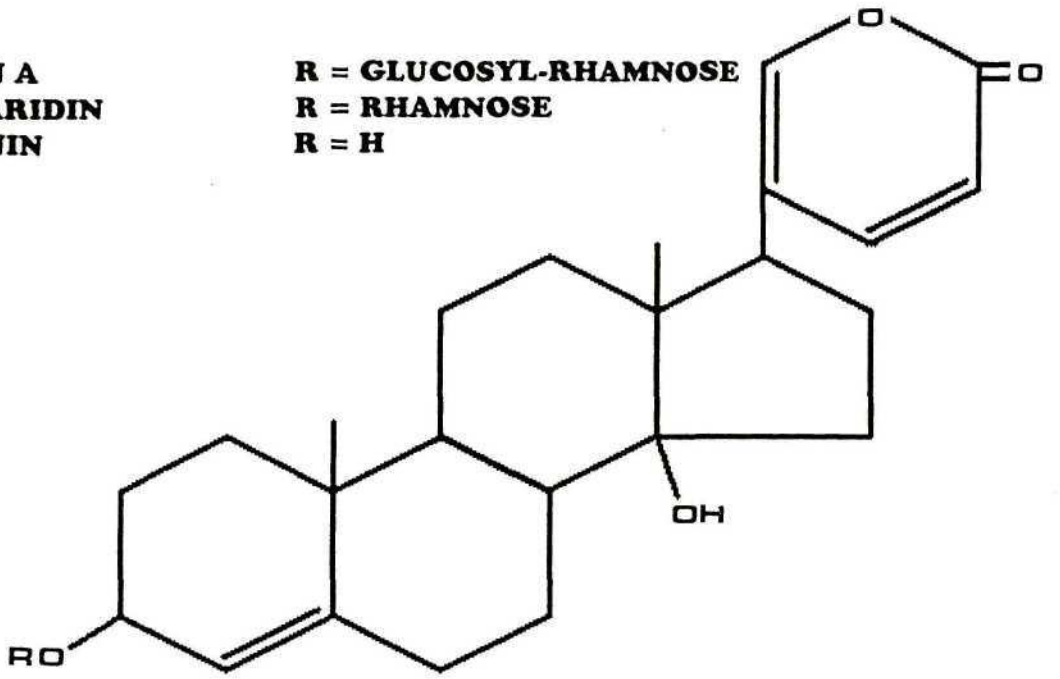
Scilla natalensis is one of the top-ten most popular medicinal plants in South Africa (CUNNINGHAM,1988; WILLIAMS,1996; MANDER,1997). Despite its specially-protected conservation status, approximately 95 tons of these bulbs are sold illegally in Durban annually. The price of these bulbs is relatively low ranging from R1.89 to R6.80 per kilogram. This is mainly due to the occurrence of large populations of these bulbs locally. The price of another popular medicinal plant, *Bowiea volubilis* is substantially higher ranging from R11.74 to R27.80 per kilogram (MANDER, 1997). The price of these bulbs is increasing steadily with the decline in the availability and size of the bulbs (CUNNINGHAM, 1988).

Although the active ingredients of many of these bulbs have not yet been identified, several bufadienolides have been isolated from members of the Hyacinthaceae including *Bowiea volubilis* (JHA, 1988; FINNIE, DREWES & VAN STADEN, 1994), *Drimia robusta* (HUTCHINGS, SCOTT, LEWIS & CUNNINGHAM, 1996), *Urginea altissima* (HUTCHINGS, SCOTT, LEWIS & CUNNINGHAM, 1996), *U. indica* (JHA & SEN 1981& 1983; JHA 1988) and *U. maritima* (SHYR & STABA 1976; VERBISCAR, PATEL, BANIGAN & SCHATZ, 1986; GENTRY, VERBISCAR & BANIGAN, 1987; JHA, 1988). Bufadienolides such as proscillaridin A are valuable since they can be

administered to digitalin sensitive/intolerant patients (JHA, 1988). These bufadienolides, however, are usually not used as cardiotonics due to their low therapeutic indices (JÄGER & VAN STADEN, 1995), although some bufadienolides identified in certain organs of *Bowiea volubils* are thirty to sixty times more active than compounds from *Digitalis* (HUTCHINGS, SCOTT, LEWIS & CUNNINGHAM, 1996). Proscillaridin A, which is derived by enzyme hydrolysis from scillaren A, is produced under several trade names including Caradin®, Cardion®, Proscillan®, Sandoscill®, Scillacrist®, Talusin® and Urgilan® (BUDAVARI, 1996)(Fig. 2). These cardiac glycosides increase the force of the systolic contraction, inhibit the atrioventricular conduction, and enhance the 'automacy' of the cardiac tissue, and therefore, are used to treat heart failure and certain arrhythmias (WALTON, BARONDESS, LOCK, 1994).

Bufadienolides such as scilliroside and scillaren A also are effective rodenticides with the French Pharmacopoeia Codex describing a raticidal paste composed of powdered bulb of Red Squill (*Urginea maritima)*, flour and sugar (BALBAA, KHAFAGY, KHAYYAL, & GIRGIS, 1979). The efficiency of these strongly emetic rodenticides is largely due to the inability of rodents to regurgitate. This renders these rodenticides extremely safe and specific since humans and domestic animals including chickens, cats and dogs readily regurgitate accidentally-ingested baits (CRABTREE, 1947). In the 1940's, several tons of powdered bulb of Red Squill (*Urginea maritima)* were imported into the USA. This ceased with the introduction of warfarin and other anticoagulant-type rodenticides. The development of warfarin-resistance in rodents, however, has led to renewed interest in Red Squill-formulated products as co-rodenticides (VERBISCAR, PATEL, BANIGAN & SCHATZ, 1986). This also has led to the identification and quantification of bufadienolides in various clones or cytotypes of Indian Squill (*U. indica*)(JHA & SEN 1981 & 1983) and Red Squill (*U. maritima*)(GENTRY, VERBISCAR & BANIGAN, 1987) to select high-yielding strains suitable for commercial exploitation.

SCILLAREN A **R = GLUCOSYL-RHAMNOSE**
PROSCILLARIDIN **R = RHAMNOSE**
SCILLARENIN **R = H**



SCILLIROSIDE **R = GLUCOSE**
SCILLIROSIDIN **R = H**
GLUCOSYL SCILLIROSIDE **R = GLUCOSYL-GLUCOSE**

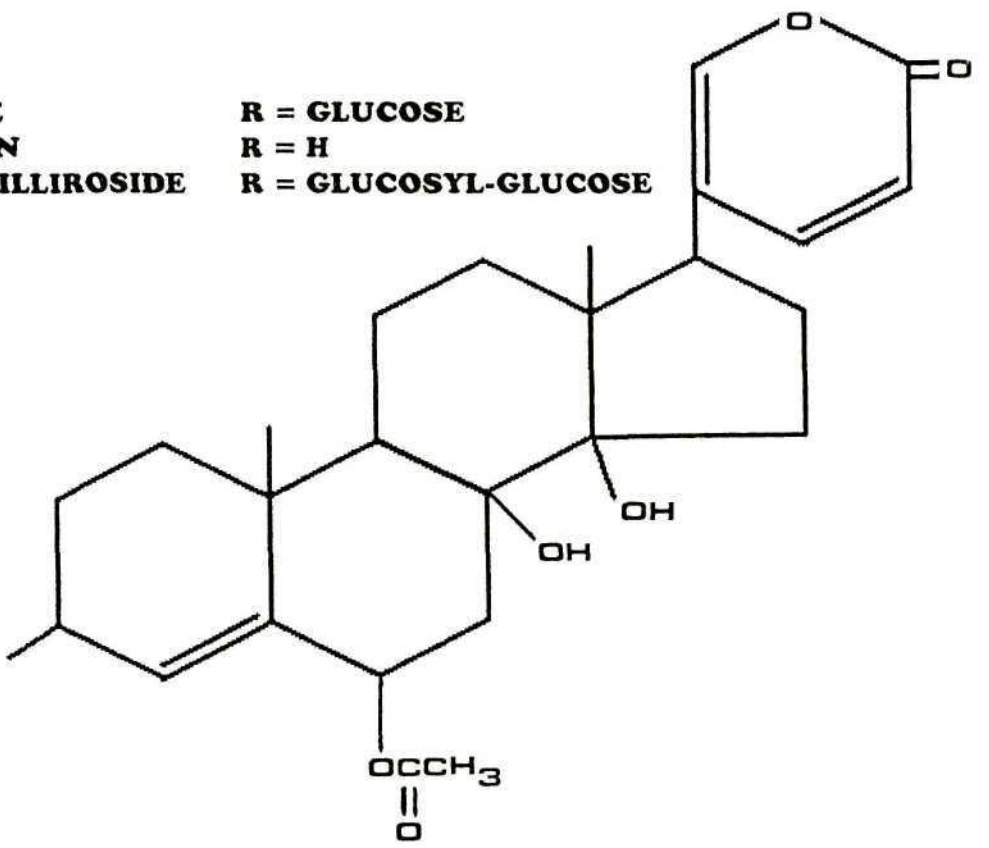


Fig. 2: Scilla compounds in Urginea maritima (Red Squill)(VERBISCAR, PATEL,, BANIGAN & SCHATZ, 1986)

Table 1: Medicinal uses of members of the Hyacinthaceae in South Africa.

PLANT NAME[1]	MEDICINAL USES	REFERENCES
Albuca canadensis (L.) Leighton syn. *A. major*	As an anthelmintic, thirst quencher and to treat venereal diseases	Watt & Breyer-Brandwijk (1962); Hutchings (1989)
Albuca cooperi Bak.	As an anthelmintic, lotion for washing wounds and to treat venereal diseases	Watt & Breyer-Brandwijk (1962); Hutchings (1989)
Albuca fastigata (L.f.) Dryand.	To treat illnesses caused by poisons	Hutchings *et al.* (1996)
Albuca setosa Jacq.	As an anthelmintic, lotion for washing wounds in animals and to treat venereal diseases	Jacot Guillamod (1971); Hutchings (1989)
Albuca shawii Bak. syn. *A. trichophylla*	As an anthelmintic and to treat constipation and gonorrhoea	Watt & Breyer-Brandwijk (1962); Jacot Guillamod (1971); Hutchings (1989)
Bowiea volubilis Harv.	To treat dropsy, barrenness in women and headaches	Watt & Breyer-Brandwijk (1962); Batten & Bokelmann (1966); Hutchings (1989); Hutchings *et al* (1996)
Dipcadi breviofolium (Thunb.) Fourc.	To treat heart pains and breathlessness	Hutchings (1989); Hutchings *et al* (1996)
Dipcadi gracillimum Bak. syn. *D. polyphyllum*	To treat gonorrhoea and pimples	Watt & Breyer-Brandwijk (1962)
Dipcadi viride (L.) Moench	To treat gonorrhoea and flatulence	Watt & Breyer-Brandwijk (1962); Batten & Bokelmann (1966); Hutchings (1989); Hutchings *et al* (1996)
Drimia ciliaris Jacq.	As an emetic, expectorant and diuretic	Watt & Breyer-Brandwijk (1962)
Drimia elata Jacq.	To treat high blood pressure and stabbing pains	Hutchings *et al* (1996)
Drimia nerinformis Bak.	To treat external tumours that have been lanced	Watt & Breyer-Brandwijk (1962); Jacot Guillamod (1971)
Drimia robusta Bak. syn. *D. alta*	As an expectorant, emetic, enema and to treat feverish colds	Hutchings (1989 & 1996)

Table 1 cont.: Medicinal uses of members of the Hyacinthaceae in South Africa.

PLANT NAME[1]	MEDICINAL USES	REFERENCES
Eucomis autumnalis (Mill.) Chitt.	To treat colic, flatulence, syphilis and hangovers	Hutchings *et al* (1996)
Eucomis bicolor Bak.	For colic and as a purgative	Watt & Breyer-Brandwijk (1962); Batten & Bokelmann (1966); Hutchings *et al* (1996)
Eucomis comosa (Houtt.) var. *comosa* syn. *E. punctata*	To treat rheumatism and teething infants	Watt & Breyer-Brandwijk (1962); Batten & Bokelmann (1966); Hutchings (1989); Hutchings *et al* (1996)
Eucomis poleevansii N.E. Br.	For people suffering from mental disease	Watt & Breyer-Brandwijk (1962)
Eucomis regia (L.) L'Herit	To treat venereal disease, lumbago, diarrhoea, respiratory conditions especially cough and biliousness and to prevent premature childbirth	Watt & Breyer-Brandwijk (1962)
Ledebouria cooperi (Hook.f.) Jessop syn. *Scilla cooperi, S. inandensis, S. satura*	Used in the initiation ceremony of boys; used to treat gastro-intestinal, gynaecological and psychological ailments	Watt & Breyer-Brandwijk (1962); Batten & Bokelmann (1966); Hutchings (1989); Hutchings *et al* (1996)
Ledebouria ovatifolia (Bak.) Jessop syn. Scilla ovatifolia	To treat gastro-intestinal and gynaecological ailments, backache and influenza	Hutchings (1989); Hutchings *et al* (1996)
Ledebouria revoluta (L.f.) Jessop syn. *Scilla lanceaefolia*	For treating lumbago and gall sickness in animals; used to bathe skin eruptions and as an ointment for wounds and sores	Watt & Breyer-Brandwijk (1962); Jacot Guillamod (1971)
Massonia echinata L.f. *syn. M. bowkeri*	For ophthalmic applications, sterility and toothache	Watt & Breyer-Brandwijk (1962); Jacot Guillamod (1971)
Ornithogalum longibracteatum Jacq.	To treat swellings or growths	Hutchings (1989); Hutchings *et al* (1996)
Ornithogalum dubium Houtt. syn. *O. miniatum*	As an anthelmintic	Batten & Bokelmann (1966)

Table 1 cont.: Medicinal uses of members of the Hyacinthaceae in South Africa.

PLANT NAME[1]	MEDICINAL USES	REFERENCES
Ornithogalum tenufolium Delaroche subsp. *tenuifolium* syn. *O. ecklonii* & *O. virens*	As a rat poison	Watt & Breyer-Brandwijk (1962); Jacot Guillamod (1971)
Ornithogalum thyrsoides Jacq.	To treat diabetes mellitus	Watt & Breyer-Brandwijk (1962)
Schizobasis intricata	To treat chest complaints	Hutchings *et al* (1996)
Scilla natalensis Planch. syn. *S. kraussii* & *S. dracomontana*	As a purgative and to treat sprains, fractures, boils, veldsores, and lung sickness in cattle	Hutchings *et al* (1996)
Scilla nervosa (Burch.) Jessop syn. *S. rigidifolia*	To treat fevers, gastro-intestinal ailments and insanity	Bryant (1966); Hutchings (1989); Hutchings *et al* (1996)
Urginea altissima (L.f.) Bak.	To treat rheumatic swellings, gastro-intestinal and respiratory ailments	Watt & Breyer-Brandwijk (1962); Hutchings (1989); Hutchings *et al* (1996)
Urginea epigea R.A. Dyer	As a treatment for colds and headaches	Watt & Breyer-Brandwijk (1962)
Urginea macrocentra Bak.	As an anthelmintic	Hutchings *et al* (1996)
Urginea physodes (Jacq.) Bak.	To treat gynaecological ailments and to facilitate delivery during birth	Hutchings (1989); Hutchings *et al* (1996)
Urginea rubella Bak.	For the treatment of colic	Watt & Breyer-Brandwijk (1962); Jacot Guillamod (1971)
Urginea sanguinea Schinz. syn. *U. burkei*	As an abortifacient and to treat paralysis, circulatory diseases and rheumatism pains	Watt & Breyer-Brandwijk (1962)

[1] Plant name and synonyms (Arnold & De Wet, 1993).

1.2.2. ORNAMENTAL POTENTIAL.

Approximately 16000 ha are allotted to ornamental bulb production in the Netherlands, representing 55 % of the world's total production area, with significantly smaller areas allotted to ornamental bulb production in the USA (4449 ha), the UK (4300 ha), Japan (1622 ha), France (1285 ha) and South Africa (425 ha)(DE HERTOGH & LE NARD, 1993a). Despite the ornamental potential of the Hyacinthaceae, the area allotted to the production of *Eucomis* (2 ha), *Galtonia* (1 ha), *Hyacinthus* (955 ha), *Ornithogalum* (50 ha), *Scilla* (20 ha) and *Urginea* (3.2 ha) is small (DE HERTOGH & LE NARD, 1993a & b; LE NARD & DE HERTOGH, 1993), representing approximately 3 % of the world's total ornamental bulb production area. Many of these genera including *Hyacinthus, Lachenalia* and *Scilla* are worthwhile pot plants, while others including *Eucomis, Ornithogalum* and *Urginea* are useful garden plants (DE HERTOGH & LE NARD, 1993b; LE NARD & DE HERTOGH, 1993). A breeding programme for *Lachenalia* comprising five phases including the establishment of a genebank, and the development and subsequent evaluation of hybrids for commercialization has been established over the past thirty years (NIEDERWIESER, ANANDAJAYASEKERAM, COETZEE, MARTELLA, PIETERSE & MARASAS, 1998). The successful introduction of these hybrids onto the international flower market could initiate other breeding programmes, increasing the popularity of these and other plants.

1.3. CONVENTIONAL PROPAGATION AND MICROPROPAGATION.

1.3.1. CONVENTIONAL PROPAGATION.

Several members of the Hyacinthaceae have potential as alternative agricultural crops, providing crops of both bulbs and inflorescences. Seed, however, is seldom used for commercial propagation, except where the species or cultivars produce large quantities of reliable seed with short juvenile periods (REES, 1992). Some members of the Hyacinthaceae including *Scilla siberica, S. siberica* cv 'Alba', and *S. bifolia,* however, are only propagated by seed, while others including *S. siberica* cv 'Spring Beauty' and *S. tubergeniana* [=*S. mischtschenkoana* (BRYAN, 1989)] are propagated vegetatively (LE NARD & DE HERTOGH, 1993). Although offsets are used to propagate many

members of the Hyacinthaceae, they are generally too slow for commercial propagation. Several artificial techniques, therefore, are used to increase the rate of natural multiplication. These techniques include scaling, basal cuttage (scooping and scoring) and bulb cuttings.

1.3.2. MICROPROPAGATION.

Several members of the Hyacinthaceae have been micropropagated using various techniques (Fig. 3a - h), which include the proliferation of axillary shoots, the initiation of adventitious shoots and the induction of somatic embryos (Table 2). These are discussed below.

1.3.2.1. Axillary shoots.

Although many workers use twin-scales as explants, few stipulate whether the resulting shoots are axillary or adventitious in origin. In *Eucomis*, axillary shoots proliferated from twin-scale explants, which were cultured on medium containing combinations of BA and NAA. These shoots, which were then sub-cultured onto the same medium, proliferated more shoots resulting in a continuous culture system. After 8 - 10 weeks, the shoots were then rooted on medium with or without NAA (AULT, 1995b). Although axillary shoots are genetically-stable, the limited availability of such shoots restricts the potential of this technique (HUSSEY, 1980). Furthermore, 'mixed' cultures comprising axillary and adventitious shoots do occur occasionally (HUSSEY, 1976a).

1.3.2.2. Direct adventitious shoots.

In several members of the Hyacinthaceae, the explant source, orientation, position and size have influenced adventitious shoot initiation (NIEDERWIESER & VAN STADEN, 1990a & 1992; NIEDERWIESER & VCELAR, 1990; LANDBY & NIEDERWIESER, 1992). In *Scilla*, the explant source influenced shoot initiation with leaf explants producing more shoots than bulb explants (MCCARTAN & VAN STADEN, 1998). In *Lachenalia*, the age of the explants influenced shoot initiation with young explants

producing more shoots than old explants. This may have been linked to endogenous cytokinin levels particularly in the intermediate and old explants (NIEDERWIESER & VAN STADEN, 1990a). In *Ornithogalum*, the orientation of the explants also influenced shoot initiation with apolar explants producing more shoots than polar explants (LANDBY & NIEDERWIESER, 1992), while in *Lachenalia*, callus and deformed shoots were formed when explants were cultured with the adaxial surface down (NIEDERWIESER & VCELAR, 1990). In *Lachenalia*, the explant size influenced shoot initiation with small explants producing more shoots than large explants. The optimum size explant was 3.3 x 15 mm (NIEDERWIESER & VCELAR, 1990). This may have been related to the wound surface, which is proportionally larger in smaller explants. In *Ornithogalum*, however, additional wounding on the surface of the explant inhibited shoot initiation (LANDBY & NIEDERWIESER, 1992). In *Hyacinthus*, the explant size also influenced shoot initiation, which decreased linearly with a decrease in explant size (PIERIK & POST, 1975).

These adventitious shoots originated from single cells or from groups of cells. In *Lachenalia*, the shoots were initiated mainly from single epidermal cells primarily derivatives of the stomatal mother cells, although a few shoots were initiated from groups of cells (NIEDERWIESER & VAN STADEN, 1990b). The frequency of mutations in shoots initiated from single cells is usually fairly high, resulting in "solid mutations" (HUSSEY, 1980). The adventitious shoots also have been restricted to certain surfaces of explants. In *Bowiea,* the shoots were initiated on the adaxial surfaces of bulb-scales. This may have been associated with anatomical differences between these surfaces, which included thicker cuticles, more chloroplasts and stomata on the abaxial surfaces, as well as the presence of unidentified globular bodies on the adaxial surfaces (VAN STADEN, ELMER-ENGLISH & FINNIE, 1991).

Some adventitious shoots have been initiated on medium containing no plant growth regulators, although most adventitious shoots have been initiated on medium containing various cytokinins and auxins. In *Lachenalia*, the addition of BA had no influence on shoot initiation in hybrids with high cytokinin-like activity, but increased shoot initiation in hybrids with low cytokinin-like activity (NIEDERWIESER & VAN

STADEN, 1992). The substitution of cytokinin derivatives ([3G]BA, [7G]BA & [9G]BA) for BA inhibited shoot initiation (VAN STADEN & DREWES, 1994). In *Hyacinthus*, the addition of cytokinins had no influence on shoot initiation, although the addition of auxins, particularly IAA and IBA, increased shoot initiation (PIERIK & STEEGMANS, 1975; BACH & CECOT, 1988 & 1989). In *Scilla,* the addition of NAA inhibited shoot initiation (MCCARTAN & VAN STADEN, 1998), while in *Hyacinthus*, the addition of NAA promoted callus formation (PIERIK & STEEGMANS, 1975). In *Hyacinthus*, the carbohydrate source also influenced shoot initiation with glucose and sucrose producing more shoots than fructose (BACH, CECOT & GAWEDA, 1992). In *Muscari*, the addition of charcoal promoted bulblet formation and inhibited callus formation (PECK & CUMMING, 1986), while in *Hyacinthus*, the addition of paclobutrazol promoted bulblet formation (BACH, CECOT & GAWEDA, 1992). Some adventitious shoots produced roots and/or bulbs spontaneously. Other adventitious shoots have been rooted on medium containing no plant growth regulators (COOK, CUNNINGHAM & VAN STADEN, 1988), or various auxins, usually IBA (NEL, 1981 & 1983; TAYLOR & VAN STADEN, 1997) or NAA (DREWES, BAYLEY & VAN STADEN, 1993; DREWES & VAN STADEN, 1993). Although, adventitious shoots are genetically less stable than axillary shoots, particularly when they originate from single cells (HUSSEY, 1980), this technique is frequently used to clone medicinal and ornamental plants rapidly and economically.

1.3.2.3. Callus and indirect adventitious shoots.

In *Bowiea*, callus was initiated on medium containing 2,4-D and coconut milk, and then transferred to medium containing 2,4-D and casein hydrolysate. This callus comprised green nodules, which produced adventitious shoots, when transferred to medium containing low concentrations of 2,4-D (JHA & SEN, 1985). In *Ornithogalum*, callus was initiated on medium containing NAA alone (HUSSEY, 1976B; NAYAK & SEN, 1995) or in combination with BA (VAN RENSBURG, VCELAR & LANDBY, 1989). The frequency and size of the callus decreased with increasing sucrose concentration (VAN RENSBURG, VCELAR & LANDBY, 1989). HUSSEY (1976b) found that the callus comprised a mixture of diploid and tetraploid cells. He also found that the frequency of tetraploid cells increased with callus age (HUSSEY, 1976b). In contrast,

NAYAK & SEN (1995) found that the callus was genetically-stable for several years. The callus produced adventitious shoots when transferred to medium containing no plant growth regulators (HUSSEY 1976b) or medium containing combinations of BA and NAA (NAYAK & SEN, 1995). In *Urginea*, callus was initiated on medium containing 2,4-D, NAA and kinetin. The frequency of callus initiation was increased by the addition of yeast extract. This callus produced small adventitious shoots and roots when transferred to medium containing low concentrations of auxins and vitamins (JHA & SEN, 1984). Although large quantities of adventitious shoots could be produced from callus, the genetic-stability of the callus should be determined to ensure that normal plantlets are produced. In certain breeding programmes, however, genetically-aberrant plantlets could be a useful source of somaclonal variation.

1.3.2.4. Direct and indirect somatic embryos.

In *Scilla*, embryogenic callus was initiated on medium containing NAA and coconut milk. The embryogenic callus, which comprised small thin-walled cells with large nuclei, formed shoots and roots when transferred to medium containing no plant growth regulators (CHAKRAVARTY & SEN, 1989). In *Urginea*, embryogenic and non-embryogenic callus were initiated on medium containing 2,4-D and coconut milk. The embryogenic callus, which comprised large, vacuolated parenchyma cells, formed greenish zones of small cells with large nuclei when exposed to 2,4-D for prolonged periods. These greenish zones produced globular embryos, which elongated to form banana-shaped bipolar embryos when transferred to medium containing low concentrations of BA with or without coconut milk. The bipolar embryos produced bulbous plantlets when transferred to medium containing kinetin and NAA. In *Urginea*, the age of the callus, the frequency of sub-culturing and the cytological state of the callus influenced the initiation and subsequent development of the callus (JHA & SEN, 1986). The ploidy of the callus (diploid & tetraploid) also influenced medium requirements (JHA, MITRA & SEN, 1991). Despite the potential of this technique, somatic embryos have not been induced in many members of the Hyacinthaceae.

1.4. APPLICATIONS OF MICROPROPAGATION IN THE HYACINTHACEAE.

The Hyacinthaceae comprises several genera, which are widely exploited for their medicinal, pharmaceutical and ornamental potential. In South Africa, several members of the Hyacinthaceae are harvested from wild populations without permits, processed and then sold as traditional medicine. This is reducing the density, distribution and genetic diversity of wild populations. Furthermore, the enforcement of existing legislation has proved ineffective with vast quantities of plants being traded locally and internationally. Consequently, it has been suggested that *ex situ* conservation through cultivation may alleviate pressures on natural resources, whilst meeting the demand for these plants. Conventional vegetative propagation, however, is usually fairly slow. Micropropagation, therefore, provides a rapid means to propagate these endangered plants, whilst supplying an alternative source of plants for the consumer market.

Many bufadienolides have been isolated from members of the Hyacinthaceae. These bufadienolides are used as cardiotonics and therefore have pharmaceutical potential. This has led to the identification and quantification of bufadienolides in various clones and cytotypes *in vitro* and *in situ* to select high yielding strains suitable for commercial exploitation. In *Bowiea*, the same bufadienolides were accumulated at similar concentrations for both *in vitro* and *in situ* plants (FINNIE, DREWES & VAN STADEN, 1994). In *Urginea*, however, the accumulation of bufadienolides was linked to the formation of specific organs *in vitro*, but was independent of the genotype, explant source and regeneration system (JHA, SAHU & MAHATO, 1991). Micropropagation, therefore, not only provides a rapid means to propagate selected chemotypes, but also a means to produce these bufadienolides *in vitro.* This is important economically since it circumvents the need to cultivate these plants *ex vitrum.*

Several members of the Hyacinthaceae are cultivated as ornamentals, although the total area allotted to the production of these bulbs is relatively small. Breeding programs, which have been established for certain genera, may increase the popularity of these plants. Micropropagation, therefore, not only provides a rapid means to propagate newly-developed cultivars, but also a means to eliminate viruses such as

Hyacinthus Mosaic Virus (BLOM-BARNHOORN, VAN AARTRIJK & VAN DER LINDE, 1986) and Ornithogalum Mosaic Virus (VCELAR, FERREIRA & NIEDERWIESER, 1992), thus reducing crop losses and facilitating the export of these plants. Micropropagation also provides a means to conserve valuable germplasm (LOUW, 1995), therefore retaining useful traits for future breeding programmes.

1.5. AIMS.

Scilla natalensis has potential as an alternative agricultural crop for medicinal, pharmaceutical and/or ornamental purposes. Conventional vegetative propagation, however, is usually fairly slow. *In vitro* propagation, therefore provides a rapid means of propagating selected chemotypes or cultivars, serving both conservation and commercial interests.

Thus, the aims of this study were:

1) to determine the effect of carbohydrates, plant growth regulators and gelling agents on adventitious shoot initiation in *Scilla natalensis sensu stricto, S. natalensis* syn. *S. kraussii* and *S. natalensis* syn. *S. dracomontana;* and

2) to determine the effect of carbohydrates and activated charcoal on shoot growth in *Scilla natalensis sensu stricto*.

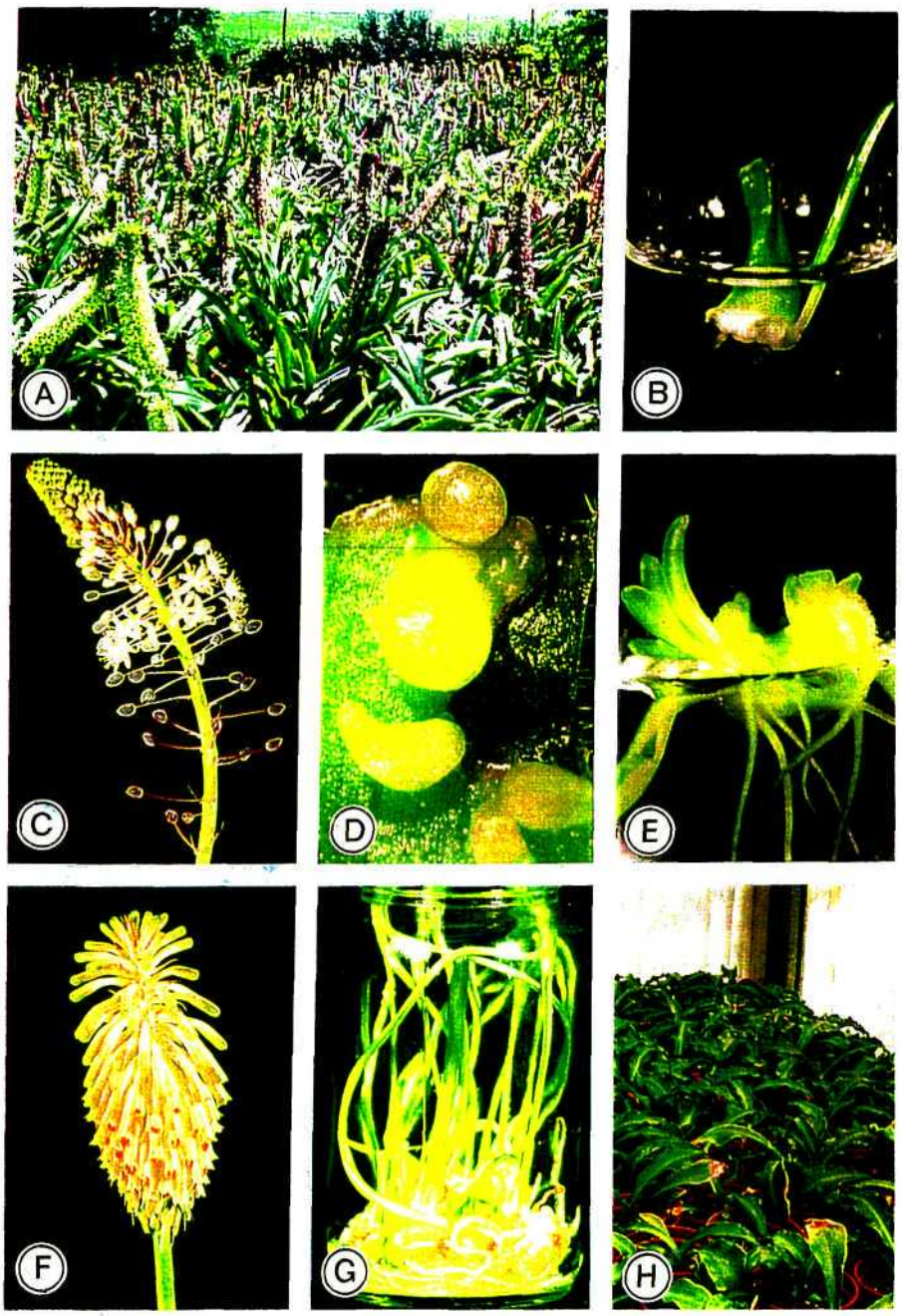


Fig. 3 A: Inflorescences of *Eucomis* hybrids and species; B: Adventitious shoot initiated along the periphery of leaf explant of *E. poleevansii*; C: Inflorescence of *Scilla natalensis* Form A; D: Adventitious buds initiated on the adaxial surface of leaf explant of *S. natalensis* Form A; E: Adventitious shoots initiated on leaf explant of *S. natalensis* Form A; F: Inflorescence of *Veltheimia bracteata* cv. Lemon Flame; G: Plantlets of *V. bracteata* cv. Lemon Flame H: Acclimatized plants of *V. bracteata* cv. Lemon Flame.

Table 2: Micropropagation of some members of the Hyacinthaceae.

PLANT NAME	1° & 2° EXPLANTS	MEDIUM AND SUPPLEMENTS[1]	GROWTH RESPONSE	REFERENCES
Bowiea volubilis	Bulb scales Shoots	MS, Sucrose (30000), BA (1), NAA (1), Agar (8000) MS, Sucrose (30000), Agar (8000)	Shoots Plantlets	Cook *et al.* (1988)
Bowiea volubilis	Inflorescence Buds and callus	MS, Sucrose (30000), BA (1), 2,4-D (1), Agar (10000) MS, Sucrose (30000), Agar (10000)	Shoots Bulblets	Hannweg *et al.* (1996)
Bowiea volubilis	Inflorescence	Modified MS, Sucrose (30000), CM (150), 2,4-D (1), Agar (6000)	Callus	Jha & Sen (1985)
	Bulb scales and split shoots	Modified MS, Sucrose (30000), CH (100), 2,4-D (0.5), Agar (6000)	Shoots	
	Shoots	Modified MS, Sucrose (30000), Agar (6000)	Plantlets	
	Shoots	Modified MS, Sucrose (30000), BA (2), 2,4-D (0.05)	Plantlets	
Bowiea volubilis	Twin-scales	MS, Sucrose (30000), BA (10), NAA (1), Agar (8000)	Shoots	Van Staden *et al.* (1991)
Drimia robusta	Bulb-scales Bulblets	MS, Sucrose (30000), BA (2), NAA (1), Agar (10000) MS, Sucrose (30000), NAA (1), Agar (10000)	Bulblets Rooted bulblets	Ngugi *et al.* (1998)
Eucomis autumnalis, E. comosa & *E. zambesiaca*	Twin-scales from bulbs Shoots	MS, Sucrose (30000), BA (0-5), NAA (1), Agar (7000) MS, Sucrose (30000), NAA (0-2), Agar (7000)	Shoots Plantlets	Ault (1995b)
Eucomis autumnalis, E. comosa, E. bicolor, E. humilis, E. zambesiaca (Fig. 3a)	Leaves and bulb-scales Shoots	MS, Sucrose (20000), BA (1-2), NAA (1-2), Gelrite (2000) MS, Sucrose (20000), IAA (1-2) or IBA (1-2), Gelrite (2000)	Shoots Plantlets	Taylor & Van Staden (1998)

Table 2 cont.: Micropropagation of some members of the Hyacinthaceae.

PLANT NAME	1° & 2° EXPLANTS	MEDIUM AND SUPPLEMENTS[1]	GROWTH RESPONSE	REFERENCES
Eucomis poleevansii	Seeds	Modified MS, Sucrose (20000), Gelrite (2000)	Seedlings	McCartan & Van Staden (1995)
	Leaves of seedlings	Modified MS, Sucrose (20000), NAA (1), Gelrite (2000)	Shoots (Fig. 3b)	
	Shoots	Modified MS, Sucrose (20000), IAA (1), Gelrite (2000)	Plantlets	
Eucomis vandermerwei	Leaves	Modified MS, Sucrose (20000), BA (1-2), IAA (1), Gelrite (2000)	Shoots	McCartan *et al.* (In Press)
	Shoots	Modified MS, Sucrose (20000), IBA (1), Gelrite (2000)	Plantlets	
Galtonia candicans & *G. viridiflora*	Pedicels & ovary bases	MS, Sucrose (30000), BA (0.3), NAA (1), Agar (8000)	Shoots	Drewes & Van Staden (1993)
	Split shoots	MS (½), Sucrose (30000), NAA (0.5), Agar (8000)	Plantlets	
Hyacinthus orientalis cv. Delft's Blue	Leaves	MS, Glucose or Sucrose (30000), BA (1), NAA (0.1), ± PAC (8), Agar (8000)	Bulblets	Bach *et al.* (1992)
Hyacinthus orientalis cv. Pink Pearl	Basal parts of bulbs	Modified Knops (½), Glucose (20000), IAA (10), Agar (6000)	Bulblets	Pierik & Steegmans (1975)
Hyacinthus sp cv. Ostara & Prinses Irene	Twin-scales from bulbs	MS, Sucrose (20000), Agar (7000)	Shoots	Hussey (1975)
	Leaves, stems & ovary	MS, Sucrose (20000), IAA (2-8) or NAA (0.03-0.12), Agar (7000)	Shoots & callus	
	Split shoots and callus	MS, Sucrose (20000), Agar (7000)	Plantlets	

Table 2 cont.: Micropropagation of some members of the Hyacinthaceae.

PLANT NAME	1° & 2° EXPLANTS	MEDIUM AND SUPPLEMENTS[1]	GROWTH RESPONSE	REFERENCES
Lachenalia arbuthnotiae, L. bulbifera, L. purpureo-coerulea	Leaves	MS, Sucrose (30000), BA (2), K-NAA (0.1), Agar (6000)	Shoots	Ault (1995a)
	Shoots	MS, Sucrose (30000), K-IBA (0-2) or K-NAA (0-2), Agar (6000)	Plantlets	
Lachenalia cv. Robyn, Rolina, Romargo & Romaud	Leaves	Modified MS, Sucrose (10000-90000), BA (2), NAA (0.1), Agar (7000)	Buds	Van Rensburg & Vcelar (1989)
Lachenalia cv. Romaud	Leaves	Modified MS, Sucrose (50000), BA (4), NAA (2), Agar (7000)	Buds	Van Staden & Drewes (1994)
Lachenalia hybrids	Leaves	MS, Sucrose (30000), BA (2), NAA (0.1). Agar (6000)	Buds	Niederwieser & Van Staden (1992)
Lachenalia hybrids	Leaves	Modified MS, Sucrose (30000), BA (2), NAA (0.1), Agar (7000)	Shoots	Nel (1983)
	Shoots	Modified MS, Sucrose (30000), IBA (2), Agar (7000)	Plantlets	
Lachenalia spp. & hybrids	Leaves	Modified MS, Sucrose (50000), BA (1), NAA (1), Agar (6000)	Buds	Niederwieser & Van Staden (1990b)
Muscari armeniacum cv. Early Giant	Bulb scales	Modified MS, Sucrose (30000), BA (5), NAA (2), AC (1000), Gelrite (2000)	Bulblets	Peck & Cumming (1986)
Ornithogalum cv. Rollow	Leaves	Modified MS, Sucrose (30000), BA (2), NAA (0.1), Agar (7000)	Shoots	Landby & Niederwieser (1992)
Ornithogalum cv. Rogel	Leaves	Modified MS, Sucrose (30000), BA (2), NAA (0.1), Gelrite (2000)	Buds	Vcelar *et al.* (1992)
	Meristems from buds	Modified MS, Sucrose (30000), BA (1), NAA (1), adenine arabinose (1),Gelrite (2000)	Virus-indexed planlets	

Table 2 cont.: Micropropagation of some members of the Hyacinthaceae.

PLANT NAME	1° & 2° EXPLANTS	MEDIUM AND SUPPLEMENTS[1]	GROWTH RESPONSE	REFERENCES
Ornithogalum hybrid	Leaves	Modified MS, Sucrose (30000), BA (2), NAA (0.1), Agar (7000)	Shoots	Nel (1981)
	Shoots	Modified MS, Sucrose (30000), Agar (7000)	Plantlets	
Ornithogalum maculatum	Leaves	Modified MS, Sucrose (30000), BA (2), NAA (0.1), Agar (7000)	Callus & shoots	Van Rensburg *et al.* (1989)
Ornithogalum thyrsoides	Stem, Leaves, Ovary, Sepals, Bulb scales	MS, Sucrose (20000), Agar (7000)	Plantlets	Hussey (1976b)
Ornithogalum umbellatum	Twin-scales of bulbs	MS, Sucrose (30000), NAA (8), Agar (5000)	Callus	Nayak & Sen (1995)
	Callus	MS, Sucrose (30000), BA (0.5), NAA (2), Agar (5000)	Shoots	
	Shoots	MS (½), Sucrose (30000), Agar (5000)	Plantlets	
Schizobasis intricata	Bulb scales	MS, Sucrose (30000), BA (2), NAA (2), Agar (8000)	Shoots and callus	Drewes *et al.* (1993)
	Shoots	MS (½), Sucrose (30000), NAA (1), Agar (8000)	Plantlets	
Scilla hyacinthiana	Leaves	MS, Sucrose (30000), Agar (10000)	Shoots	Nair (1989)
	Shoots	MS, Sucrose (30000), kinetin (5), NAA (1), Agar (10000)	Shoots	
	Shoots	MS, Sucrose (20000)	Plantlets	
Scilla natalensis (Fig. 3c)	Bulbs	Modified MS, Sucrose (20000), Gelrite (2000)	Shoots	McCartan & Van Staden (1998)
	Leaf and bulb scales of shoots	Modified MS, Sucrose (20000), Kinetin (1-2), IAA (1- 2), Gelrite (2000)	Shoots (Figs. 3d & e)	
	Shoots	Modified MS, Sucrose (20000), IAA (1), Gelrite (2000)	Plantlets	

Table 2 cont.: Micropropagation of some members of the Hyacinthaceae.

PLANT NAME	1° & 2° EXPLANTS	MEDIUM AND SUPPLEMENTS[1]	GROWTH RESPONSE	REFERENCES
Thuranthos basuticum	Bulb scales	MS, Sucrose (30000), BA (0.1), NAA (5), Agar (8000)	Plantlets	Jones *et al.* (1992)
Urginea indica (2n,3n & 4n)	Twin-scales of bulbs	Modified MS, Sucrose (30000), kinetin (1-2), 2,4-D (1-4), ± NAA (2), ± CM (150), ± YE (1000), Agar (6000)	Callus depending upon ploidy	Jha *et al.* (1991)
	Callus	Modified MS, Sucrose (30000), kinetin (2), 2,4-D (0.5), ± NAA (0.5-1), ± CM (150), ± YE (1000), Agar (6000)	Shoots depending upon ploidy	
	Callus	Modified MS, Sucrose (30000), kinetin (1) or 2,4-D (1), Agar (6000)	Somatic embryos depending upon ploidy	
Urginea indica	Twin-scales of bulbs	Modified MS, Sucrose (30000), 2,4-D (2), CM (150), Agar (6000)	Callus and somatic embryos	Jha *et al.* (1986)
	Somatic embryos	Modified MS, Sucrose (30000), BA (0.1), CM (100), Agar (6000)	Plantlets	
	Plantlets	Modified MS, Sucrose (30000), kinetin (0.05), NAA (0.01), Agar (6000)	Bulbous plantlets	
Urginea indica	Twin-scales of bulbs	Modified MS, Sucrose (30000), 2,4-D (2), CM (150), OR , kinetin, (2), 2,4-D (4), NAA (2), YE (1000), Agar (6000)	Callus and shoots	Jha *et al.* (1984)
	Shoots	MS (½), Sucrose (5000), Agar (6000)	Plantlets	
Urginea maritima	Bulb scales	Modified MS, Sucrose (?), BA (0.1-0.3), NAA (0.1-0.3), Agar (7500)	Bulblets	El Grari & Backhaus (1987)
	Bulblets	Modified MS, Sucrose (?), NAA (0.1-0.3), Agar (7500)	Plantlets	

Table 2 cont.: Micropropagation of some members of the Hyacinthaceae.

PLANT NAME	1° & 2° EXPLANTS	MEDIUM AND SUPPLEMENTS[1]	GROWTH RESPONSE	REFERENCES
Veltheimia bracteata cv. Lemon Flame & Rosalba & *V. capensis*	Leaves and floral stems Shoots	MS, Sucrose (30000), BA (2), NAA (0.1), Agar (7000)	Shoots	Ault (1996)
		MS, Sucrose (30000), K-NAA (0-2), Agar (7000)	Plantlets	
Veltheimia bracteata (Fig. 3f)	Leaves and bulb-scales	MS, Sucrose (30000), BA (2), NAA (0.1), Agar (8000)	Shoots (Fig. 3g)	Taylor & Van Staden (1997)
	Shoots	MS, Sucrose (30000), IBA (2), Agar (8000)	Plantlets (Fig. 3h)	

Figures in parentheses indicate $mg.l^{-1}$ or $ml.l^{-1}$.

CHAPTER TWO

EFFECT OF CARBOHYDRATES, PLANT GROWTH REGULATORS AND GELLING AGENTS ON ADVENTITIOUS SHOOT INITIATION.

2.1. INTRODUCTION.

The process of adventitious shoot initiation is divided into four stages: dedifferentiation; cell division; shoot initiation; and growth of the adventitious shoot (PIERIK, 1985). These shoots may arise from single cells or groups of cells (NIEDERWIESER & VAN STADEN, 1990b). The frequency of mutations in shoots initiated from single cells, however, is usually quite high resulting in "solid mutations" (HUSSEY, 1980). The shoots also may be initiated directly from the explant or indirectly from callus. Many factors influence adventitious shoot initiation. These include: genetic factors such as genotype (LANDBY & NIEDERWIESER, 1992; NIEDERWIESER & VAN STADEN, 1992); and epigenetic factors such as the age (NIEDERWIESER & VAN STADEN, 1990a); orientation (GEORGE & SHERRINGTON, 1984; NIEDERWIESER & VCELAR, 1990; LANDBY & NIEDERWIESER, 1992); shape (PIERIK & POST, 1975); size (PIERIK & POST, 1975; NIEDERWIESER & VCELAR, 1990); and type of explant (MCCARTAN & VAN STADEN, 1998); nutrient medium (Table 2) and environmental conditions. Several members of the Hyacinthaceae have been successfully propagated *in vitro* (Table 2).

The aims of this part of the study were:

1) to determine the effect of form and explant type on adventitious shoot initiation in *Scilla natalensis sensu stricto, S. natalensis* syn. *S. kraussii* and *S. natalensis* syn. *S. dracomontana* respectively; and

2) to determine the effect of carbohydrate source, plant growth regulators and gelling agents on adventitious shoot initiation in *Scilla natalensis sensu stricto, S. natalensis* syn. *S. kraussii* and *S. natalensis* syn. *S. dracomontana* respectively.

2.2. MATERIALS AND METHODS.

The plants used in this study were *Scilla natalensis sensu stricto* Planch., *S. natalensis* syn. *S. kraussii* Bak. and *S. natalensis* syn. *S. dracomontana* Hilliard & Burtt, which are referred to as Form A, Form B and Form C respectively. The stock plants were grown in pots in a 20 % shade-house for the duration of the study.

The primary explants were determined by the availability and size of the plants. For the large form, Form A, the bulbs were washed thoroughly in tap-water, divided horizontally into three sections, and then divided vertically into quarters. For the smaller forms, Form B and Form C respectively, the leaves were washed thoroughly in tap-water. The bulbs and leaves were sterilized in 80 % EtOH for 1 minute, 1 g l^{-1} Benlate® for 5 minutes, 1 g l^{-1} $HgCl_2$ and Tween 20 for 10 minutes, and then rinsed thoroughly in sterile distilled water. The primary explants (10 x 10 mm) were transferred aseptically onto basal media (BM) comprising Murashige & Skoog (1962) medium modified by the addition of 20 mg l^{-1} glycine. The BM was supplemented with 20 g l^{-1} sucrose. The pH of the medium was adjusted to 5.8 with KOH, NaOH or HCl. The medium was solidified with 2 g l^{-1} Gelrite®. The medium was heated (to dissolve the gelling agent), hot-dispensed into test-tubes (100 x 25 mm) and capped with either metal or plastic lids, and then autoclaved at 121 °C for 20 minutes (103.4 kPa). The cultures were incubated under 16:8 light/dark cycles (45 µmol m^{-2} s^{-1}) provided by a mixture of cool white fluorescent tubes and incandescent lights and maintained at 25±2 °C. Shoots were subcultured repeatedly onto BM at 8 to 12 week intervals. Shoots produced bulbs spontaneously after several weeks *in vitro.*

Subsequent secondary leaf and bulb explants (10 x 5 mm) were obtained from *in vitro* plantlets. For Form A, leaf and bulb explants were used, while for Form B and Form C, only leaf explants were used. The secondary explants were determined by the availability and size of the plantlets. These explants were transferred aseptically onto shoot initiation media comprising BM and supplements:

1 a-f) BM, 20 g l^{-1} D(+)-glucose, 20 g l^{-1} D(-)-fructose, 20 g l^{-1} lactose, 20 g l^{-1} maltose, 20 g l^{-1} D(-)-mannitol or 20 g l^{-1} sucrose, 2 g l^{-1} Gelrite®;

2 a-i) BM, 20 g l^{-1} sucrose, 0, 1 and 2 mg l^{-1} kinetin in factorial combination with 0, 1 and 2 mg l^{-1} indole-3-acetic acid (IAA), 2 g l^{-1} Gelrite®;

3 a-i) BM, 20 g l^{-1} sucrose, 0, 1 and 2 mg l^{-1} kinetin in factorial combination with 0, 1 and 2 mg l^{-1} α-napthaleneacetic acid (NAA), 2 g l^{-1} Gelrite®;

4 a-i) BM, 20 g l^{-1} sucrose, 0, 0.1 and 0.2 mg l^{-1} thidiazuron (TDZ) in factorial combination with 0, 1 and 2 mg l^{-1} NAA, 2 g l^{-1} Gelrite®; and

5 a-c) BM, 20 g l^{-1} sucrose, 2 g l^{-1} Gelrite®, 10 g l^{-1} ACE Agar (Associated Chemical Enterprises Agar 9465/826) or 10 g l^{-1} Unilab agar (47672).

For Form A and Form B, twenty-five replicates were used per treatment and each treatment was repeated twice. For Form C, ten replicates were used per treatment.

The shoots (8 weeks-old) were then sub-cultured onto root initiation media comprising BM and supplements:

1 a-c) BM, 20 g l^{-1} sucrose, 1 mg l^{-1} indole-3-butyric acid (IBA), 1 mg l^{-1} IAA or 1 mg l^{-1} NAA, 2 g l^{-1} Gelrite®.

Twenty-five replicates were used per treatment. Micropropagated plantlets (12-14 weeks-old for Form A and Form B; 23 weeks-old for Form C) were planted out into vermiculite and kept in the misthouse for several weeks. They were then transferred to a 20 % shadehouse.

The frequency of shoot initiation and the mean number of shoots initiated per explant were recorded. The data for each form and explant type was subjected to ANOVA and Tukey HSD multiple range tests using Minitab Xtra 10.51 to determine the effect of carbohydrate source, plant growth regulators and gelling agents on shoot initiation. The data for the frequency of shoot initiation (%) was arcsined prior to the ANOVA. The frequency of shoot deformities and hyperhydricity was recorded. The data was also pooled to determine the interactions between: form and carbohydrate source; form, cytokinin and auxin; form and gelling agents; explant type and carbohydrates; explant type; cytokinin and auxin; and explant type and gelling agent. This pooled data was also subjected to ANOVA.

2.3. RESULTS AND DISCUSSION.

2.3.1. STERILE EXPLANTS.

Only 20 to 30 % of the primary bulb explants of Form A were sterile, while 90 to 100 % of the primary leaf explants of Form B and Form C were sterile. Some of the primary explants initiated shoots, which were sub-cultured repeatedly onto BM to provide a source of sterile secondary bulb and leaf explants for use in subsequent experiments. For Form A, leaf and bulb explants were used, while for Form B and Form C, only leaf explants were used. This was determined by the availability and size of the plantlets. The effect of carbohydrate source, plant growth regulators and gelling agents on adventitious shoot initiation was then determined for secondary explants of Form A (*Scilla natalensis sensu stricto),* Form B (*S. natalensis* syn. *S. kraussii*) and Form C (*S. natalensis* syn. *S. dracomontana*) respectively.

2.3.2. EFFECT OF FORM ON ADVENTITIOUS SHOOT INITIATION.

The carbohydrate source data, the plant growth regulator data and gelling agent data were pooled (as described below) to determine the mean number of shoots initiated for the three forms (Table 3). The form, Form A, Form B or Form C respectively, influenced shoot initiation with the larger forms generally producing more shoots than the smaller forms (Form A - *Scilla natalensis sensu stricto* > Form B - *S. natalensis* syn. *S. kraussii* Bak. > Form C - *S. natalensis* syn. *S. dracomontana* (Table 3).

The carbohydrate source data for the three forms were pooled to determine the interaction between form and carbohydrate source. The form significantly influenced the mean number of shoots initiated with Form A producing more shoots than Form B or Form C (Tables 3 & 4). The large SE's for the carbohydrate data were due to the response of the forms to the various carbohydrate sources particularly mannitol, which significantly reduced the number of shoots initiated (data presented in 2.3.4). The carbohydrate source and the interaction between form and carbohydrate source also significantly influenced the number of shoots initiated (Table 4). This may be due to the ability of the forms to utilize the carbohydrate sources.

The plant growth regulator data for the three forms were pooled to determine the interactions between form, cytokinin and auxin. The form significantly influenced the mean number of shoots initiated with Form A producing more shoots than Form B or Form C (Tables 3 & 5). The large SE's for the plant growth regulator data (kinetin:NAA & TDZ:NAA) were due to NAA, which significantly reduced the number of shoots initiated when used alone or in combination with cytokinins (data presented in 2.3.5). The interactions between form and cytokinin or form and auxin significantly influenced the number of shoots initiated. The mean number of shoots initiated also was significantly influenced by the interactions between form, cytokinin and auxin. This was significant for combinations of kinetin: NAA or TDZ: NAA although this was not significant for combinations of kinetin: IAA (Table 5). This data suggests that the effect of form could not be compensated for by the addition of plant growth regulators. In *Lachenalia*, genotypes were divided into two broad groups based on the number of shoots initiated (NIEDERWIESER & VAN STADEN, 1992). This was linked to endogenous cytokinin levels where genotypes with low endogenous cytokinin-like activity initiated more shoots than those with high endogenous cytokinin-like activity (NIEDERWIESER & VAN STADEN, 1992). The effect of the genotype also could not be compensated for by the addition of plant growth regulators, which may be due to the ability of the genotypes to metabolize the exogenous plant growth regulators or the availability of these plant growth regulators (NIEDERWIESER & VAN STADEN, 1992).

The gelling agent data for the three forms were pooled to determine the interaction between form and gelling agent. The form significantly influenced the mean number of shoots initiated with Form A producing more shoots than Form B or Form C (Table 3 & 6). The gelling agent also significantly influenced the number of shoots initiated athough this was not significantly influenced by the interaction between form and gelling agents (Table 6).

The data confirmed that the three forms, Form A, Form B and Form C respectively, are significantly different in terms of their physiological response to carbohydrates, plant growth regulators and gelling agents *in vitro.* Since the effect of form on the mean number of shoots initiated per explant could not be compensated for by the addition of either carbohydrates, plant growth regulators or gelling agents, this may provide some support for the reinstatement of these forms as three species, *Scilla natalensis* Planch.,

S. kraussii Bak. and *S. dracomontana* Hilliard & Burtt.

Table 3: The average number of shoots initiated per leaf explant of Form A, Form B and Form C for the pooled carbohydrate, plant growth regulator and gelling agent data.

Pooled data	Number of shoots per explant ± SE		
	Form A	Form B	Form C
Carbohydrates	3.0±0.9	1.1±0.3	1.0±0.3
Kinetin: IAA	9.3±3.8	2.9±0.6	1.0±0.6
Kinetin:NAA	3.5±0.3	1.±1.9	0.7±0.7
TDZ:NAA	2.3±3.1	0.2±0.3	1.0±1.1
Gelling agents	1.6±0.2	1.3±0.3	0.9±0.6

Table 4: ANOVA showing the effect of form and carbohydrate source on the number of shoots initiated. NS, *,**, *** : Non-significant or significant at the 5 %, 1 % or 0.1 % level respectively.

SOURCE OF VARIATION	VARIANCE RATIO
Main effects:	
Form	11.70***
Carbohydrate source	2.47*
Interactions:	
Form x Carbohydrate source	2.42**

Table 5: ANOVA showing the effect of form, cytokinin and auxin on the number of shoots initiated. NS, *,**, *** : Non-significant or significant at the 5 %, 1 % or 0.1 % level respectively.

SOURCE OF VARIATION	VARIANCE RATIO		
	Kinetin: IAA	Kinetin: NAA	TDZ: NAA
Main effects:			
Form	195.95***	32.19***	62.55***
Cytokinin	35.27***	1.14NS	13.23***
Auxin	2.16NS	41.67***	56.17***
Interactions:			
Form x Cytokinin	10.91***	2.51*	13.59***
Form x Auxin	3.43***	8.07***	46.35***
Form x Cytokinin x Auxin	1.36NS	11.53***	8.11***

Table 6: ANOVA showing the effect of form and gelling agent on the number of shoots initiated. NS, *,**, *** : Non-significant or significant at the 5 %, 1 % or 0.1 % level respectively.

SOURCE OF VARIATION	VARIANCE RATIO
Main effects:	
Form	4.90**
Gelling agent	3.39*
Interactions:	
Form x Gelling agent	0.32NS

2.3.3. EFFECT OF EXPLANT TYPE ON ADVENTITIOUS SHOOT INITIATION.

The carbohydrate source data, the plant growth regulator data and gelling agent data were pooled (as described below) to determine the mean number of shoots initiated for bulb or leaf explants of Form A (Table 7). The explant type, that is bulb or leaf explants, significantly influenced shoot initiation (Tables 7, 8, 9 & 10). This may have been influenced by other factors. In *Hyacinthus* (PIERIK & POST, 1975; PIERIK & STEEGMANS, 1975), *Lachenalia* (NIEDERWIESER & VAN STADEN, 1990b & 1992) and *Ornithogalum* (LANDBY & NIEDERWIESER, 1992), the explant size influenced shoot initiation. In *Hyacinthus*, the explant shape also influenced shoot initiation, which increased linearly with increasing explant length (PIERIK & POST, 1975).

The carbohydrate source data for the bulb and leaf explants of Form A were pooled to determine the interactions between explant type and carbohydrate source. The explant type significantly influenced the mean number of shoots initiated with bulb explants producing more shoots than leaf explants (Tables 7 & 8). This may be due to the higher levels of nutrient reserves in bulb explants than in leaf explants. Some workers found a positive correlation between the sites of starch accumulation and shoot initiation (VAN AARTRIJK & BLOM-BARNHOORN, 1980; GEORGE & SHERRINGTON, 1984). The large SE's for the carbohydrate data were due to the responses of the explant types to the various carbohydrate sources particularly mannitol, which significantly reduced the number of shoots initiated (data presented in 2.3.4). The carbohydrate source also significantly influenced the number of shoots initiated (Table 8). The number of shoots initiated, however, was not significantly influenced by the interaction between explant type and carbohydrate source (Table 8).

The plant growth regulator data for the bulb and leaf explants of Form A were pooled to determine the interactions between the explant type, cytokinin and auxin. The explant type significantly influenced the mean number of shoots initiated with leaf explants producing more shoots than bulb explants (Tables 7 & 9). The large SE's for the plant growth regulator data (kinetin:NAA & TDZ:NAA) were due to NAA, which significantly reduced the number of shoots initiated (data presented in 2.3.5) when used alone in combination with cytokinins. The cytokinin and auxin significantly influenced

the number of shoots initiated (Table 9). The number of shoots initiated also was significantly influenced by interactions between the explant type and cytokinin and the interaction between explant type, cytokinin and auxin (except for kinetin:IAA). Thus, the number of shoots initiated was significantly influenced by combinations of kinetin:NAA or TDZ:NAA, but was not significantly influenced by the interactions between explant type and combinations of kinetin: IAA (Table 9). This may be due to the ability of the explants to metabolize exogenous plant growth regulators. Other factors such as the age, orientation (apolar or polar), position (distal or proximal) and polarity of the explants also may have influenced shoot initiation. In *Lachenalia*, the explant age influenced shoot initiation with young explants producing more shoots than old explants. This may be linked to endogenous cytokinin levels particularly in the intermediate and old explants (NIEDERWIESER & VAN STADEN, 1990a). In *Ornithogalum*, the explant orientation also influenced shoot initiation with apolar explants producing more shoots than polar explants (LANDBY & NIEDERWIESER, 1992). In *Lachenalia*, explants produced callus and deformed shoots when cultured with the adaxial surface downwards (NIEDERWIESER & VCELAR, 1990). In *Lilium*, however, explants produced more bulblets and roots when cultured with the adaxial surface upwards (GEORGE & SHERRINGTON, 1984). This may be due to explant polarity. Shoot initiation in bulb explants is often influenced by explant polarity with more shoots forming at the proximal end rather than the distal end. This has been attributed to the transport of various plant growth regulators particularly the polar transport of IAA within the explant (GEORGE & SHERRINGTON, 1984).

The gelling agent data for the leaf and bulb explants of Form A were pooled to determine the interaction between explant type and gelling agent. The explant type influenced the mean number of shoots initiated per explant with bulb explants producing more shoots than leaf explants (Tables 7 & 10). This was not significantly influenced by the interaction between the explant type and gelling agent (Table 10). The gelling agents, however, may have influenced the gel-strength. The gel-strength influences the degree of contact between the explant and the media, decreasing with increasing gelling agent concentration. This also influences the availability of plant growth regulators, salts and water (DEBERGH, 1983). These factors may have masked any interaction between explant type and gelling agent.

Table 7: The average number of shoots initiated per bulb or leaf explant of Form A for the pooled carbohydrate, plant growth regulator and gelling agent data.

Pooled data	Number of shoots per explant ± SE	
	Bulb explants	Leaf explants
Carbohydrates	4.1±1.0	3.0±0.9
Kinetin: IAA	6.0±1.7	9.3±3.8
Kinetin:NAA	2.6±2.0	3.5±0.3
TDZ:NAA	3.1±2.9	2.3±3.1
Gelling agents	2.8±0.2	1.6±0.2

Table 8: ANOVA showing the effect of explant type and carbohydrate source on the number of shoots initiated. NS, *,**, *** : Non-significant or significant at the 5 %, 1 % or 0.1 % level respectively.

SOURCE OF VARIATION	VARIANCE RATIO
Main effects:	
Explant type	5.80*
Carbohydrate source	17.55***
Interactions:	
Explant type x Carbohydrate source	0.52NS

Table 9: ANOVA showing the effect of explant type, cytokinin and auxin on the number of shoots initiated. NS, *,**, *** : Non-significant or significant at the 5 %, 1 % or 0.1 % level respectively.

SOURCE OF VARIATION	VARIANCE RATIO		
	Kinetin: IAA	Kinetin: NAA	TDZ: NAA
Main effects:			
Explant type	71.93***	7.94**	4.95*
Cytokinin	64.88***	0.24NS	25.15***
Auxin	14.78***	107.71***	194.36***
Interactions:			
Explant type x Cytokinin	16.6***	3.27*	8.59***
Explant type x Auxin	0.89NS	5.37**	2.35NS
Explant type x Cytokinin x Auxin	0.99NS	9.93***	6.57***

Table 10: ANOVA showing the effect of explant type and gelling agent on the number of shoots initiated. NS, *,**, *** : Non-significant or significant at the 5 %, 1 % or 0.1 % level respectively.

SOURCE OF VARIATION	VARIANCE RATIO
Main effects:	
Explant type	16.08***
Gelling agent	0.47NS
Interactions:	
Explant type x Gelling agent	1.46NS

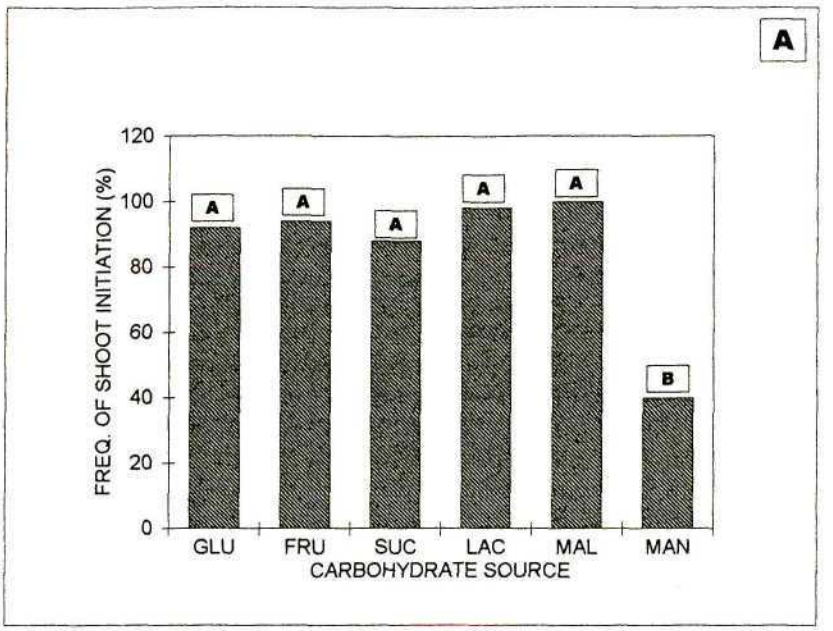
2.3.4. EFFECT OF CARBOHYDRATES ON ADVENTITIOUS SHOOT INITIATION.

2.3.4.1. Effect of carbohydrates on adventitious shoot initiation of Form A.

The secondary explants viz. bulb and leaf explants of Form A, responded differently to the carbohydrate sources. Shoot initiation was generally higher for bulb explants than leaf explants (Tables 7 & 8). The carbohydrate source also significantly influenced shoot initiation. The frequency of shoot initiation for bulb explants was significantly influenced by the carbohydrate source (Fig 4a). The frequency of shoot initiation for bulb explants on medium containing sucrose was 88 % (Fig. 4a). When glucose, fructose, lactose or maltose were substituted for sucrose, the frequency of shoot initiation was moderately increased although this was not significant. The frequency of shoot initiation, however, was significantly reduced by the substitution of mannitol for sucrose. The mean number of shoots initiated per explant on medium containing sucrose was 5.0 shoots per explant (Fig. 4b). When glucose or fructose were substituted for sucrose, the number of shoots initiated per explant was increased to 7.1 and 6.5 shoots per explant respectively. This was significant for medium containing glucose but not significant for medium containing fructose. The mean number of shoots initiated per explant, however, was significantly reduced by the substitution of lactose or mannitol for sucrose, and considerably reduced by the substitution of maltose for sucrose. The explants, which were cultured on media containing glucose, fructose or sucrose, produced long shoots with robust leaves. In contrast, the explants, which were cultured on media containing lactose, maltose or mannitol, produced short shoots with spindly leaves. Most explants, which were cultured on medium containing mannitol, were achlorophyllous or necrotic, although some short shoots were produced. Optimal shoot initiation in terms of frequency, number and quality for bulb explants occurred on media containing glucose or fructose.

The frequency of shoot initiation for leaf explants of Form A was significantly influenced by the carbohydrate source (Fig. 5a). The frequency of shoot initiation medium containing sucrose was 88 % (Fig. 5a). When glucose, fructose or maltose were substituted for sucrose, the frequency of shoot initiation was moderately reduced. The frequency of shoot initiation, however, was significantly reduced by the substitution of

lactose and particularly mannitol for sucrose. The mean number of shoots initiated per explant on medium containing sucrose was 4.9 shoots per explant (Fig. 5b). When fructose was substituted for sucrose, the number of shoots initiated per explant was moderately increased to 5.6 shoots per explant. The mean number of shoots initiated, however, was moderately reduced by the substitution of glucose and maltose for sucrose. The substitution of lactose or mannitol for sucrose also reduced the number of shoots initiated per explant although this was not significant. The explants, which were cultured on media containing fructose, glucose or sucrose, produced long shoots with robust leaves. In contrast, the explants, which were cultured on media containing lactose or maltose, produced short, and often hyperhydric shoots. No shoots were produced by the explants that were cultured on medium containing mannitol. Optimal shoot initiation in terms of frequency, numbers and quality for leaf explants occurred on media containing fructose, sucrose or glucose.



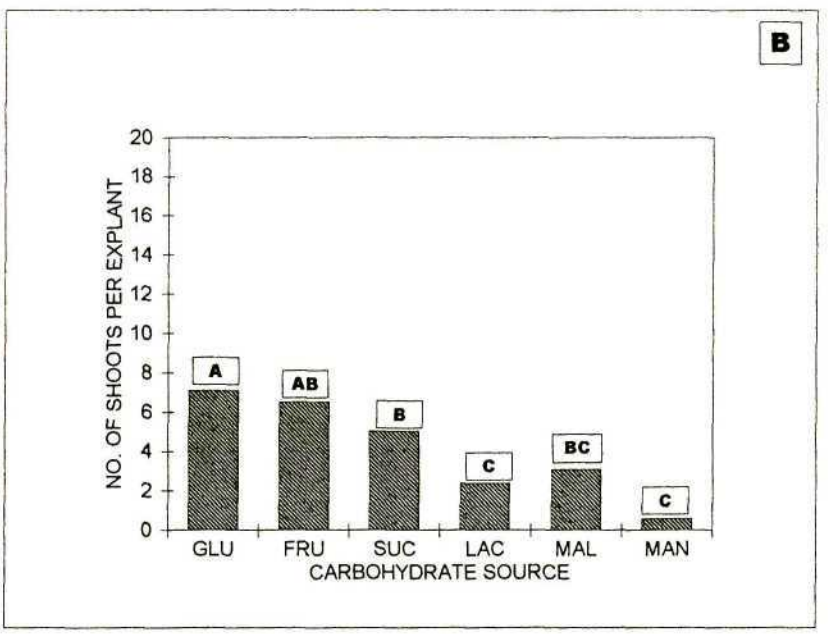
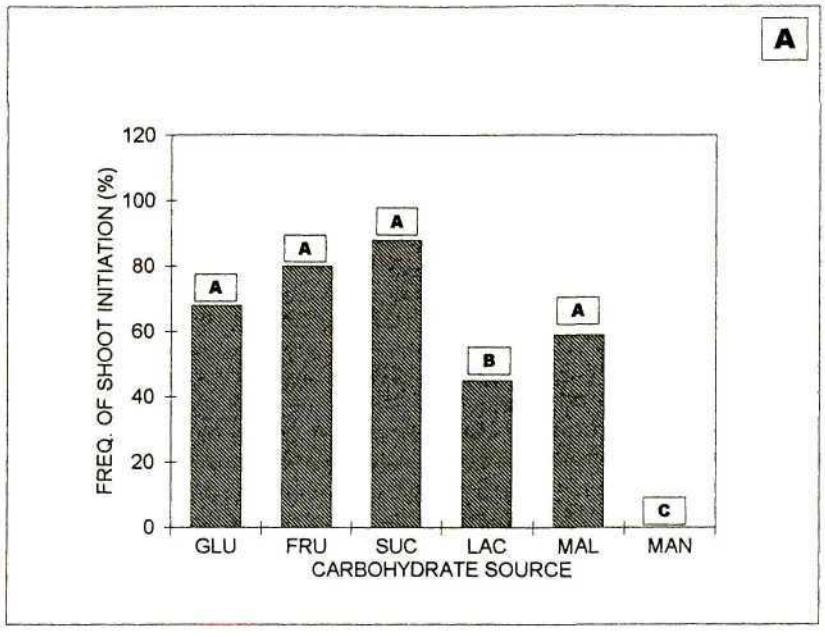


Fig. 4: The frequency of shoot initiation (A) and average number of shoots initiated per bulb explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various carbohydrates. Bars with the same letters are not significantly different at P=0.05.



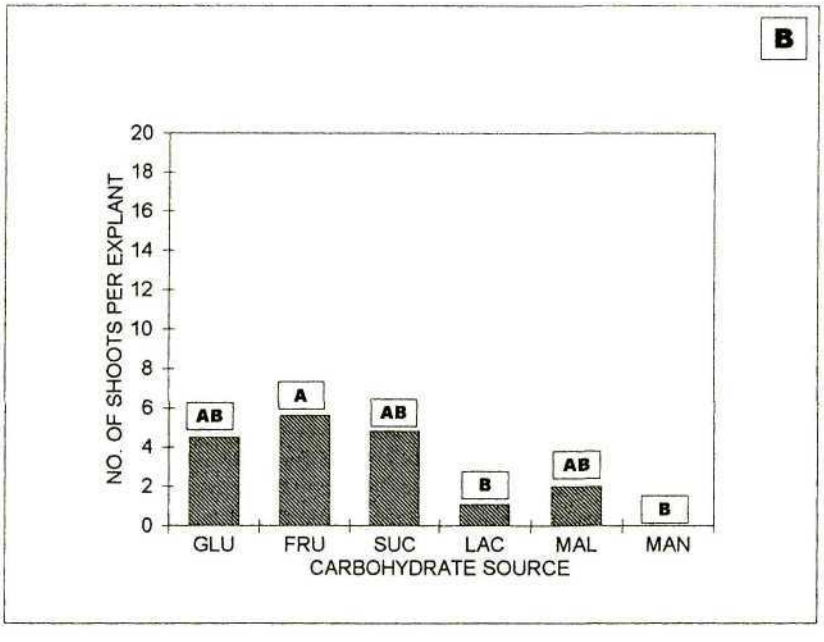
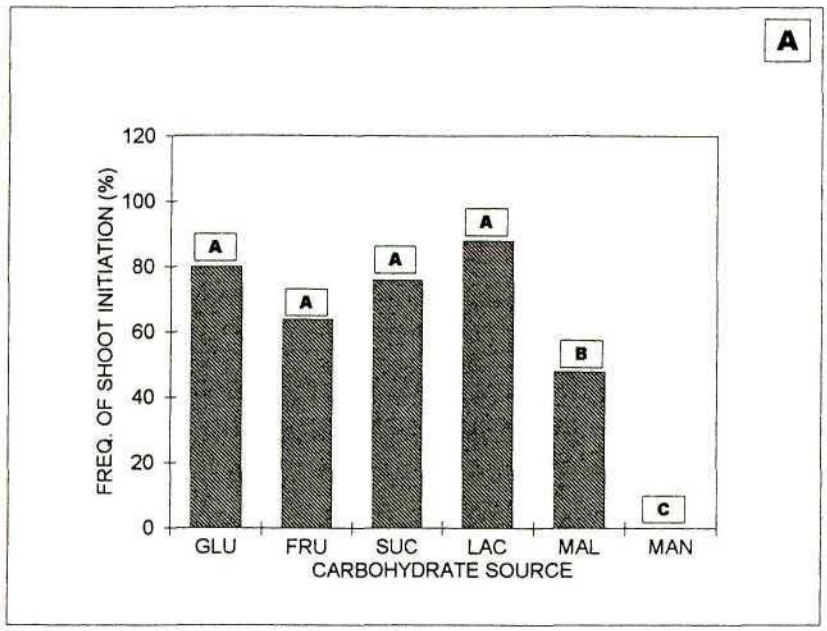


Fig. 5: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various carbohydrates. Bars with the same letters are not significantly different at P=0.05.

2.3.4.2. Effect of carbohydrates on adventitious shoot initiation of Form B.

The carbohydrate source significantly influenced shoot initiation of leaf explants of Form B. The frequency of shoot initiation was significantly influenced by the carbohydrate source (Fig. 6a). The frequency of shoot initiation on medium containing sucrose was 76 %. When glucose or lactose were substituted for sucrose, the frequency of shoot initiation increased moderately although this was not significant (Fig. 6a). The frequency of shoot initiation was moderately reduced by the substitution of fructose for sucrose but was significantly reduced by the substitution of maltose and particularly mannitol for sucrose. The mean number of shoots initiated per explant on medium containing sucrose was 1.0 shoot per explant (Fig. 6b). The substition of glucose or lactose significantly increased the number of shoots initiated per explant to 2.3 and 1.8 shoots per explant respectively. The mean number of shoot initiated per explant, however, was significantly reduced by the substition of mannitol for sucrose. The explants, which were cultured on media containing glucose, lactose or sucrose, produced long shoots with spindly leaves and well-developed roots. In contrast, the explants, which were cultured on media containing fructose, produced long shoots with spindly leaves and stunted, brown roots. No shoots were produced by the explants, which were cultured on medium containing mannitol although these explants produced some wound callus along the cut edge. Optimal shoot initiation in terms of frequency, number and quality occurred on media containing glucose.



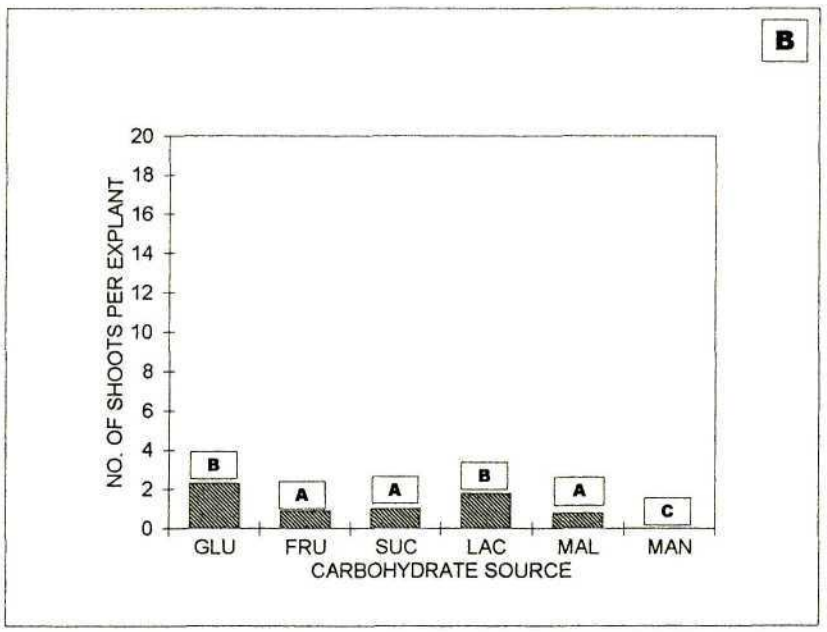
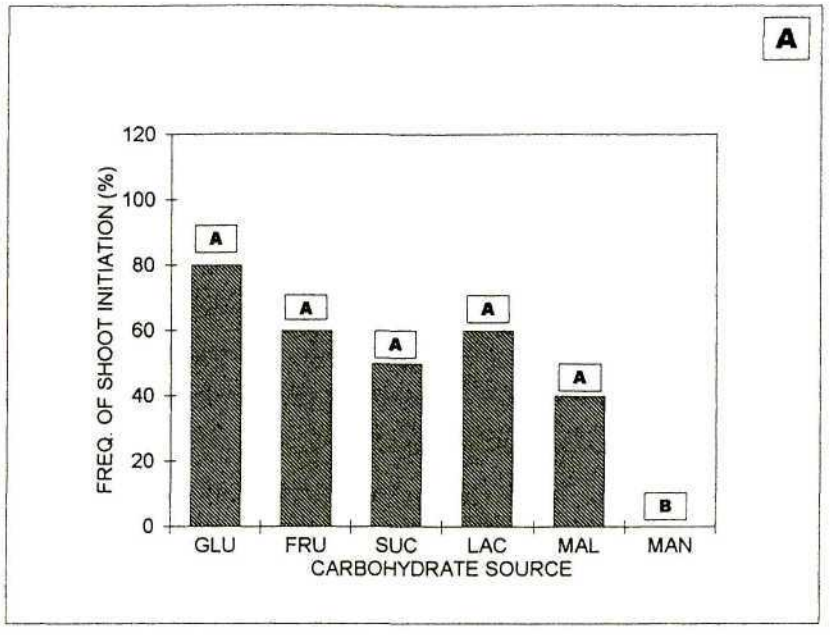


Fig. 6: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form B after 8 weeks on media containing various carbohydrates. Bars with the same letters are not significantly different at P=0.05.

2.3.4.3. Effect of carbohydrates on adventitious shoot initiation in Form C.

The carbohydrate source significantly influenced shoot initiation in leaf explants of Form C. The frequency of shoot initiation was significantly influenced by the carbohydrate source (Fig. 7a). The frequency of shoot initiation on medium containing sucrose was 50 %. When glucose, fructose or lactose were substituted for sucrose, the frequency of shoot initiation was increased moderately although this was not significant. The frequency of shoot initiation was moderately reduced by the substitution of maltose for sucrose but was significantly reduced by the substitution of mannitol for sucrose. The mean number of shoots initiated per explant on medium containing sucrose was 1.6 shoots per explant (Fig. 7b). When glucose was substituted for sucrose, the number of shoots initiated per explant was increased to 1.8 shoots per explant although this was not significant. The mean number of shoots initiated per explant was considerably reduced by the substitution of mannitol for sucrose although this also was not significant. The explants, which were cultured on medium containing glucose, produced long shoots with robust leaves. In contrast, the explants, which were cultured on media containing fructose, lactose, maltose or sucrose, produced short shoots. No shoots were produced by explants cultured on medium containing mannitol. Optimal shoot initiation in terms of frequency, number and quality occurred on medium containing glucose.



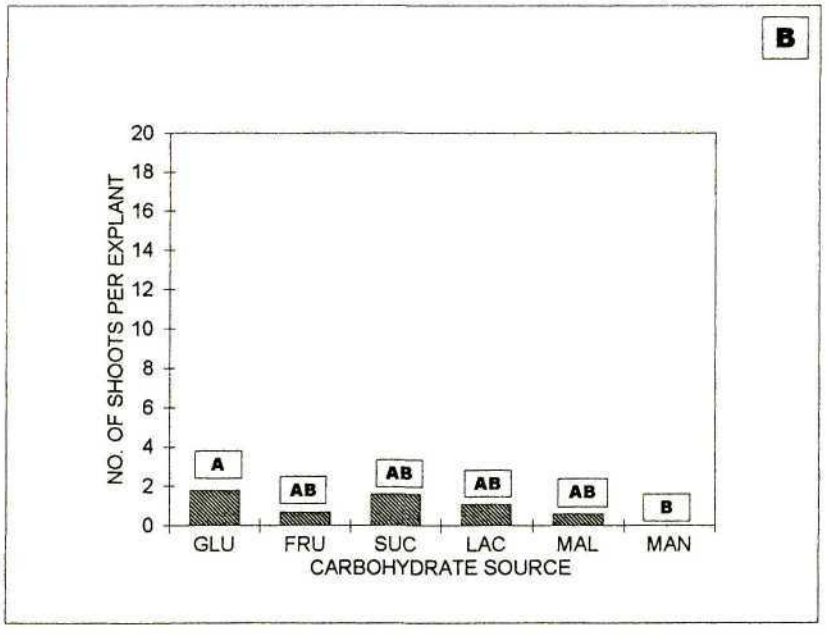


Fig. 7: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form C after 8 weeks on media containing various carbohydrates. Bars with the same letters are not significantly different at P=0.05.

2.3.4.4. Overall effect of carbohydrates on adventitious shoot initiation in three forms of Scilla natalensis.

The carbohydrate source significantly influenced shoot initiation for the forms, Form A, Form B and FormC in terms of frequency, number (Table 3) and quality. The explants generally produced more shoots when cultured on media containing glucose or sucrose than on media containing fructose, lactose, maltose and particularly mannitol. This may be due to the ability of the explants to utilize the carbohydrate source. This also may be influenced by the endogenous levels of carbohydrates. Some workers found a positive correlation between sites of starch accumulation, the starch content, the mode of glucose oxidation, the respiration rate and shoot initiation (VAN AARTRIJK & BLOM-BARNHOORN, 1980). Thus, the availability of sucrose, which is the main carbon source for starch accumulation, may have influenced shoot initiation. The beneficial effects of sucrose also may be partially or totally due to other processes such as osmosis (VAN AARTRIJK & BLOM-BARNHOORN, 1980). Some workers suggested that sucrose may be used in the ratios 1:3 and 2:3 for osmoregulation and as a carbon source respectively. The dual role for sucrose was indicated by the inability of mannitol to substitute for sucrose below a critical threshold (BROWN, LEUNG & THORPE, 1979). In contrast, others reported that the addition of sorbitol promoted shoot initiation, while the addition of fructose, galactose or sucrose promoted callus. These workers proposed that the accumulation of sorbitol resulted in carbon starvation, providing the correct plant growth regulator concentration and internal osmotic balance, and thus leading to the initiation of new meristematic sites. It was also proposed that shoot initiation is probably not due to an osmotic effect because the addition of galactose, glucose or sucrose alone or in combination with sorbitol promoted callus (LEMOS & BAKER, 1998). The frequency and number of shoots initiated per explant also may have been influenced by the hydrolysis of various carbohydrates during autoclaving and the subsequent effect on the physical properties of the media. The breakdown of fructose into 5-(hydroxymethyl)-2-furaldehyde (HMF), hydroxy-acetal furan, levulinic acid and various other minor compounds, and the subsequent decrease in post-autoclaving pH may influence the availability of certain salts (RÉDEI, 1974). These breakdown compounds have been implicated in the "fructose effect or syndrome" of plantlets, which are characterized by chlorosis and root inhibition (RÉDEI, 1974). Optimal shoot initiation in terms of frequency, number and quality for the forms, Form A, Form B and Form C occurred on media containing glucose or sucrose.

2.3.5. EFFECT OF PLANT GROWTH REGULATORS (PGR's) ON ADVENTITIOUS SHOOT INITIATION OF THREE FORMS OF *SCILLA NATALENSIS*.

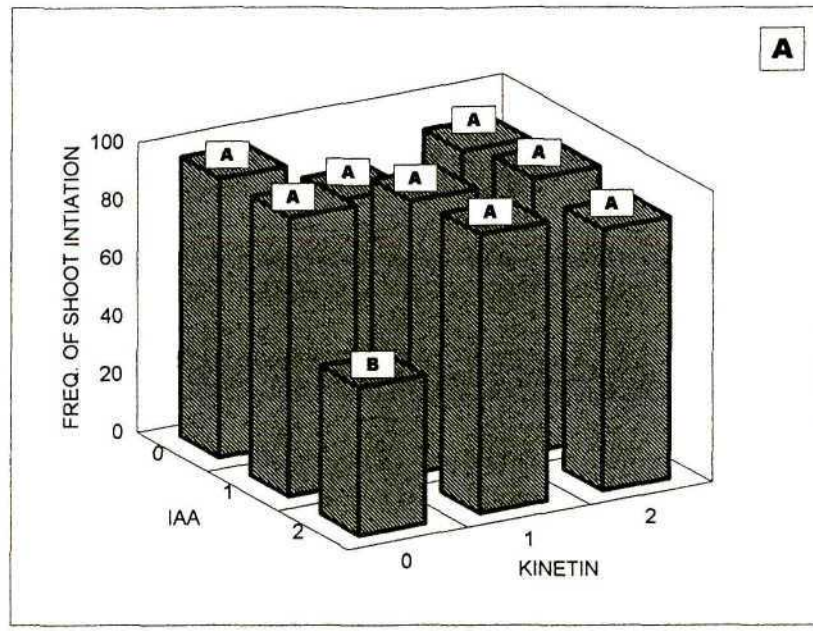
2.3.5.1. Effect of PGR's on adventitious shoot initiation of Form A.

The secondary bulb and leaf explants of Form A responded similarly to the various combinations of plant growth regulators. The frequency of shoot initiation on media containing no plant growth regulators (control) ranged from 96 to 100 % for bulb explants (Figs. 8a - 10a) and 76 to 92 % for leaf explants (Figs. 11a-13a). The number of shoots initiated per explant on control media ranged from 3.2 to 5.2 per bulb explant (Figs. 8b-10b) and 3.9 to 10.4 for leaf explants (Figs. 11b -13b). The shoots were long with spindly leaves and few roots. These were initiated directly on the adaxial surfaces or along the periphery of the explants (Figs. 14a &b). Some crystalline wound callus was produced along the cut edges of the explants.

The frequency of shoot initiation was moderately reduced by the addition of kinetin or TDZ alone for bulb (Figs. 8a - 10a) and leaf explants (Figs. 11a - 13a). The addition of kinetin or TDZ alone also significantly increased the number of shoots initiated per explant for bulb (Figs. 8b -10b) and leaf explants (Figs. 11b -13b). The shoots were small with robust leaves. These were often initiated directly on the adaxial surfaces of explants (Fig. 14e), although some shoots were initiated indirectly from hard speckled or white callus.

The frequency of shoot initiation was moderately reduced by the addition of IAA alone for bulb (Fig. 8a) and leaf explants (Fig. 11a), decreasing with increasing concentration. The frequency of shoot initiation was significantly reduced by the addition of NAA alone or in combination for bulb (Figs. 9a - 10a) and leaf explants (Figs. 12a - 13a). The mean number of shoots initiated was also moderately reduced by the addition of IAA alone for bulb (Fig. 8b) and leaf explants (Fig. 11b). The mean number of shoots initiated, however, was significantly reduced by the addition of NAA alone or in combination for bulb (Figs. 9b - 10b) and leaf explants (Figs. 12b - 13b). The explants, which were cultured on media containing IAA or NAA alone, produced large quantities of white callus and numerous roots (Fig. 14f). A few shoots with short, robust and sometimes deformed or fused leaves were initiated indirectly from callus.

The frequency of shoot initiation and mean number of shoots initiated were higher for combinations of kinetin: IAA than for combinations of kinetin: NAA or TDZ: NAA for bulb and leaf explants. The interaction between kinetin: IAA considerably increased the number of shoots initiated. The mean number of shoots initiated on media containing combinations of kinetin: IAA ranged from 5.9 to 8.5 shoots per bulb explant and 5.9 to 14.2 shoots per leaf explant. These explants produced large quantities of white callus and robust shoots (Figs. 14c & d). Some shoots were fused, deformed or stunted. The frequency of fused and deformed leaves ranged from 8 to 24 %. The interactions between kinetin: NAA and TDZ: NAA significantly reduced the number of shoots initiated for bulb (Figs. 9b - 10b) and leaf explants (Figs. 12b - 13b). The explants produced large quantities of brown or speckled callus often as a thick band along the cut edge of the explant. Numerous roots, which were sometimes deformed, also were produced. Optimal shoot initiation in terms of frequency, number and quality for bulb and leaf explants of Form A occurred on media containing 1 to 2 mg l^{-1} kinetin and 1 to 2 mg l^{-1} IAA.



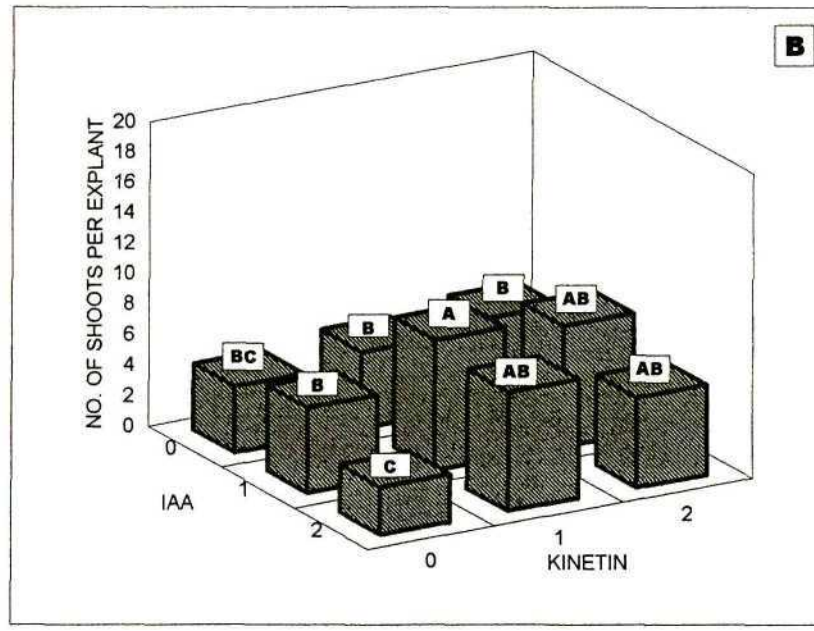
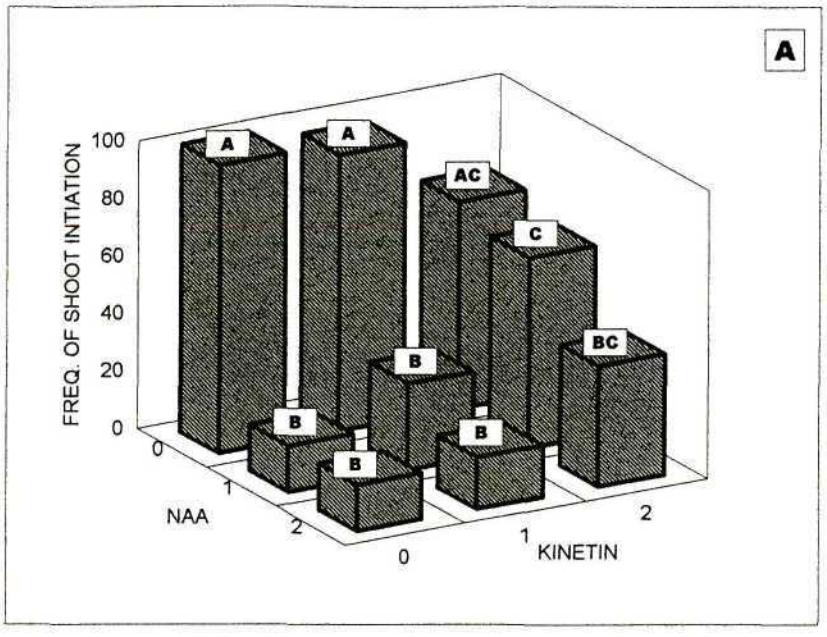


Fig. 8: The frequency of shoot initiation (A) and average number of shoots initiated per bulb explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various combinations of kinetin: IAA. Bars with the same letters are not significantly different at P=0.05.



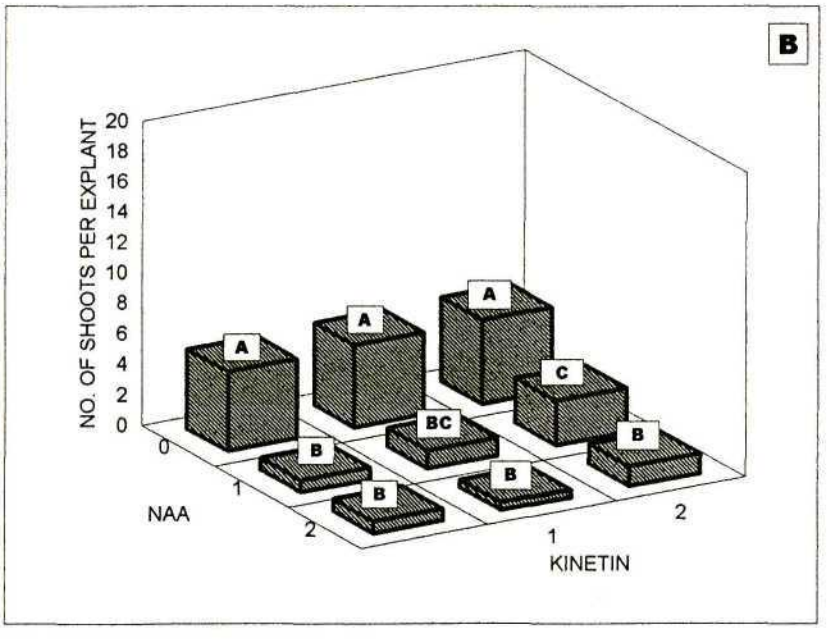
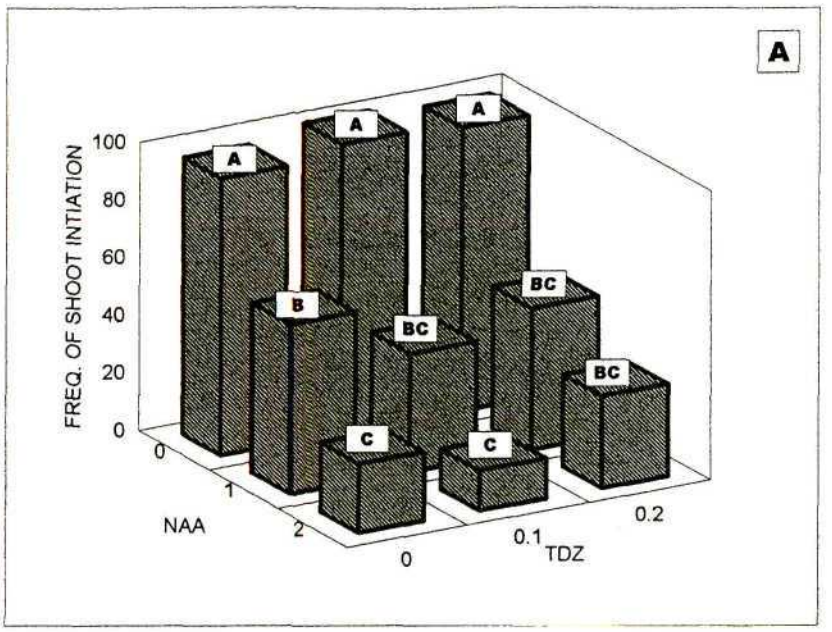


Fig. 9: The frequency of shoot initiation (A) and average number of shoots initiated per bulb explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various combinations of kinetin: NAA. Bars with the same letters are not significantly different at P=0.05.



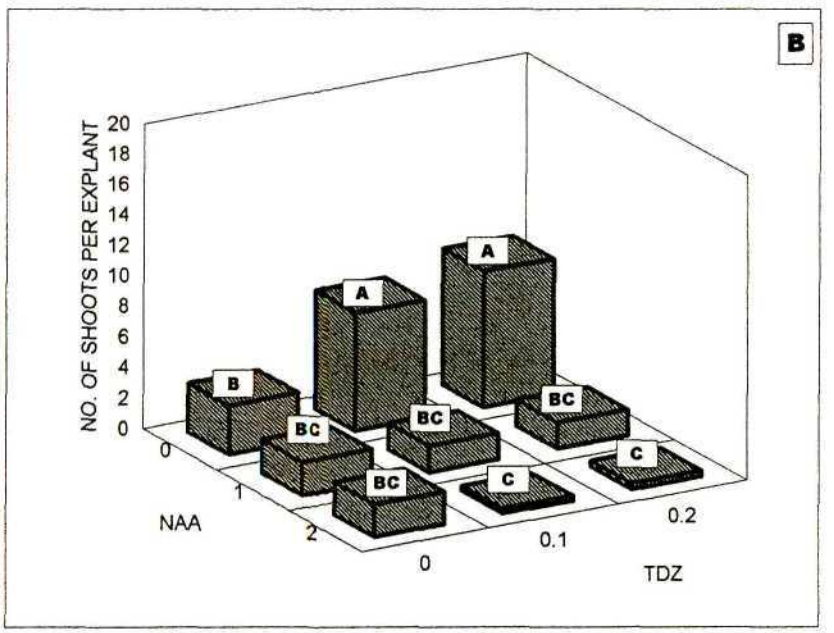
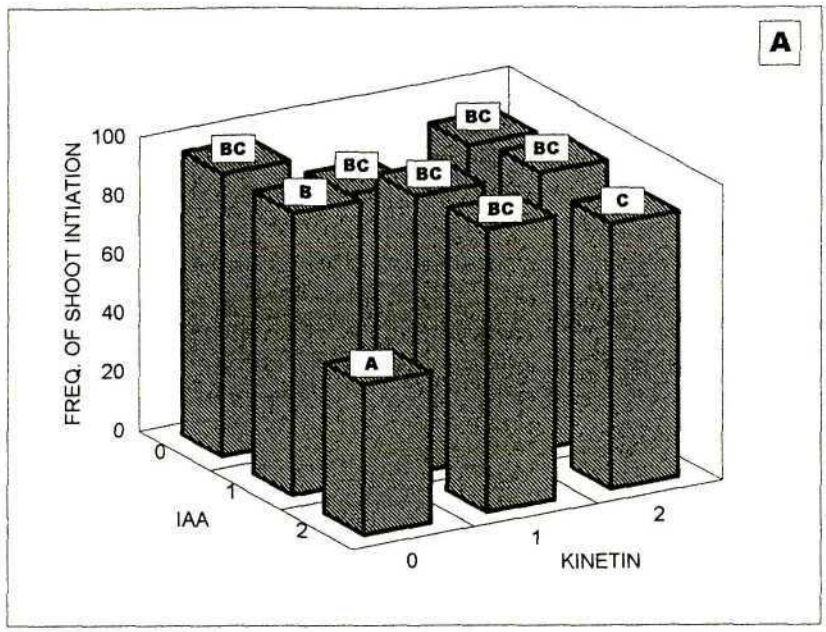


Fig. 10: The frequency of shoot initiation (A) and average number of shoots initiated per bulb explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various combinations of TDZ: NAA. Bars with the same letters are not significantly different at P=0.05.



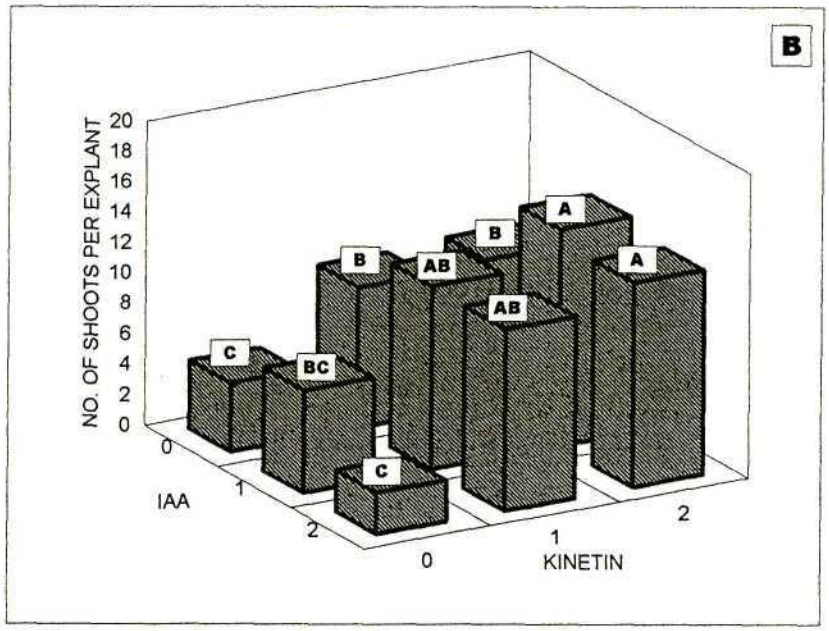
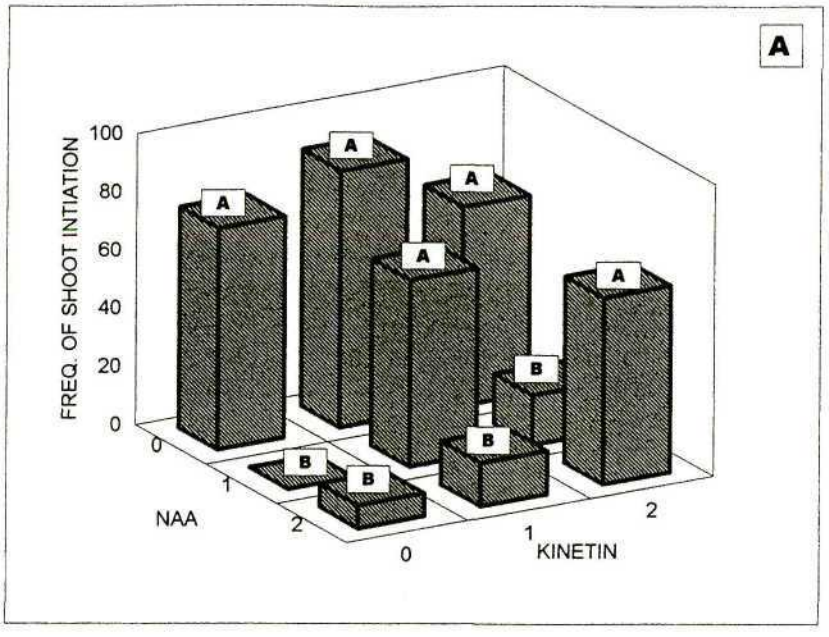


Fig. 11: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various combinations of kinetin: IAA. Bars with the same letters are not significantly different at P=0.05.



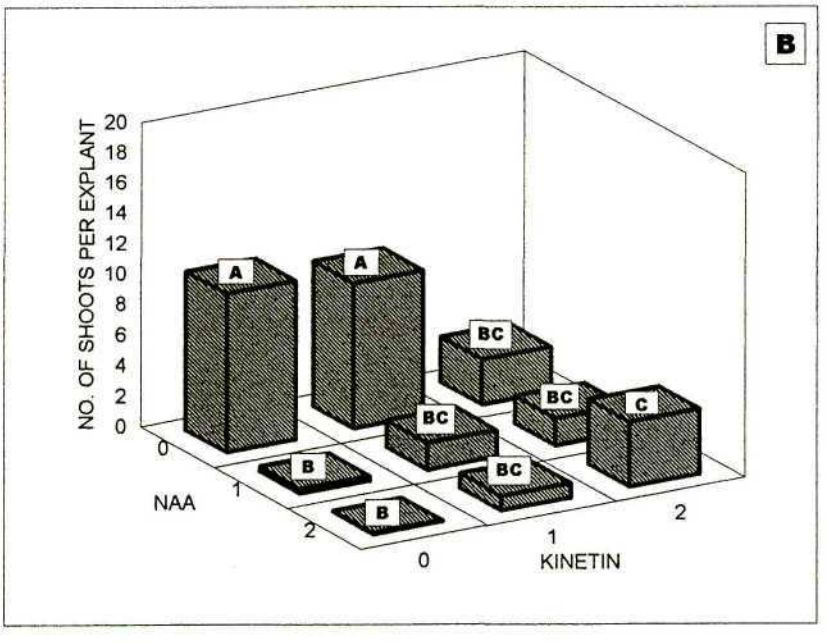
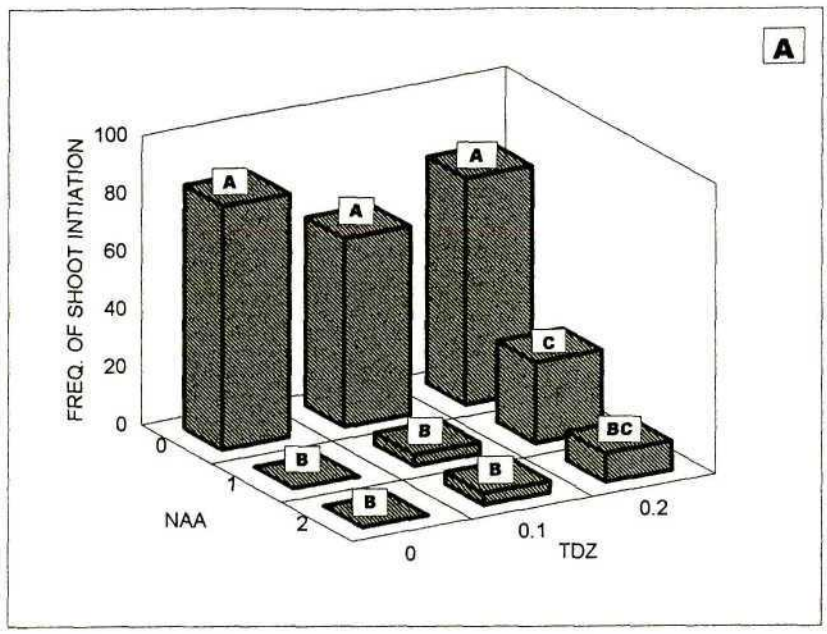


Fig. 12: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various combinations of kinetin: NAA. Bars with the same letters are not significantly different at P=0.05.



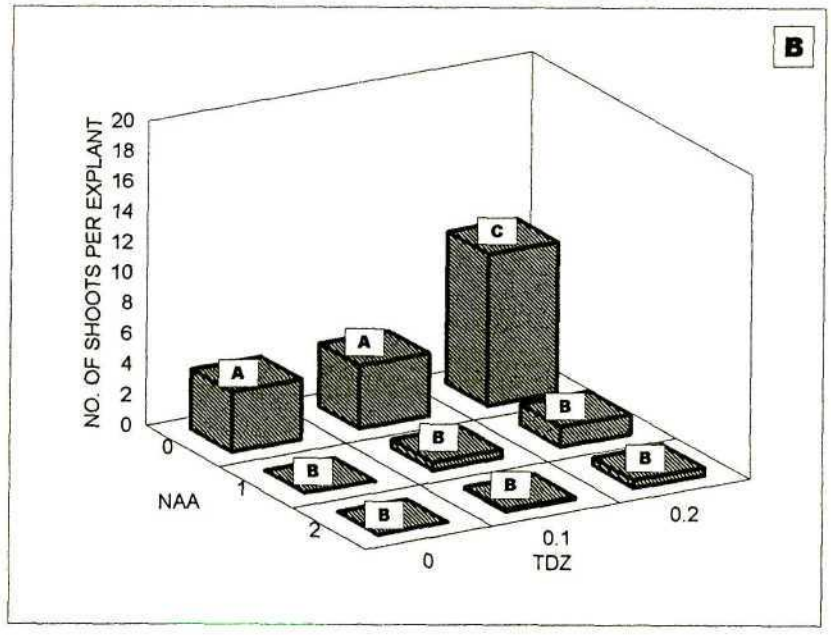


Fig. 13: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form A after 8 weeks on media containing various combinations of TDZ: NAA. Bars with the same letters are not significantly different at P=0.05.

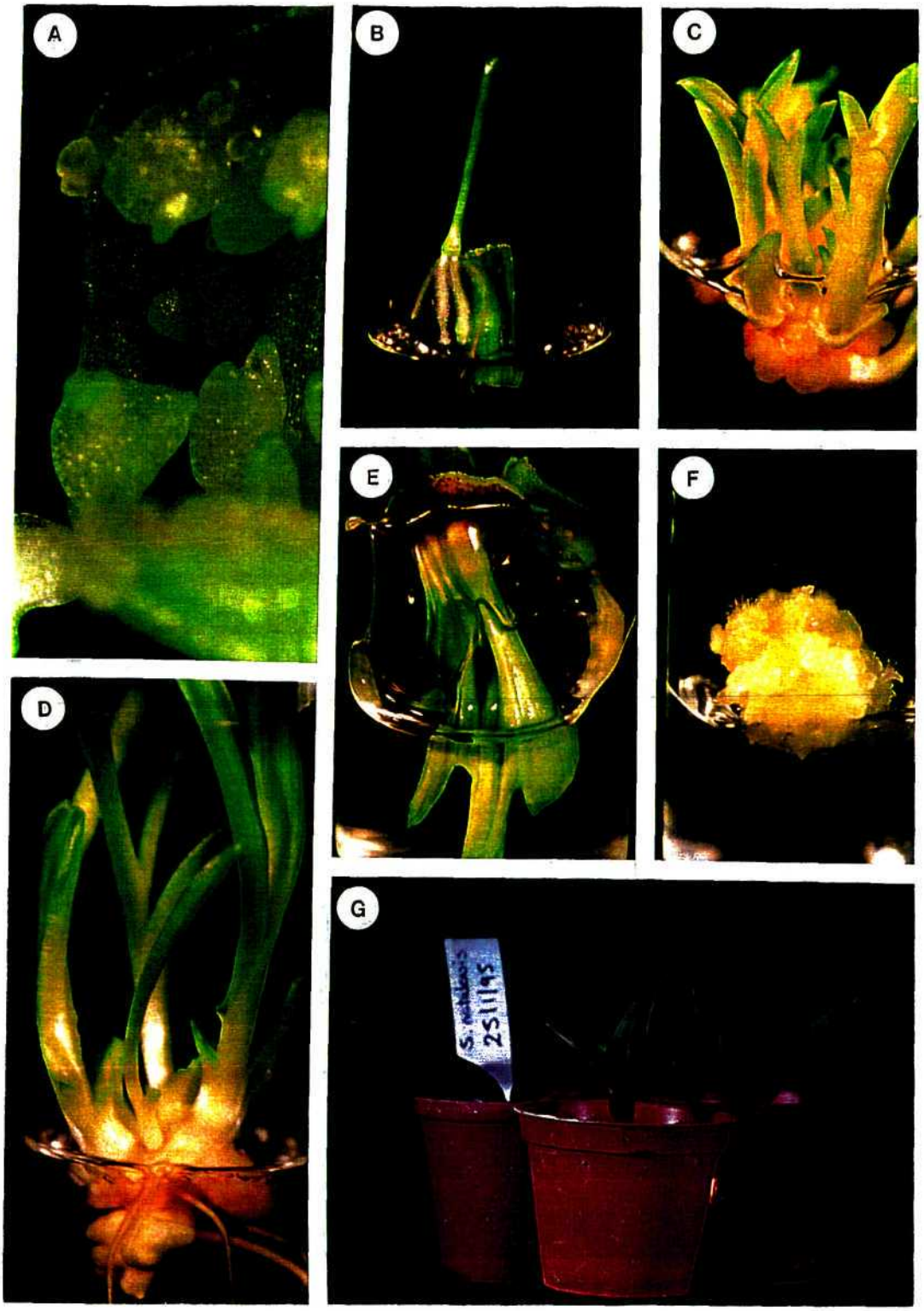


Fig. 14A: Adventitious buds initiated on leaf explants of *Scilla natalensis* Form A on medium without plant growth regulators; B: Shoot initiated along cut edge of leaf explant of *S. natalensis* Form A on medium without plant growth regulators; C: Shoots initiated indirectly from callus of *S. natalensis* Form A on medium containing 1 mg l^{-1} kinetin and 1 mg l^{-1} IAA; D: Shoots initiated indirectly from callus of *S. natalensis* Form A on medium containing 2 mg l^{-1} kinetin and 1 mg l^{-1} IAA; E: Fused shoot of *S. natalensis* Form A on medium containing 1 mg l^{-1} kinetin; F: Callus and roots of *S. natalensis* Form A on medium containing 2 mg l^{-1} NAA; G: Acclimatised plant of *S. natalensis* Form A.

2.3.5.2. Effect of PGR's on adventitious shoot initiation of Form B.

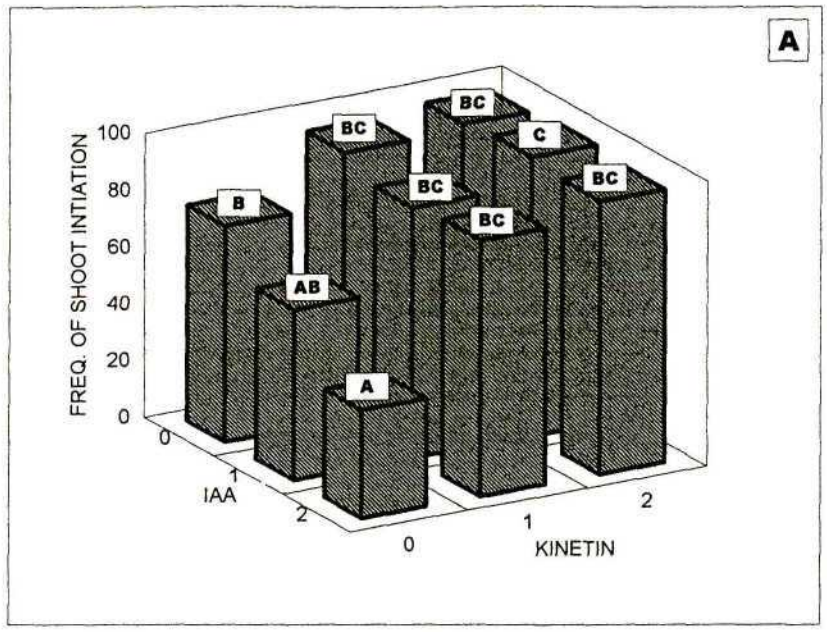
The frequency of shoot initiation on control media ranged from 8 to 78 % per leaf explant of Form B (Figs. 15a-17a). This may be due edge effects in the growth room caused by small differences in temperature and particularly light on the various shelves. The mean number of shoots initiated on control media ranged from 0.1 to 2.0 shoots per explant (Figs. 15b-17b). The shoots were long with spindly leaves and few roots (Fig. 18a). These were initiated on the adaxial surfaces or along the periphery of the explants. The explants also produced limited quantities of callus.

The frequency of shoot initiation was increased by the addition of kinetin or TDZ alone (Figs. 15a - 17a). The mean number of shoots initiated was also increased by the addition of kinetin or TDZ alone (Figs. 15b - 17b). The addition of kinetin alone significantly increased the number of shoots initiated, increasing moderately with increasing concentration (Figs. 15b & 16b). The addition of TDZ alone also increased the number of shoots initiated although this was not significant (Fig. 17b). These shoots resembled the controls (Fig. 18b).

The frequency of shoot initiation was reduced by the addition of IAA and particularly NAA alone (Figs . 15a - 17a). The mean number of shoots initiated was also reduced by the addition of IAA or NAA alone (Figs. 15b - 17b). Some explants, which were cultured on media containing IAA alone, produced callus usually as a band along the cut edge of the explant. Most explants, which were cultured on media containing NAA alone, were necrotic with the lower end of the explant turning black. A few explants, however, produced roots (Fig. 18e).

The frequency of shoot initiation was higher for combinations of kinetin: IAA than for combinations of kinetin: NAA or TDZ: NAA. The mean number of shoots initiated was also higher for combinations of kinetin: IAA than for combinations of kinetin: NAA or TDZ: NAA (Figs. 15b - 17b). The interaction between kinetin: IAA considerably increased the number of shoots initiated. The mean number of shoots initiated on media containing combinations of kinetin: IAA ranged from 2.5 to 6.0 shoots per explant. The shoots were small with short, often robust leaves. These were initiated long the

periphery of the explants or from brown callus (Figs. 18c & d). The interaction between the kinetin: NAA or TDZ: NAA significantly reduced the number of shoots initiated. Some explants produced callus, usually as a band along the cut edge of the explant, but most explants were necrotic. Optimal shoot initiation for leaf explants of Form B in terms of frequency, number and quality occurred on media containing 2 mg l^{-1} kinetin and 1 to 2 mg l^{-1} IAA.



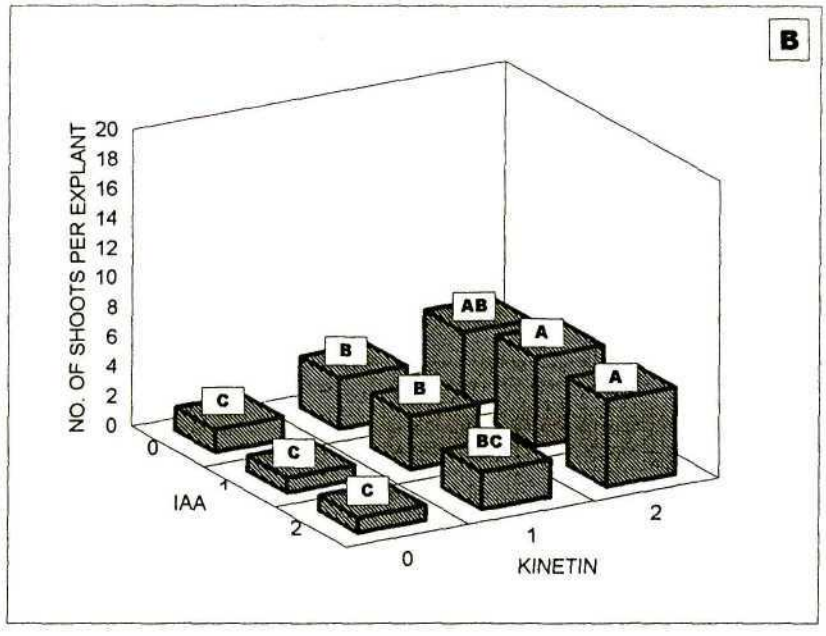
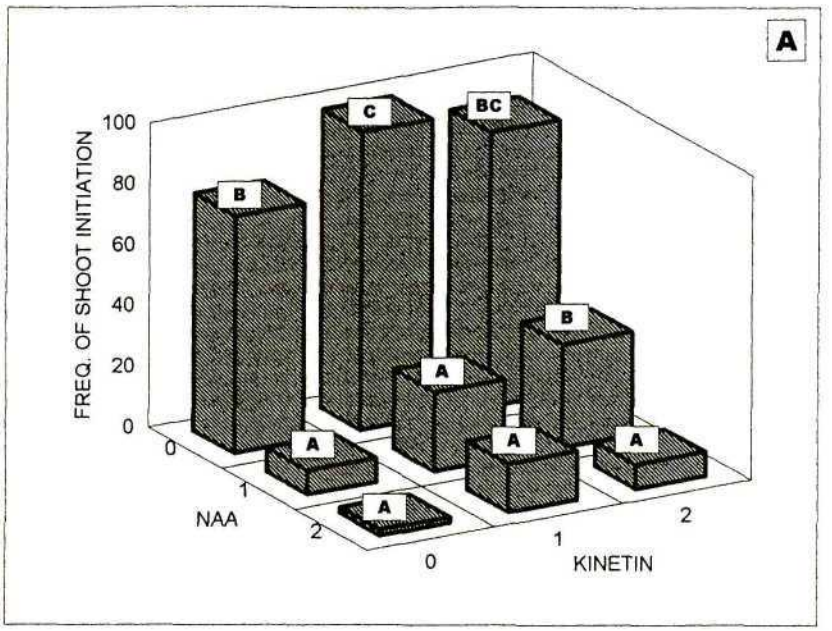


Fig. 15: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form B after 8 weeks on media containing various combinations of kinetin: IAA. Bars with the same letters are not significantly different at P=0.05.



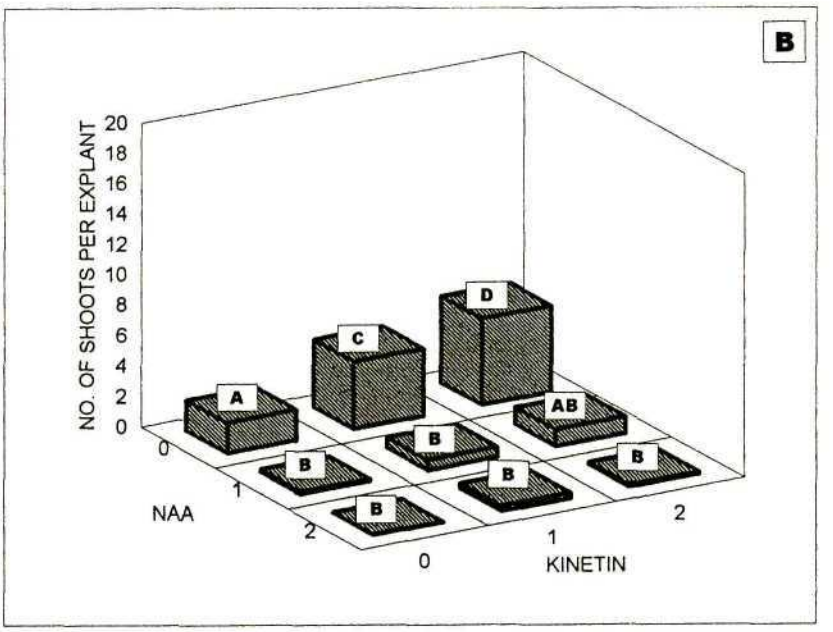
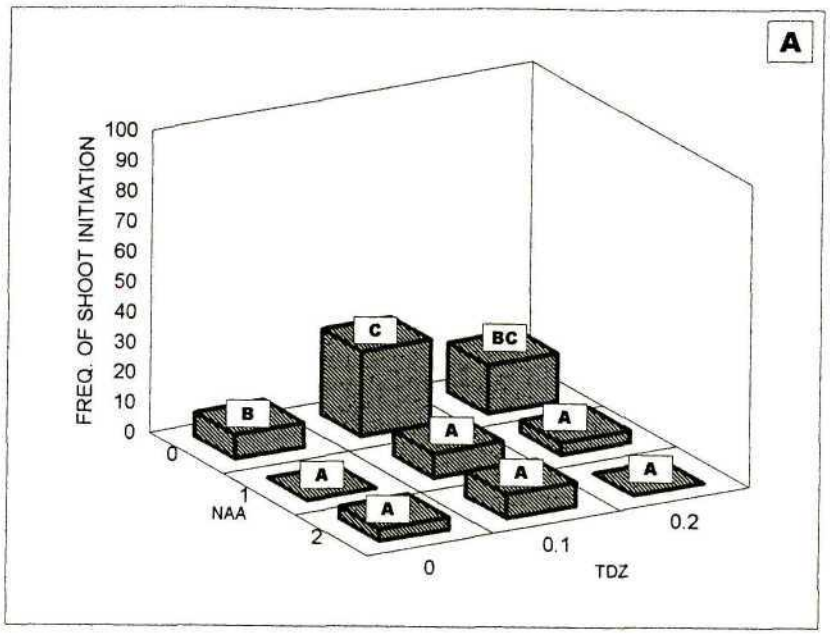


Fig. 16: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form B after 8 weeks on media containing various combinations of kinetin: NAA. Bars with the same letters are not significantly different at P=0.05.



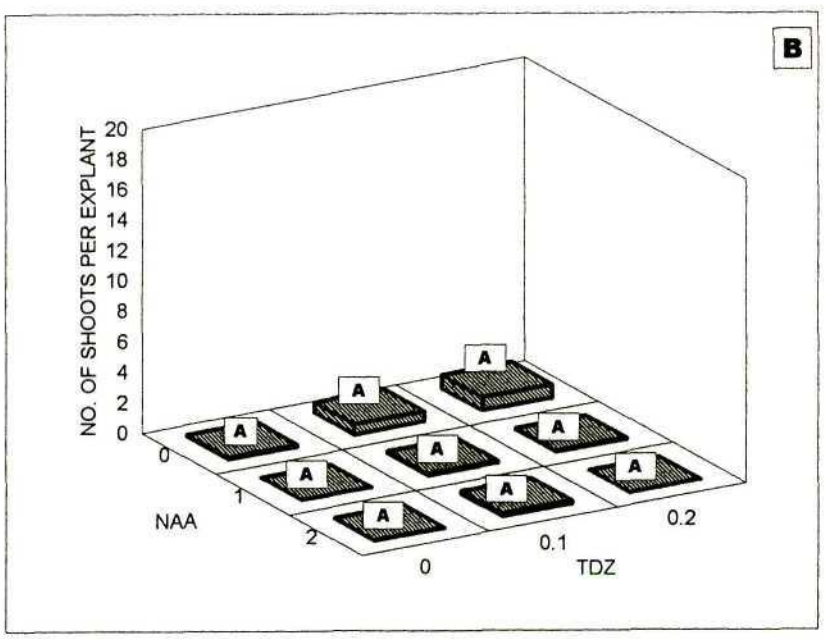


Fig. 17: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form B after 8 weeks on media containing various combinations of TDZ: NAA. Bars with the same letters are not significantly different at P=0.05.

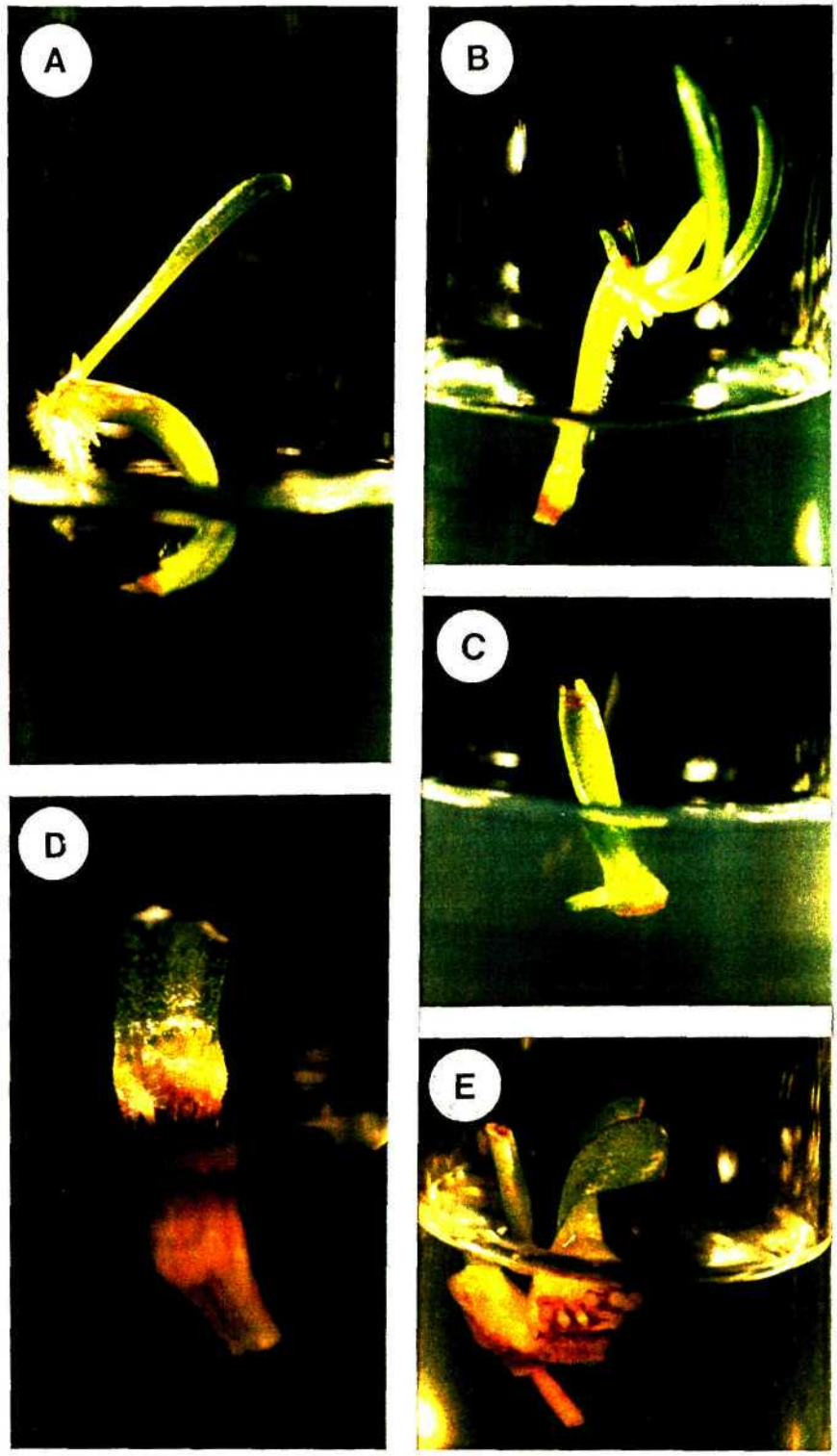


Fig. 18A: Shoot initiated along cut edge of leaf explant of *Scilla natalensis* Form B on medium without plant growth regulators; B: Shoots initiated along cut edge of leaf explant of *S. natalensis* Form B on medium containing 1 mg l^{-1} kinetin; C: Shoots initiated along cut edge of leaf explant of *S. natalensis* Form B on medium containing 1 mg l^{-1} kinetin and 1 mg l^{-1} IAA; D: Shoots initiated on necrotic leaf explant of *S. natalensis* Form B on medium containing 1 mg l^{-1} NAA; E: Roots initiated on leaf explant of *S. natalensis* Form B on medium containing 0.1 mg l^{-1} TDZ and 1 mg l^{-1} NAA.

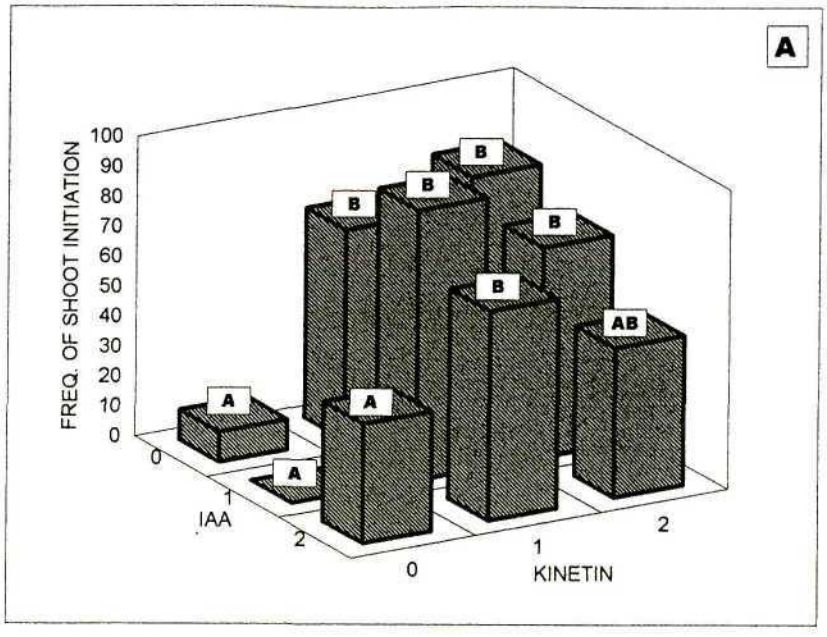
2.3.5.3. Effect of PGR's on adventitious shoot initiation of Form C.

The frequency of shoots initiation on control media ranged from 10 to 80 % (Figs. 19a-21a). This may be due edge effects in the growth room caused by small differences in temperature and particularly light on the various shelves. The mean number of shoots initiated on control media ranged from 0.1 to 1.5 shoots per explant (Figs. 19b - 21b). The shoots were small with spindly leaves and few roots. These were initiated directly on the adaxial surfaces or along the periphery of the explants.

The frequency of shoot initiation was increased by the addition of kinetin or TDZ alone (Figs. 19a - 21a). The mean number of shoots initiated was also moderately increased by the addition of kinetin or TDZ alone (Figs. 19b - 21b). These shoots resembled the controls.

The frequency of shoot initiation was reduced by the addition of IAA and particularly NAA alone (Figs. 19a - 21a). The mean number of shoots initiated was also reduced by the addition of IAA and NAA alone (Figs. 19b - 21b). The explants, which were cultured on media containing IAA, initiated only a few shoots, while those cultured on media containing NAA were necrotic (Fig. 22d).

The frequency of shoot initiation was higher for combinations of kinetin: IAA than for combinations of kinetin: NAA or TDZ: NAA. The interaction between kinetin: IAA moderately increased the number of shoots initiated, decreasing with increasing IAA concentration. The average number of shoots initiated on media containing combinations of kinetin: IAA ranged from 0.7 to 1.7 shoots per explant. The interactions between kinetin: NAA or TDZ: NAA significantly reduced the number of shoots initiated per explant. These explants were often necrotic, although some explants produced short shoots. Optimal shoot initiation in terms of frequency, number and quality occurred on media containing 1 to 2 mg l^{-1} kinetin and 1 to 2 mg l^{-1} IAA (Fig. 22c).



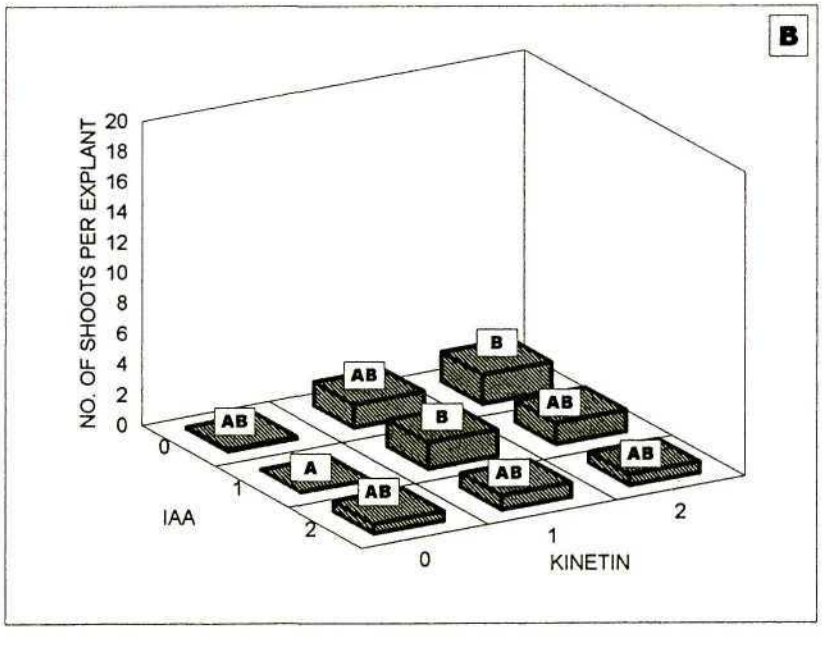
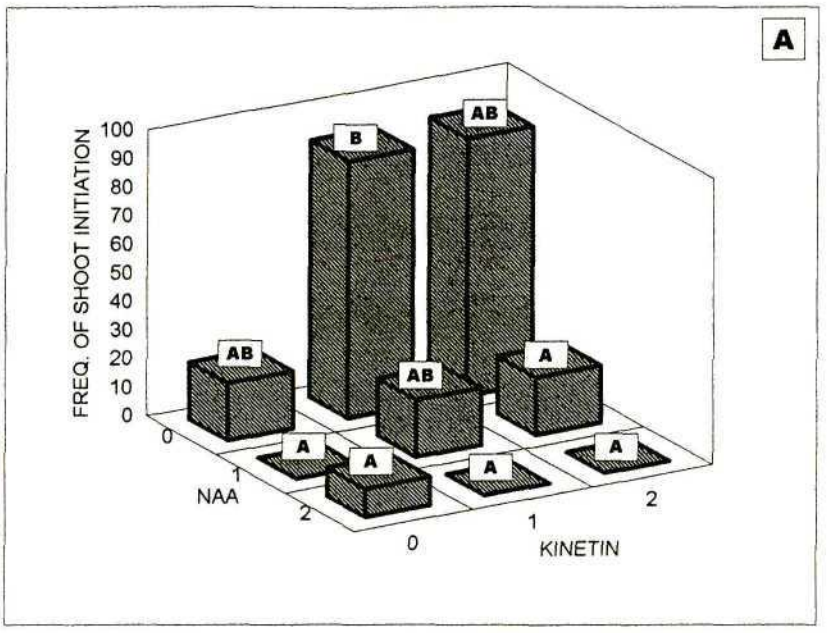


Fig 19: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form C after 8 weeks on media containing various combinations of kinetin: IAA. Bars with the same letters are not significantly different at P=0.05.



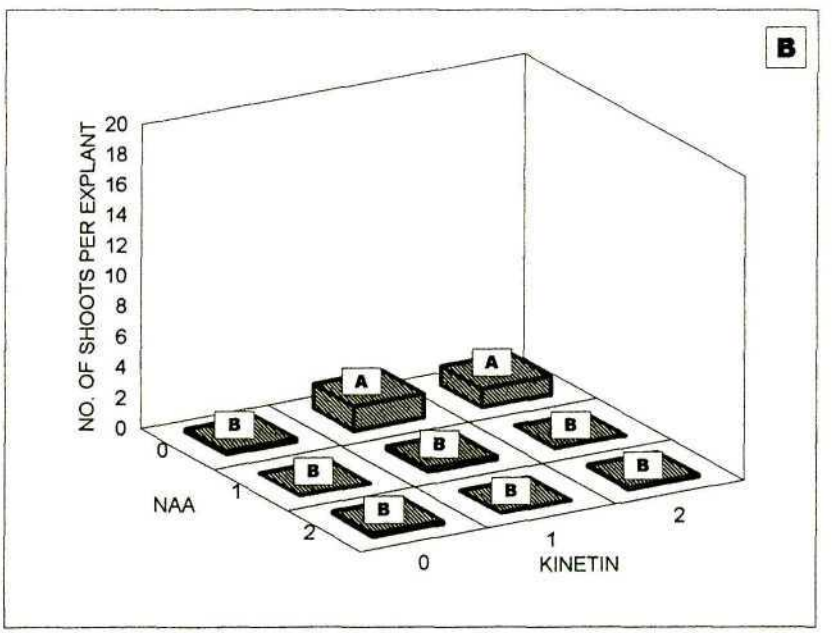


Fig. 20: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form C after 8 weeks on media containing various combinations of kinetin: NAA. Bars with the same letters are not significantly different at P=0.05.

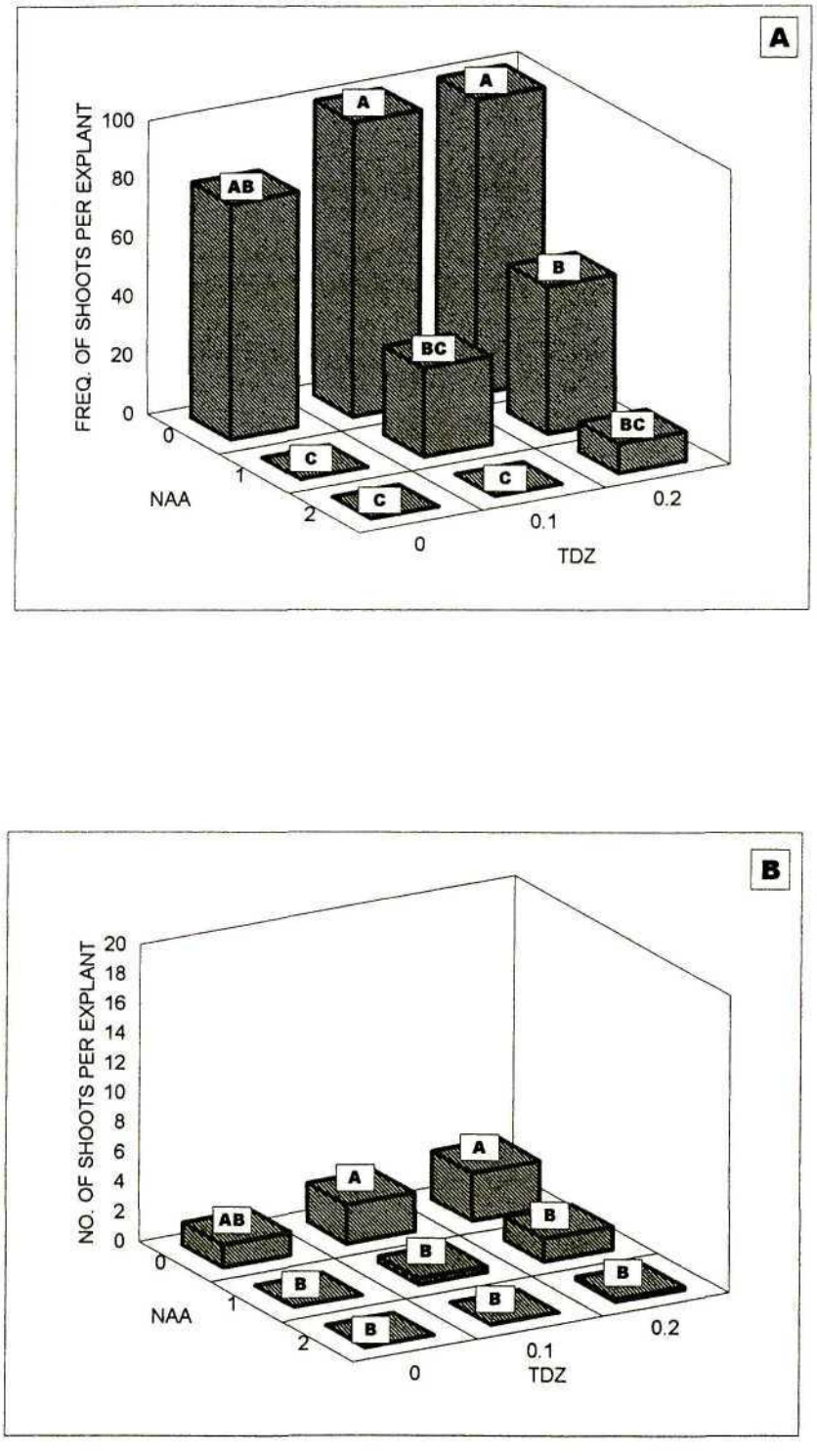


Fig. 21: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form C after 8 weeks on media containing various combinations of TDZ: NAA. Bars with the same letters are not significantly different at P=0.05.

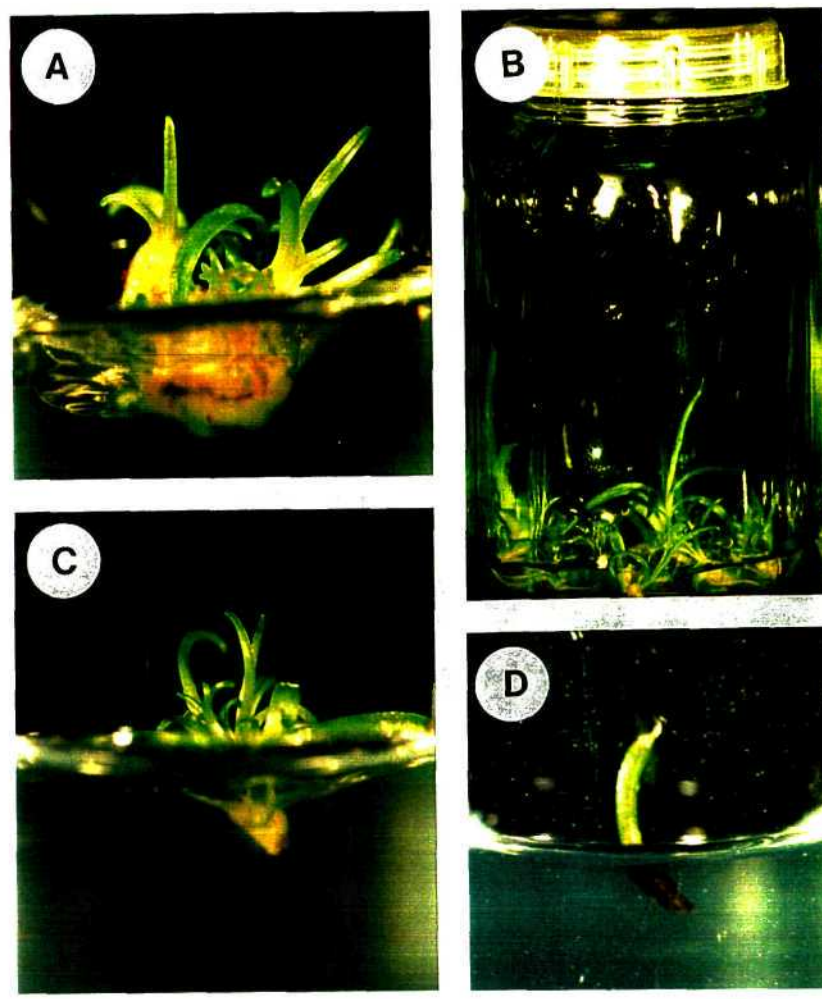


Fig. 22A: Shoots initiated from leaf explant of *Scilla natalensis* Form C after 8 weeks on medium containing 1 mg l^{-1} kinetin; B: Shoots initiated from leaf explant of *S. natalensis* Form C after 11 weeks on medium containing 1 mg l^{-1} kinetin; C: Shoots initiated from leaf explant of *S. natalensis* Form C on medium containing 1 mg l^{-1} kinetin and 1 mg l^{-1} IAA; D: Necrotic leaf explant of *S. natalensis* Form C on medium containing 1 mg l^{-1} NAA.

2.3.5.4. Overall effect of PGR's on adventitious shoot initiation in three forms of Scilla natalensis.

In *Scilla,* some shoots were initiated on medium containing no plant growth regulators. The frequency of shoot initiation and the mean number of shoots initiated, however, was generally increased by the addition of cytokinins particularly kinetin. In contrast, the addition of various cytokinins did not significantly influence shoot initiation in *Hyacinthus* (PIERIK & STEEGMANS, 1975; BACH & CECOT, 1988), *Ornithogalum* (HUSSEY, 1976) and *Lilium* (VAN AARTRIJK & BLOM-BARNHOORN, 1981). In *Lilium*, the addition of various cytokinins caused aberrant growth characterised by thin, green leafy scales instead of reddish-brown, thick and fleshy scales (VAN AARTRIJK & BLOM-BARNHOORN, 1981). In *Scilla,* the frequency of shoot initiation and mean number of shoots initiated per explant was generally reduced by the addition of auxins particularly NAA alone or in combination with cytokinins. The addition of NAA increased shoot initiation in *Lachenalia* (NIEDERWIESER & VAN STADEN, 1992) but reduced shoot initiation in *Hyacinthus* (PIERIK & STEEGMANS, 1975; BACH & CECOT, 1989) and *Ornithogalum* (HUSSEY, 1976a). In *Hyacinthus* and *Ornithogalum*, the addition of NAA promoted callus formation, while IAA promoted shoot initiation. In *Lilium*, the inhibitory effects of NAA were correlated with auxin-induced ethylene synthesis (VAN AARTRIJK & BLOM-BARNHOORN, 1981). Optimal shoot initiation for *Scilla natalensis* Form A, Form B and Form C occurred on media containing 1 to 2 mg l^{-1} kinetin and 1 to 2 mg l^{-1} IAA. In contrast, optimal shoot initiation for *Scilla hyacinthiana* occurred on media containing 5 mg l^{-1} kinetin and 1 mg l^{-1} NAA (NAIR, 1989).

2.3.6. EFFECT OF GELLING AGENTS ON ADVENTITIOUS SHOOT INITIATION.

2.3.6.1. Effect of gelling agents on adventitious shoot initiation of Form A.

The frequency of shoot initiation was considerably higher for bulb explants than for leaf explants (Figs. 23a & 24a). For bulb explants, the frequency of shoot initiation ranged from 96 to 100 %. This was not significantly influenced by the gelling agent (Fig. 23a). For leaf explants, the frequency of shoot initiation ranged from 64 to 80 % (Fig. 24a). This was significantly influenced by the gelling agent with Gelrite® or Unilab agar resulting in a higher frequency of shoot initiation than Oxoid agar. The number of shoots initiated was considerably higher for bulb explants than for leaf explants (Figs. 23b & 24b) although this was not significant (Table 7). For bulb explants, the mean number of shoots initiated ranged from 2.4 to 2.9 shoots per explant. This was not significantly influenced by the gelling agent although explants on media solidified with Oxoid agar produced more shoots than those on media solidified with Unilab agar or Gelrite®. For leaf explants, the mean number of shoots initiated ranged from 1.2 to 1.8 shoots per explant (Fig. 24b). This was not significantly influenced by the gelling agent although explants on media solidified with Gelrite® produced more shoots than those on media solidified with Oxoid or Unilab agar.

2.3.6.2. Effect of gelling agents on adventitious shoot initiation of Form B.

The frequency of shoot initiation, which ranged from 80 to 100 %, was significantly influenced by the gelling agent (Fig. 25a). This was significantly higher for explants on media solidified with Gelrite® or Oxoid agar than for those on media solidifed with Unilab agar. The mean number of shoots initiated, which ranged from 0.7 to 1.8 shoots per explant, was also influenced by the gelling agents with explants on media solidified with Gelrite® or Oxoid agar producing significantly more shoots than those on media solidified with Unilab agar (Fig. 25b).

2.3.6.3. Effect of gelling agents on adventitious shoot initiation of Form C.

The frequency of shoot initiation, which ranged from 30 to 50 %, was not significantly influenced by the gelling agent (Fig. 26a). This was, however, considerably higher for

explants on media solidified with Gelrite® than for those on media solidified with Oxoid or Unilab agar. The mean number of shoots initiated ranged from 0.3 to 2 shoots per explant although this was not significant (Fig. 26b).

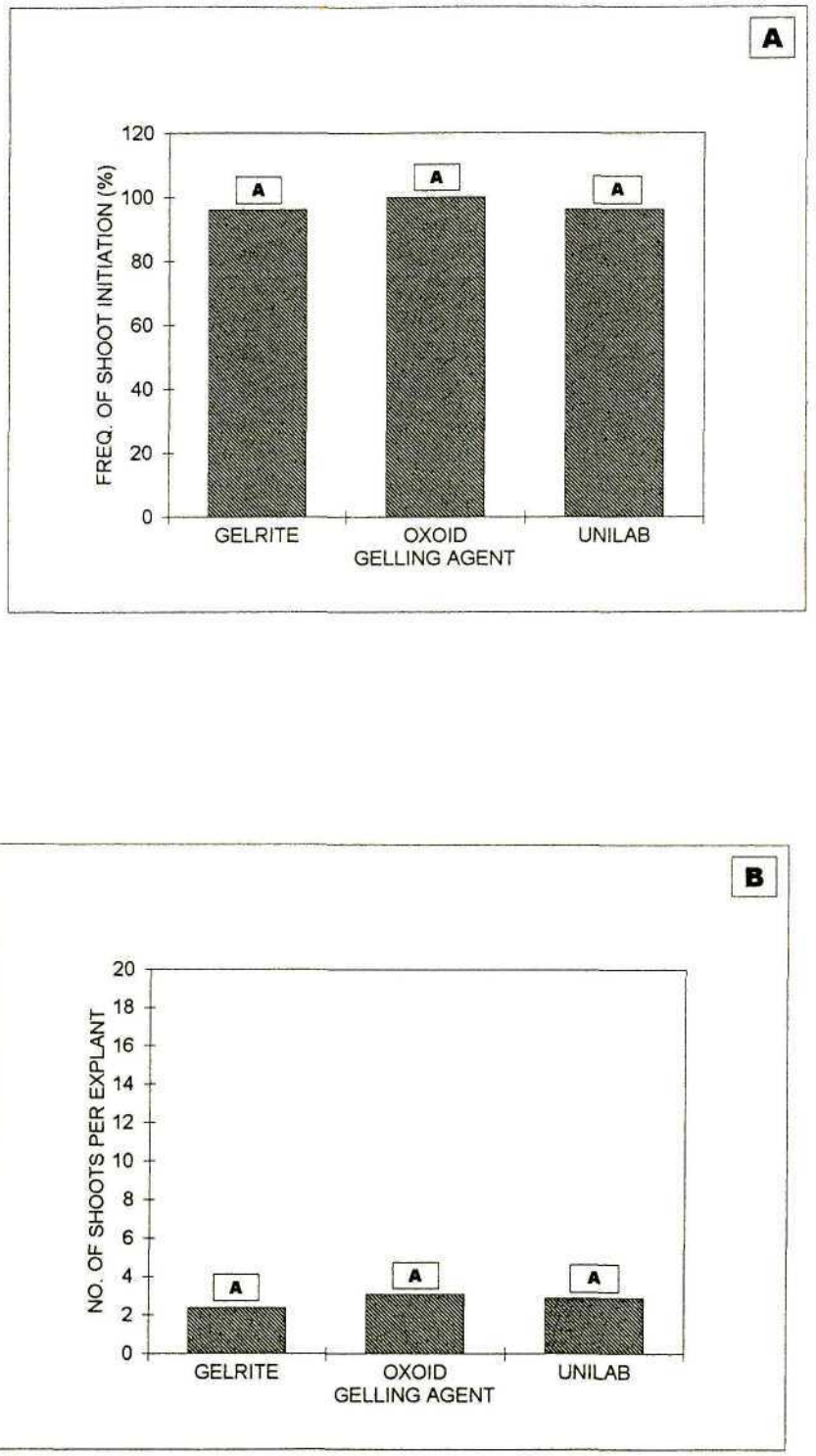


Fig. 23: The frequency of shoot initiation (A) and average number of shoots initiated per bulb explant (B) of *Scilla natalensis* Form A after 8 weeks on media solidified with various gelling agents. Bars with the same letters are not significantly different at P=0.05.

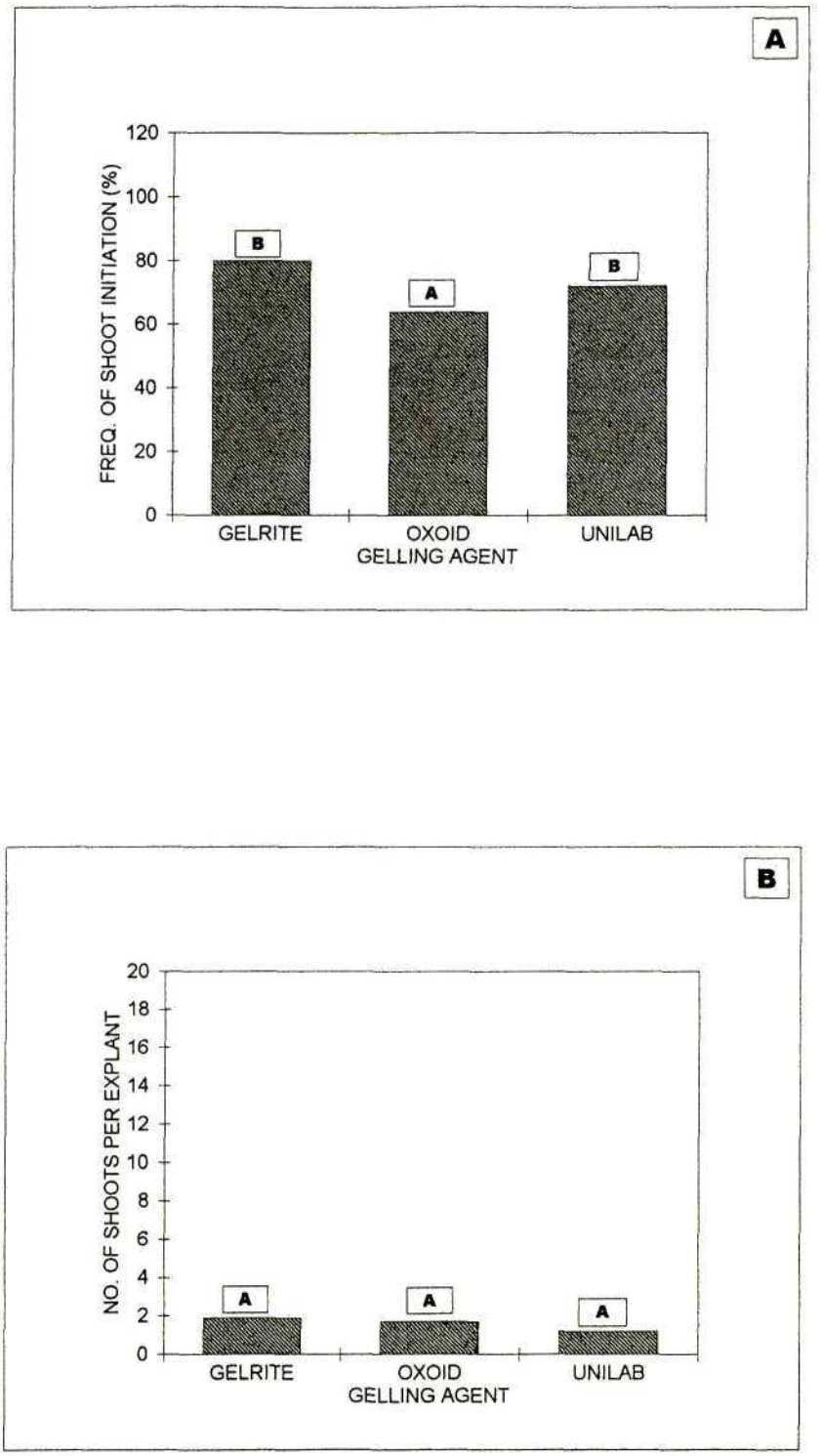
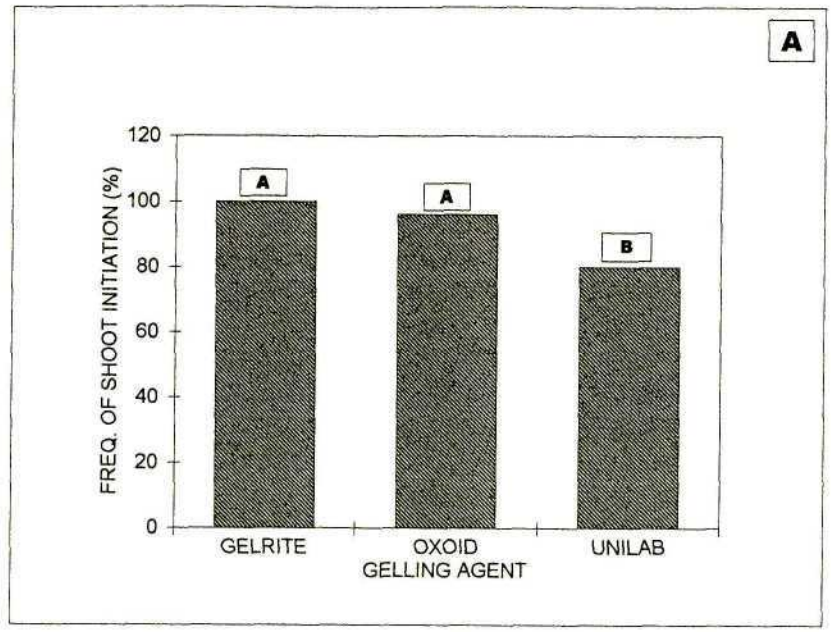


Fig. 24: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form A after 8 weeks on media solidified with various gelling agents. Bars with the same letters are not significantly different at P=0.05.



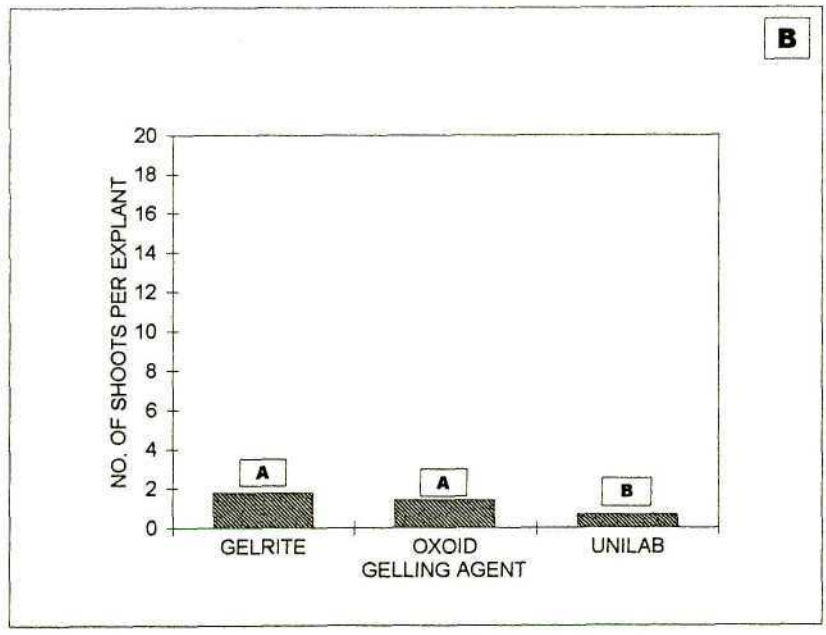


Fig. 25: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form B after 8 weeks on media solidified with various gelling agents. Bars with the same letters are not significantly different at P=0.05.

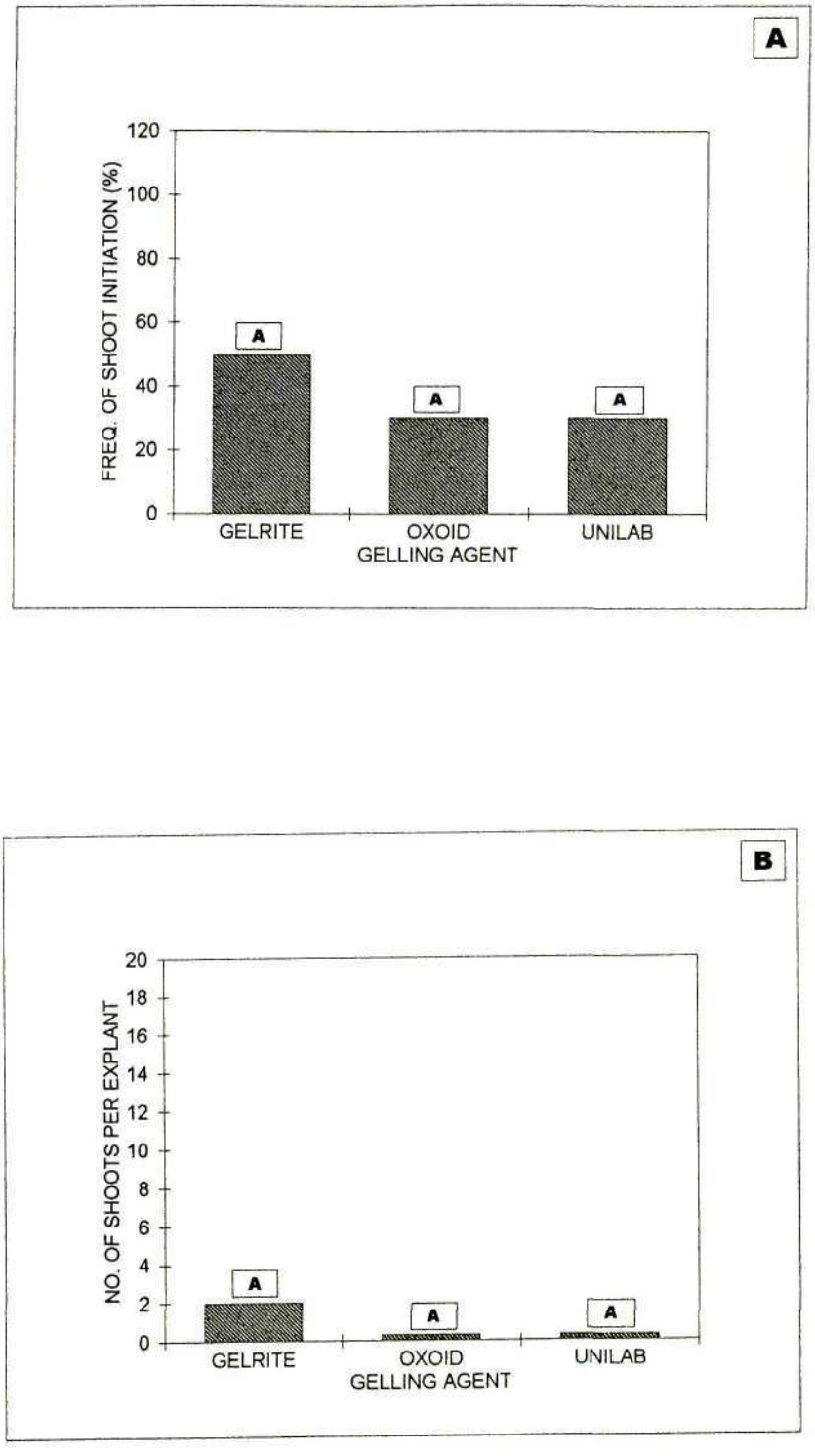


Fig. 26: The frequency of shoot initiation (A) and average number of shoots initiated per leaf explant (B) of *Scilla natalensis* Form C after 8 weeks on media solidified with various gelling agents. Bars with the same letters are not significantly different at P=0.05.

2.3.6.4. Effect of gelling agents on adventitious shoot initiation in three forms of Scilla natalensis.

The frequency of shoot initiation for the three forms was influenced by the gelling agent, which also significantly influenced the mean number of shoots initiated (Table 6). Similarly, the gelling agent influenced the growth of tomato (JARAMILLO & SUMMERS, 1990), *Eucalyptus* (MACRAE & VAN STADEN, 1990) and sugarbeet (OWENS & WOZNIAK, 1991). This may be due to the effect of gelling agents on the physical properties of media. Gel-strength, which is influenced by the brand and concentration of gelling agent, influences adventitious shoot initiation and the frequency of hyperhydricity (DEBERGH, HARBAOUI & LEMEUR, 1981; BORNMAN & VOGELMANN, 1984; SELBY, LEE & HARVEY, 1989). Gel-strength also influences water availability, which is largely determined by the matric potential (tenacity with which water is held in the solid phase of the gel) and expressibility (ease with which water is expressed in response to the mechanical deformation of the gel by the explant) of the media (OWENS & WOZNIAK, 1991). Expressibility, which is inversely related to gel-concentration and gel-strength, is markedly different for Gelrite® and various brands of agar. Furthermore, Gelrite® has a substantially lower relative matric potential (less available water) than the various brands of agar. This influenced the production of callus, which was directly proportional to matric potential and indirectly proportional to gel concentration. Gelling agents also contain various impurities and salts, which decrease the water availability by lowering the osmotic potential (OWENS & WOZNIAK, 1991). Certain gelling agents such as Gelrite® contain significantly more calcium (3 to 85 times), magnesium (3 to 85 times), potassium (2 to 170 times) and phosphorous (2 to 15 times) than various brands of agar (ANONYMOUS, 1992). Certain brands of agar also contain high levels of trace elements (SCHOLTEN & PIERIK, 1998).

2.3.7. ROOT INITIATION AND ACCLIMATIZATION.

2.3.7.1. Root initiation and acclimatization of Form A.

Although some shoots produced roots spontaneously, the shoots were sub-cultured onto root initiation media containing various auxins (Table 12). The highest number of roots were produced on media containing 1 mg l^{-1} IAA. The addition of IAA promoted the formation of long roots with numerous root hairs. In contrast, the addition of IBA, promoted the formation of short, brown roots, while the addition of NAA promoted callus formation. After 5 weeks, the plantlets were transferred to the misthouse. Approximately 90 to 100 % of the plantlets were successfully acclimatized (Fig. 14a). After 18 months, these plantlets had developed bulbs with a diameter ± SE of 21.4 ± 0.4 mm. Several thousand plantlets can be produced rapidly. Approximately 35000 to 40000 shoots can be produced from a single shoot with 5 sub-cultures at 8-week intervals. This is due to the exponential increase in the number of shoots after each sub-culture, which is limited only by the capacity of the laboratory in terms of labour and space.

2.3.7.2. Root initiation and acclimatization of Form B.

The highest number of roots were produced on media containing 1 mg l^{-1} IBA (Table 12). The addition of IBA promoted the formation of long roots with few root hairs. In contrast, the addition of IAA promoted the formation of short roots with numerous root hairs. Large quantities of white callus were produced at the base of the shoots. The addition of NAA, however, inhibited the formation of roots but promoted callus formation. After 5 weeks, the plantlets were transferred to the misthouse. Approximately 48 to 56 % of the plantlets were successfully acclimatized. Acclimatization, however, can be improved by the application of seaweed concentrate (LINDSEY, JÄGER & VAN STADEN, 1998). Approximately 700 to 1500 shoots can be produced from a single shoot with 5 sub-cultures at 8-week intervals. This is considerably lower than for Form A. This is due to the limited availability of secondary explants, which is a function of the shoot size, the slower growth rate and the lower replate ratio (mean number of shoots initiated per explant). This is reduced even further by poor acclimatization.

2.3.7.3. Root initiation and acclimatization of Form C.

The highest number of roots were produced on media containing 1 mg l^{-1} NAA (Table 12). The addition of NAA promoted the formation of long roots with numerous root hairs. Limited callus was formed at the base of the shoots. In contrast, the addition of IAA or IBA promoted the formation of fewer, shorter roots. The shoot bases were often black. After 15 weeks, the plantlets were transferred to the misthouse. Only 28 to 56 % of the plantlets were successfully acclimatized. Approximately 30 to 60 shoots can be produced from single shoot with 5 sub-cultures at 8-week intervals. This is reduced further by the extremely poor acclimatization. The small size of the bulb and the limited number of seed produced, which limits conventional propagation, may render this protocol economically viable, particularly if acclimatization is improved.

Table 12: The mean number of roots initiated per shoot on media containing various auxins.

AUXIN (1 mg l^{-1})	NO. OF ROOTS PER SHOOT ± SE		
	FORM A	FORM B	FORM C
IAA	4.6±0.8	2.8±0.6	1.1±0.4
IBA	4.2±0.7	4.6±0.7	1.7±0.5
NAA	1.6±0.5	0.8±0.2	3.5±0.7

2.4. CONCLUSIONS.

Continuous culture systems were established for the three forms of *Scilla natalensis*. The efficiency of the systems was strongly influenced by genetic factors, viz the form and epigenetic factors, viz the explant type, carbohydrate source, plant growth regulators and gelling agents.

- The form, Form A, Form B or Form C respectively, influenced shoot initiation with the larger forms generally producing more shoots than the smaller forms. The data confirmed that the three forms are significantly different in terms of their physiological response to carbohydrates, plant growth regulators and gelling agents *in vitro,* which may be due to the ability of the forms to metabolize the exogenous plant growth regulators or due to the availability of the exogenous plant growth regulators. Since the effect of form could not be compensated for by the addition of either carbohydrates, plant growth regulators or gelling agents, this may provide some support for the reinstatement of these forms as three species, *Scilla natalensis* Planch., *S. kraussii* Bak. and *S. dracomontana* Hilliard & Burtt.
- The explant type, that is bulb or leaf explants respectively, significantly influenced shoot initiation. Leaf explants generally produced more shoots than bulb explants. This may have been influenced by other factors such as the age, orientation (apolar or polar), position (distal or proximal), polarity, shape and size of the explants.
- The carbohydrate source significantly influenced shoot initiation. The explants generally produced more shoots when cultured on media containing glucose or sucrose than on media containing fructose, lactose, maltose and particularly mannitol. The number of shoots initiated, however, was not significantly influenced by the interaction between explant type and carbohydrate source although bulb explants produced more shoots than leaf explants. This may be due to the higher levels of nutrient reserves in bulb explants than in leaf explants or due to the ability of the explants to utilize the carbohydrate source.
- The cytokinin, auxin and interaction between the cytokinin and auxin significantly influenced shoot initiation. The addition of kinetin or TDZ alone increased the

number of shoots initiated although the addition of IAA and particularly NAA alone reduced the number of shoots initiated. Optimal shoot initiation for Form A, Form B and Form C occurred on media containing 1 to 2 mg l^{-1} kinetin and 1 to 2 mg l^{-1} IAA.

- The gelling agent also influenced the number of shoots initiated with media solidified with Gelrite® producing more shoots than media solidified with Oxoid or Unilab agar. The number of shoots initiated, however, was not significantly influenced by the interaction between the form and gelling agent or the interaction between explant type and gelling agent.
- Shoots were successfully rooted on media containing IAA, IBA or NAA. This was influenced by the form, Form A, Form B or Form C respectively. The plantlets were then acclimatised.

Continuous culture systems can be used to produce large quantities of plantlets. *In vitro* propagation, therefore provides a rapid means of propagating selected chemotypes or cultivars, serving both conservation and commercial interests. This may alleviate pressures on natural resources and provide an alternative source of high quality plants for the burgeoning medicinal plant market.

CHAPTER THREE

EFFECT OF CARBOHYDRATES AND ACTIVATED CHARCOAL ON THE GROWTH OF SHOOTS.

3.1. INTRODUCTION.

The physical properties of media such as gel-strength influences the growth of plants *in vitro* (DEBERGH, 1983; BORNMAN & VOGELMANN, 1984; SELBY, LEE & HARVEY, 1989; JARAMILLO & SUMMERS, 1990; KLIMASZEWSKA & SMITH, 1997). Few workers, however, have determined the effect of carbohydrates, activated charcoal (AC), gelling agents and other supplements on the physical properties of media and consequently, the availability of nutrients, plant growth regulators and water. Thus, the effects of carbohydrates, activated charcoal and gelling agents on the growth of plants and the physical properties of media are discussed below.

3.1.1.1. Carbohydrates.

Few plants are capable of meeting their total carbohydrate demands *in vitro*. It is usually necessary to incorporate a carbon source into the media. The carbon source most widely used in cultures is sucrose. Sucrose, which is a disaccharide comprised of glucose and fructose, supports a wide variety of plants in culture. Other carbon sources also used in cultures include various monosaccharides, disaccharides and trisaccharides such as glucose, maltose and raffinose respectively (GEORGE & SHERRINGTON, 1993). These carbon sources, which are usually more expensive than sucrose, are seldom used unless they result in clear-cut benefits in terms of rate or type of growth response. Some workers showed that corn syrup, which is comprised of glucose and maltose, improved androgenesis, embryogenesis and secondary metabolite production in poppy, tobacco and wild carrot (KINNERSLEY & HENDERSON, 1988), while others showed that glucose, fructose and sucrose influenced embryogenesis in asparagus (LEVI & SINK, 1990 & 1992).

Although several workers have determined the effect of carbohydrates on osmotic potential (KIMBALL, BEVERSDORF & BINGHAM, 1975; BROWN, LEUNG & THORPE, 1979), water potential (DEBERGH, HARBAOURI & LEMEUR, 1981), and quantity and quality of breakdown products (RÉDEI, 1973a & b; 1974) on media, few have determined the effect of carbohydrates on the pH, electrical conductivity (EC) and gel-strength of media.

3.1.1.2. Activated charcoal.

Activated charcoal is widely used as an adsorbing or decolourizing compound (WANG & HUANG, 1976; FRIDBORG, PEDERSÉN, LANDSTRÖM & ERIKKSON, 1978; TAKAYAMA & MISAWA, 1980). Activated charcoal is produced by the destructive distillation of bones, peat, wood and other carbonaceous matter, and the subsequent oxidation of the product in a stream of carbon dioxide. This results in activated charcoal with superior adsorbing properties, which are due to large specific adsorption areas (600 to 2000 $m^2 g^{-1}$) and pore distributions (10 to 50 µM). The adsorbing properties also are determined by the grade, purity, and density of the charcoal, and the pH, inorganic salts and temperature of the media (HALHOULI, DARWISH & AL-SHOON, 1995; PAN & VAN STADEN, 1998).

Activated charcoal is often added to media (WANG & HUANG, 1976; FRIDBORG, PEDERSÉN, LANDSTRÖM & ERIKKSON, 1978; TAKAYAMA & MISAWA, 1980). In *Phalaenopsis*, the addition of charcoal improved shoot and root growth by reducing the frequency of stunted shoots and negating ageotropic root growth. The addition of charcoal also prevented the browning of the medium by substances released from the cultures (WANG & HUANG, 1976). In *Lilium*, the addition of charcoal increased the weight of bulblets, but decreased the frequency of bulblet formation (TAKAYAMA & MISAWA, 1980).

The beneficial effects of charcoal on the growth and development of various plants have been attributed to: the creation of a dark environment in the media, thus facilitating the accumulation of photosensitive auxins or co-factors (PAN & VAN STADEN, 1998); the adsorption of inhibitory compounds such as phenolics (WEATHERHEAD, BURDON &

HENSHAW, 1978) and 5-(hydroxymethyl)-2-furaldehyde (HMF)(RÉDEI, 1974); the adsorption of plant growth regulators such as cytokinins, auxins and ethylene (WEATHERHEAD, BURDON & HENSHAW, 1978; EBERT & TAYLOR, 1990; EBERT, TAYLOR & BLAKE, 1993); and the release of naturally present- or previously adsorbed-compounds into the media (PAN & VAN STADEN, 1998). Activated charcoal preferentially adsorbs moderately polar compounds rather than highly polar or apolar compounds. Highly polar and water-soluble compounds such as glucose, inositol, mannitol and sorbitol may not be adsorbed, while aromatic compounds such as phenolics, cytokinins and auxins are readily adsorbed (PAN & VAN STADEN, 1998). The rate of adsorption is influenced by the temperature and pH of the media, increasing with high temperatures or low pH values (EBERT & TAYLOR, 1990). The rate of desorption from activated charcoal is very slow. This is dependant upon the grade of charcoal, the temperature and pH of the solution, and the type of solvent. It has been suggested that desorbed compounds may be available for active uptake by the cultures (PAN & VAN STADEN, 1998).

3.1.1.3. Gelling agents.

The gelling agent, agar is widely used as a support matrix in cultures. Agar is a water-soluble polysaccharide derived from the Rhodophyta particularly *Gelidium* and *Gracilaria* species. This polysaccharide comprises repeating units of β-D-galactose and α-L-anhydrogalactose, which are frequently substituted with methyl/sulphyl esters or pyruvate ketal groups (LAHAYE & ROCHAS, 1991; WILSON & CRITCHLEY, 1998). The position and quantity of these substitutions influence the chemical and physical properties of the agar. These chemical and physical properties of agar also are influenced by the algal species (WILSON & CRITCHLEY, 1998), as well as the seasons(WHYTE, ENGLAR, SAUNDERS & LINDSAY, 1981), the nutrients (CRAIGIE, WEN & VAN DER MEER, 1984) and environmental conditions of the algae (CHRISTIAEN, STADLER, ONDARZA & VERDUS, 1987; CHRISTELLER & LAING, 1989).

Although the gelling agent influences the growth response of cultures (BORNMAN & VOGELMANN, 1984; JARAMILLO & SUMMERS, 1990; MACRAE & VAN STADEN,

1990; SCHOLTEN & PIERIK, 1998), the availability and cost of the gelling agent usually determines the gelling agent used (DEBERGH, 1983). The cost of agar, which ranges from R500 to R650 per kilogram, has led to the use of alternative support matrices including corn-starch (HENDERSON & KINNERSLEY, 1988; ZIMMERMAN, BHARDWAJ & FORDHAM, 1995), sago, isubgol (BHATTACHARYA, DEY & BHATTACHARYYA, 1994), carrageenan (LINES, 1977) and the gellan gum, PS-60 (now known as Gelrite®)(KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982).

The gelling agent used in this study was the gellan gum, Gelrite®, which is a water-soluble polysaccharide derived from *Pseudomonas elodea*. This polysaccharide is comprised of glucuronate, rhamnose and cellobiose molecules, as well as significant quantities of calcium, magnesium, potassium and sodium (GEORGE & SHERRINGTON, 1993). This gelling agent costs approximately R800 per kilogram but is used at lower concentrations than agar rendering it more cost-effective. Although several workers have determined the effects of agar on physical properties of media (BROWN, LEUNG & THORPE, 1979; DEBERGH, 1983; DEBERGH, HARBAOUI & LEMEUR, 1983; SKIRVIN, CHU, MANN, YOUNG, SULLIVAN & FERMANIAN, 1986; SARMA, MAESATO, HARA & SONODA, 1990; WETZSTEIN, KIM & SOMMER, 1994), few have determined the effects of Gelrite® on physical properties of media (BORNMAN & VOGELMANN, 1984; OWEN & WOZNIAK, 1991).

3.2. AIMS.

The aims of this part of the study were:

1) to determine the effect of carbohydrates and activated charcoal on the growth of shoots of *Scilla natalensis* Planch. *sensu stricto* ;
2) to determine the effect of carbohydrates, activated charcoal and the gelling agent, Gelrite® on the physical properties of media; and
3) to determine whether the source and concentration of carbohydrates or the physical properties of the media influenced the growth of shoots of *Scilla natalensis* Planch. *sensu stricto*.

3.3. MATERIALS & METHODS

3.3.1. EFFECT OF CARBOHYDRATES AND ACTIVATED CHARCOAL ON THE GROWTH OF SHOOTS.

The basal medium (BM) consisted of Murashige & Skoog (MS)(1962) salts, which was modified by the addition of 20 mg l^{-1} glycine. The following BM and supplements were tested:

1 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} glucose, 2 g l^{-1} Gelrite®;
2 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} fructose, 2 g l^{-1} Gelrite®;
3 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} sucrose, 2 g l^{-1} Gelrite®;
4 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} mannitol, 2 g l^{-1} Gelrite®;
5 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} sucrose, 5 g l^{-1} Sigma activated charcoal (AC), 2 g l^{-1} Gelrite®

Media containing AC were shaken continuously for 30 minutes before the pH was adjusted. The pH of the media was then adjusted to 5.8 with 1M KOH or 1N HCl. The media was heated to dissolve the gelling agent and then the media were then autoclaved at 121 °C for 20 minutes (103.4 kPa). The medium was heated (to dissolve the gelling agent), hot-dispensed into test-tubes (100 x 25 mm) and capped with either metal or plastic lids, and then autoclaved at 121 °C for 20 minutes (103.4 kPa).

Sterile 8-week old shoots of *S. natalensis* Planch. *sensu stricto* (20 to 30 mm tall) were transferred aseptically onto the media. The cultures were incubated under 16:8 light/dark cycles (45 µmol $m^{-2}s^{-1}$) provided by a mixture of cool white fluorescent tubes and incandescent lamps and maintained at 25 ± 2 °C for 8 weeks. The shoots/plantlets were then destructively harvested. The fresh weight of the shoots/plantlets was recorded and subjected to ANOVA using Minitab 10.51 Xtra. Fifty replicates were used per medium tested. The frequency and number of roots initiated were also recorded.

3.3.2. EFFECT OF CARBOHYDRATES, ACTIVATED CHARCOAL AND GELLING AGENTS ON THE PHYSICAL PROPERTIES OF MEDIA.

3.3.2.1. Effect of activated charcoal on the pre-autoclaving pH of media.

An Orion Research Digital Ionalyzer 501 was used to measure the pre-autoclaving pH of the following BM and supplements:

1) BM, 20 g l^{-1} sucrose;
2 a-c) BM, 5, 10 or 15 g l^{-1} Sigma AC (Sigma 6289); and
3 a-c) BM, 5, 10 or 15 g l^{-1} BDH AC (BDH 330324E).

The pH of the media was adjusted to 5.8 with 1 M KOH or 1 N HCl immediately after preparation. Media were then stirred continuously using a magnetic stirrer. The changes in the pre-autoclaving pH of the media were recorded for a period of 100 minutes.

3.3.2.2. Effect of activated charcoal on the adsorption/desorption of ions from media.

A Varian-1275 Atomic Absorption Spectrophotometer was used to determine whether activated charcoal (AC) adsorbed calcium, magnesium, potassium or sodium from the media. The following standards were prepared from stock solutions of calcium nitrate, magnesium nitrate, potassium nitrate and sodium nitrate respectively:

1) 1 to 5 mg l^{-1} calcium and 1000 µg ml^{-1} lanthanum;
2) 0.1 to 1 mg l^{-1} magnesium and 1000 µg ml^{-1} lanthanum;
3) 1 to 5 mg l^{-1} potassium and 1000 µg ml^{-1} caesium chloride; and
4) 1 to 5 mg l^{-1} sodium and 2000 µg ml^{-1} potassium nitrate.

Releasing agenst such as lanthanum, potassium nitrate and caesium chloride were used to suppress ionization of the analyte in the air-acetylene flame (ANONYMOUS, 1979) . The following BM and supplements were tested:

1) BM, 20 g l^{-1} sucrose (control);
2) BM, 20 g l^{-1} l sucrose and 5 g l^{-1} Sigma AC;
3) BM, 20 g l^{-1} sucrose and 5 g l^{-1} BDH AC;
4) Distilled water, 5 g l^{-1} Sigma AC; and
5) Distilled water, 5 g l^{-1} BDH AC.

Media 2, 3, 4 and 5 containing AC were shaken continuously for 30 minutes. The pH of the media was adjusted to 5.8 with 1 M KOH or 1 N HCl. Media were autoclaved at 121 °C for 20 minutes (1 03.4 kPa). Media 2, 3, 4 and 5 containing AC were subsequently filtered through a Sartorious filter-holder fitted with a Millipore non-solvent filter (0.22µm). Standard working conditions for the atomic spectrophotometer were used for determining the calcium, magnesium, potassium and sodium concentrations in the liquid fraction of the treatments (Appendix 1). Five readings were recorded per treatment. The data was subjected to a single-factor ANOVA using Statgraphics Version 5.0.

3.3.2.3 Effect of sterilization method on the breakdown products of carbohydrates.

A thin-layer chromatography (TLC) system was used to compare the effects of autoclaving and filter-sterilization on glucose, fructose and sucrose with or without AC in the media. The following standards were prepared:

1) 10 mg ml^{-1} D(+)-glucose monohydrate;
2) 10 mg ml^{-1} D(-)-fructose;
3) 10 mg ml^{-1} sucrose;
4) double spot of 10 mg ml^{-1} D(+)-glucose monohydrate and 10 mg ml^{-1} D(-)-fructose; and
5) triple spot of 10 mg ml^{-1} D(+)-glucose monohydrate, 10 mg ml^{-1} D(-)-fructose and 10 mg ml^{-1} sucrose.

The following BM and supplements were tested:

1) BM, 20 g l^{-1} glucose (filter-sterilized);
2) BM, 20 g l^{-1} glucose (autoclaved);
3) BM, 20 g l^{-1} fructose (filter-sterilized);
4) BM, 20 g l^{-1} fructose (autoclaved);
5) BM, 20 g l^{-1} sucrose (filter-sterilized);
6) BM, 20 g l^{-1} sucrose (autoclaved);
7) BM, 20 g l^{-1} sucrose and 5 g l^{-1} Sigma AC (autoclaved); and
8) BM, 20 g l^{-1} sucrose and 5 g l^{-1} Sigma AC (autoclaved).

Media 7 and 8 containing AC were shaken continuously for 30 minutes before the pH of the media was adjusted. The pH of media 1 to 7 was adjusted to 5.8, while the pH of medium 8 was adjusted to 3.8 with 1M KOH or 1N HCl. Media 1, 3 and 5 were filter-sterilized through a Sartorious filter-holder fitted with a Millipore non-solvent filter (0.22µm), while media 2, 4, 6, 7 and 8 were autoclaved at 121 °C for 20 minutes (103.4 kPa). Media 7 and 8 containing AC were then filtered to remove the AC. Media were diluted resulting in final concentrations of 10 mg ml^{-1} of glucose, fructose or sucrose respectively. Standards and media (2 µl) were spotted onto a 20 x 20 cm TLC plate, which was coated with silica gel (Merck Silica Gel $60F_{254}$). The plate was run ascendingly in a 4 l tank containing 95 ml of n-butanol-acetic acid-ether-water (9:6:3:1) and developed for five hours. The plate was removed from the tank, dried and then flooded with anisaldehyde reagent (0.5 ml of anisaldehyde, 10 ml glacial acetic acid, 85 ml methanol, 5 ml of concentrated sulphuric acid) and then heated at 110 °C for 10 minutes. The spots were recorded under visible light and their R_f values were calculated.

3.3.2.4. Effect of carbohydrates, activated charcoal and gelling agents on post-autoclaving pH, electrical conductivity and gel-strength of media.

The following BM and supplements were tested:

1 a-g) BM, 20 g l^{-1} sucrose, 2 g l^{-1} Gelrite®;
2 a-f) BM (0, 20, 40, 60, 80 or 100 %), 20 g l^{-1} sucrose, 2 g l^{-1} Gelrite®;
3 a-g) BM, 20 g l^{-1} sucrose, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 or 4.0 g l^{-1} Gelrite®;
4 a-b) BM, 20 g l^{-1} sucrose, 2 g l^{-1} Gelrite® or 10 g l^{-1} ACE Agar (Associated Chemical Enterprises Agar 9465/826);
5 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} glucose, 2 g.l^{-1} Gelrite®;
6 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} fructose, 2 g l^{-1} Gelrite®;
7 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} sucrose, 2 g l^{-1} Gelrite®;
8 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} mannitol, 2 g l^{-1} Gelrite®;
9 a-f) BM, 0, 20, 40, 60, 80 or 100 g l^{-1} sucrose, 5 g l^{-1} Sigma AC, 2 g l^{-1} Gelrite®;
10 a-i) BM, 20 g l^{-1} sucrose, 0, 5, 10, 15 or 20 g l^{-1} BDH AC or Sigma AC, 2 g l^{-1} Gelrite®; and
11 a-l) BM, glucose and fructose ratios (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) with final carbohydrate concentrations of 20 and 40 g l^{-1} respectively, 2 gl^{-1} Gelrite®.

Media 9 and 10 containing AC were shaken continuously for 30 minutes before the pH was adjusted. The pH of media 2 to 11 was adjusted to 5.8, while the pH of media 1 was adjusted to a range of pH values (3.8, 4.4, 4.8, 5.4, 5.8, 7.8 and 9.8) with 1M KOH or 1N HCl. Media were heated (to dissolve the gelling agent) and hot-dispensed into test-tubes (100 x 25 mm). Media were then autoclaved at 121 °C for 20 minutes (103.4 kPa). Media 4, however, were subjected to an additional two autoclaving cycles.

The gel-strength of the media was measured using a custom-built gel-strength meter that comprised a plunger (diam. 2 cm^2), a gauge (0 to 10 mm) and a series of weights (10 to 125 g)(Fig. 27). The weights were applied sequentially to the plunger for 10 second intervals. The displacement of the media was recorded. These values were converted into the amount of joules (J) required to displace 10 mm of media using the equation for work (1J ≡ 1 kg m^2 s^{-2}). Five readings were recorded per medium.

The media were then homogenized and the post-autoclaving pH measured using an Orion Research Digital Ionalyzer 501. The EC was measured using a CDM 80 Conductivity meter. Five post-autoclaving pH readings and three EC readings were recorded per media. Regression lines were plotted for the means using Minitab 10.51 Xtra.

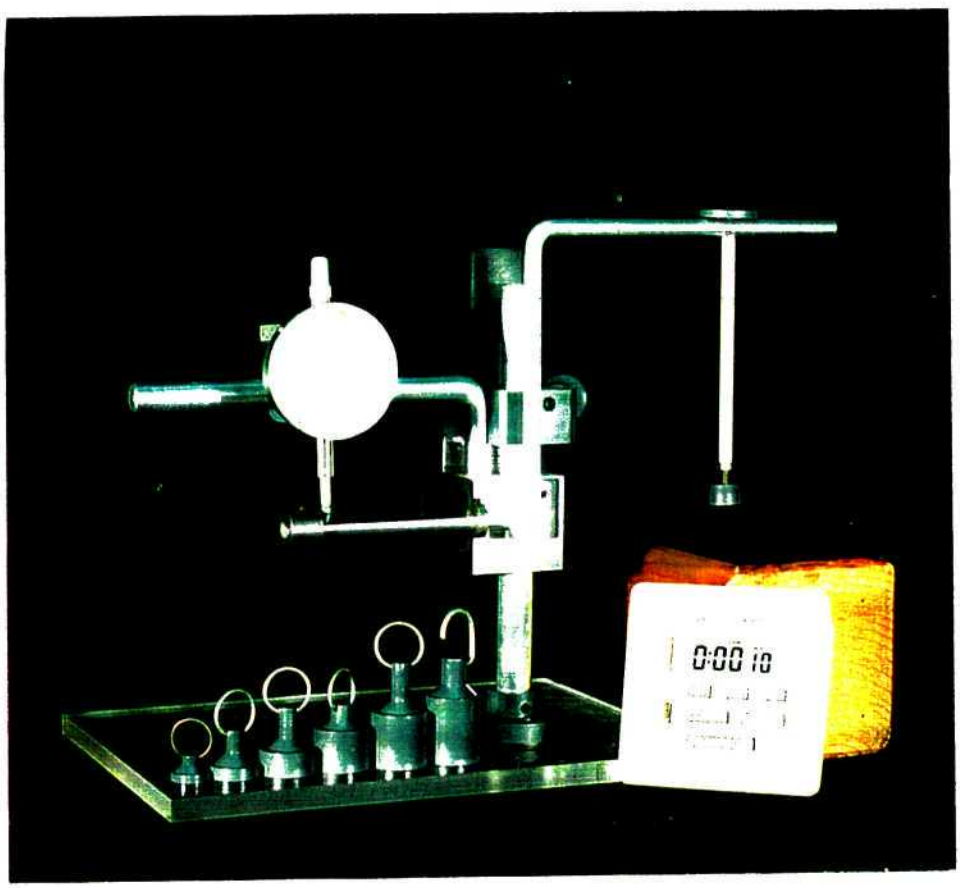


Fig. 27: Custom-built gel-strength meter that comprised a plunger (diam. 2 cm^2), a gauge (0 to 10 mm) and a series of weights (10 to 125 g).

3.4. RESULTS & DISCUSSION.

3.4.1. EFFECT OF CARBOHYDRATES AND ACTIVATED CHARCOAL ON THE GROWTH OF SHOOTS.

In the absence of carbohydrates (controls), the shoots were short with spindly leaves (< 50 mm) and occasionally produced short roots (< 10 mm)(Figs. 28a) The shoot weight (FW) was low, ranging from 0.08 to 0.10 g (Figs. 29-33). These shoots may have been unable to fully meet their total carbon requirements by photosynthesis. This may have been influenced by environmental factors such as gas composition within the containers and light quality (DESJARDINS, 1995).

When media were supplemented with glucose, the shoot weight (FW) was higher than the controls ranging from 0.19 to 0.54 g (Fig. 29). This was significant for concentrations of glucose ranging from 20 to 80 g l^{-1}. The shoot weight decreased with increasing glucose concentration. At low glucose concentrations, the shoots were long (> 50 mm) with broad leaves, small bulbs, and many short to medium roots (10 to 20 mm). At higher glucose concentrations, however, the shoots were robust and short (< 50 mm) with narrow and often red-pigmented leaves, medium-sized bulbs, and few short roots (< 10 mm). The frequency of red-pigmented leaves increased from approximately 4 to 26 % with increasing glucose concentration. The frequency and number of roots initiated also increased with increasing glucose concentration.

When fructose was substituted for glucose, the shoot weight (FW) was higher than the controls, ranging from 0.13 to 0.29 g (Fig. 30). This was significant for concentrations of fructose ranging from 40 to 80 g l^{-1}. At low fructose concentrations (20 to 40 g l^{-1}), the shoot weight increased. The shoots were long (> 50 mm) with broad leaves, small bulbs, and few short to medium length roots (10 to 20 mm)(Fig. 28b). At intermediate and high fructose concentrations (40 to 100 g l^{-1}), the shoot weight decreased. The shoots were robust and short (< 50 mm) with narrow, sometimes deformed leaves, large bulbs, and few short to medium roots (10 to 20 mm)(Figs. 28c & d). At high fructose concentrations, the roots were stunted and brown (< 10 mm). This may have been due to the breakdown of fructose into HMF, hydroxy-acetal furan, levulinic acid and various other minor compounds (RÉDEI, 1974). These compounds have been implicated in the

"fructose effect or syndrome" of plantlets, which are characterized by chlorosis and root inhibition (RÉDEI, 1974).

When sucrose was substituted for fructose, the shoot weight (FW) was higher than the controls, ranging from 0.24 to 0.47 g (Fig. 31). This was significant for concentrations of sucrose ranging from 20 to 100 g l^{-1}. At low sucrose concentrations (20 to 40 g l^{-1}), the shoot weight increased slightly. The shoots were short (< 50 mm) with many broad leaves, small bulbs, and few short to medium roots (10 to 20 mm)(Fig. 28e). Some shoots were vitrified. The frequency of hyperhydricity, however, decreased with increasing sucrose concentration. At intermediate and high sucrose concentrations (40 to 100 g l^{-1}), the shoot weight decreased significantly. The shoots were robust and long (> 50 mm) with narrow and often red-pigmented leaves, large bulbs, and many long, thick roots (> 30 mm)(Figs. 28f & g). The increase in bulb size may be due to increased respiration and starch synthesis, which increases with increasing sucrose concentration (DESJARDINS, 1995). The frequency of red-pigmented leaves increased from 6 to 32 % with increasing sucrose concentration. This may be due to the increased availability of substrate for the glycolysis pathway, and the subsequent increased production of erythrose-4-phosphate, which is essential for anthocyanin production (SALISBURY & ROSS, 1992). Nitrogen and/or phosphorous deficiencies may also contribute to this effect (SALISBURY & ROSS, 1992) The frequency and number of roots initiated also increased with increasing sucrose concentration. Optimal shoot growth and development occurred on media containing glucose or sucrose (40 to 60 g l^{-1}).

When AC was used in combination with sucrose, the shoot weight (FW) was lower than for sucrose alone, ranging from 0.08 to 0.17 g (Fig. 32). In the absence of sucrose (AC alone), the shoots were achlorophyllous or necrotic. At low and intermediate sucrose concentrations (20 to 60 g l^{-1}), the shoot weight increased significantly. The shoots were long (> 50 mm) with many broad leaves, poorly developed bulbs, and few, stunted roots (< 10 mm)(Fig. 28h). At high sucrose concentrations (60 to 100 g l^{-1}), the shoot weight decreased moderately. The shoots were robust and short (< 50 mm) with few, and occasionally red-pigmented leaves, small to medium bulbs, and few, severely stunted roots (< 5 mm)(Fig. 28i). This may be due to the release of naturally present compounds into the medium such as aluminium, copper and nickel into the media

(WEATHERHEAD, BURDON & HENSHAW, 1979). These may become toxic at certain pH values. The adsorption of certain essential compounds such as FeEDTA, pyridoxine, folic acid, thiamine and nicotinic acid (JOHANSSON, CALLEBERG & GEDIN, 1990), the hydrolysis of sucrose and subsequent decrease in the sucrose concentration (WANN, VEAZEY & KAPHAMMER, 1997), or the decrease in the pH of the media (DRUART & DE WULF, 1993; WANN, VEAZEY & KAPHAMMER, 1997) may also be contributing factors.

When mannitol was substituted for sucrose, the shoot weight (FW) was lower than the control, ranging from 0.30 to 0.70 g (Fig. 33). The shoot weight decreased with increasing mannitol concentration. This was significant for concentrations of mannitol ranging from 40 to 100 g l^{-1}. At low and intermediate mannitol concentrations (20 to 60 g l^{-1}), the shoots were short (< 50 mm) with broad, curly leaves, and very few roots (< 10 mm). At high mannitol concentrations (60 to 100 g l^{-1}), the shoots were achlorophyllous or necrotic. This may be due to the inability of the shoots to utilize mannitol as its sole carbon source. The modification of the physical properties of the media particularly the gel-strength and/or osmotic potential may also be contributing factors. This would influence the penetration of roots into the media and/or the availability of water, plant growth regulators and salts.

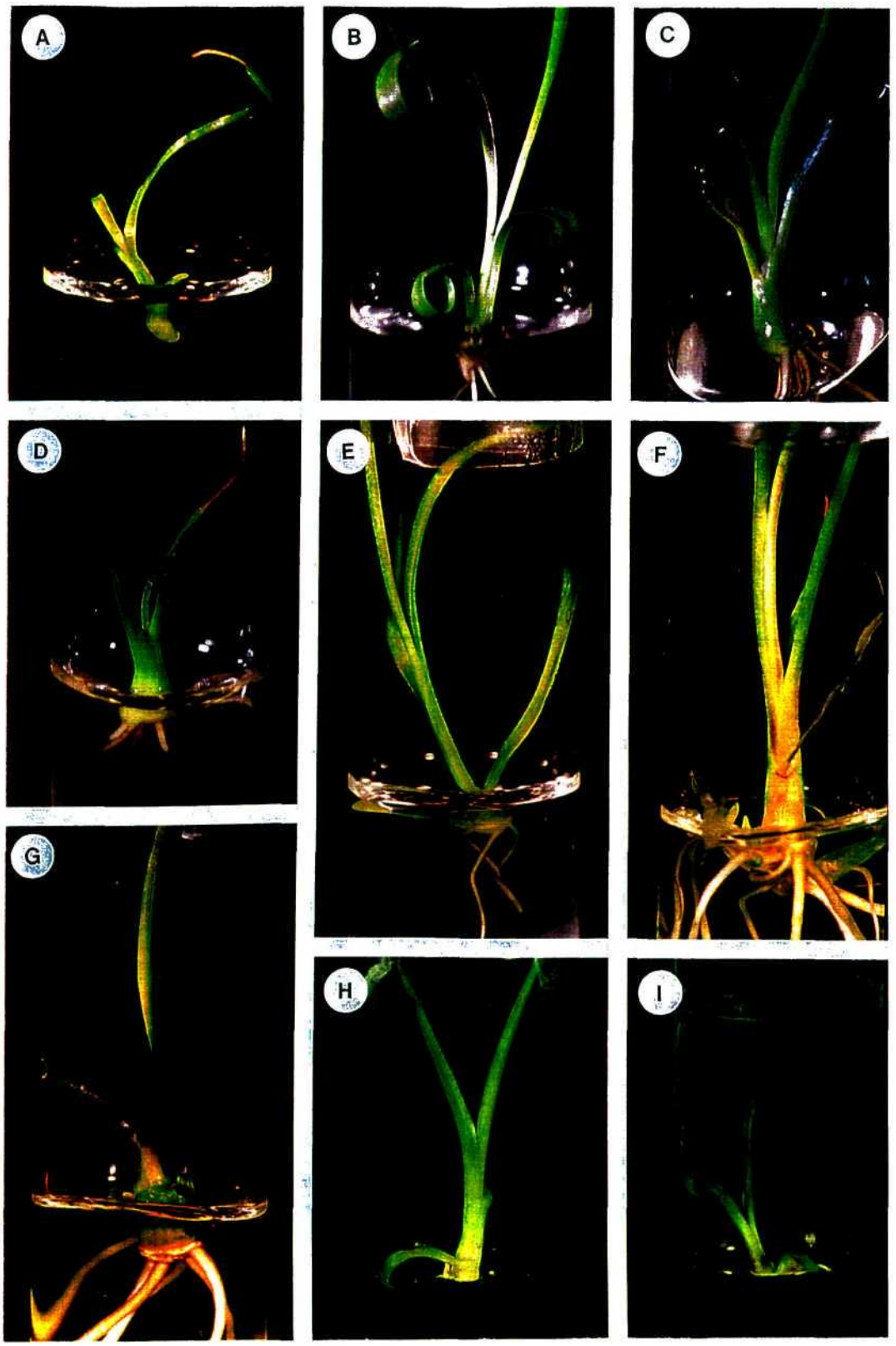


Fig. 28: Shoots of *Scilla natalensis* Form A cultured on media containing A: no carbohydrates; B: 20 g l^{-1} fructose; C: 60 g l^{-1} fructose; D: 100 g l^{-1} fructose (note the stunted roots); E: 20 g l^{-1} sucrose; F: 80 g l^{-1} sucrose; G: 100 g l^{-1} sucrose (note the red-pigmented leaves); H: 60 g l^{-1} sucrose in combination with AC; and I: 100 g l^{-1} sucrose in combination with AC for 8 weeks.

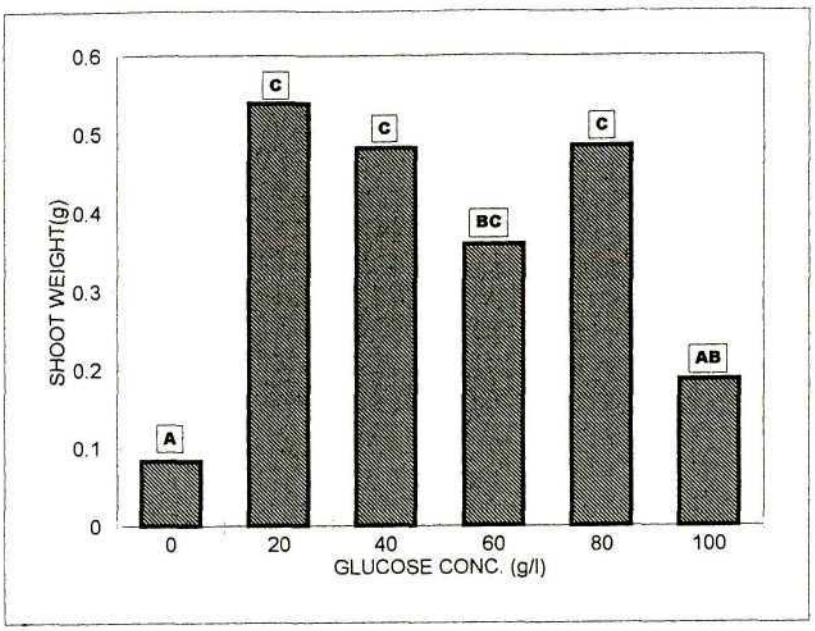


Fig. 29: The weight (FW) of shoots of *Scilla natalensis* Form A after 8 weeks on media containing various concentrations of glucose. Bars with the same letters are not significantly different at P=0.05.

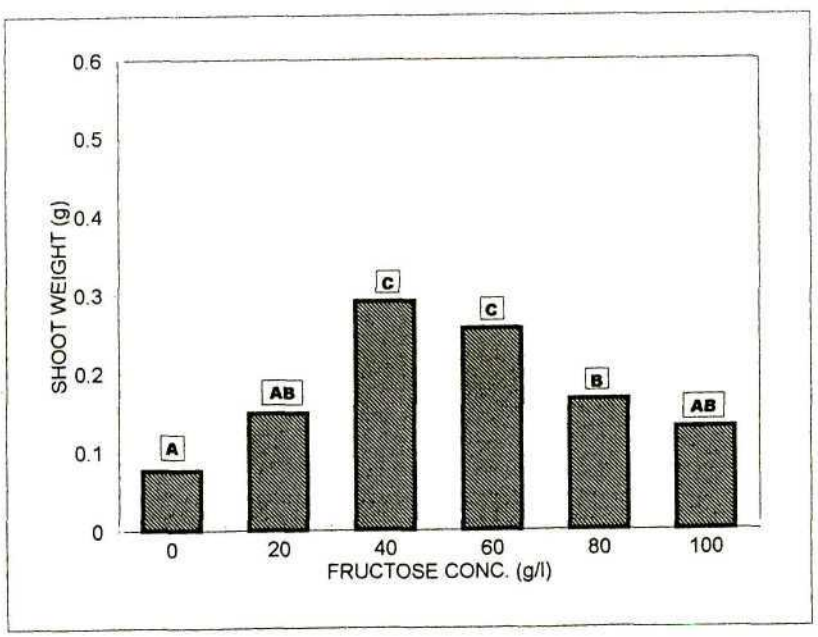


Fig. 30: The weight (FW) of shoots of *Scilla natalensis* Form A after 8 weeks on media containing various concentrations of fructose. Bars with the same letters are not significantly different at P=0.05.

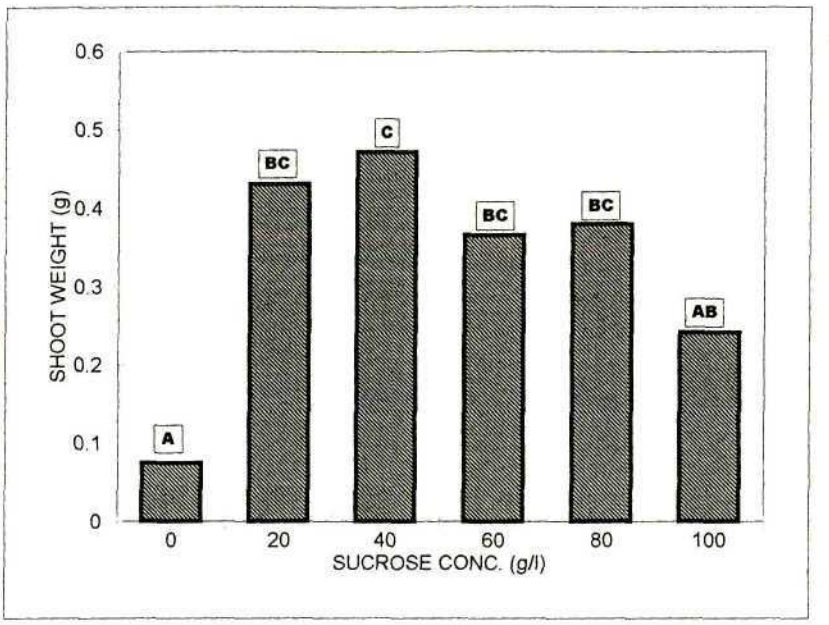


Fig. 31: The weight (FW) of shoots of *Scilla natalensis* Form A after 8 weeks on media containing various concentrations of sucrose. Bars with the same letters are not significantly different at P=0.05.

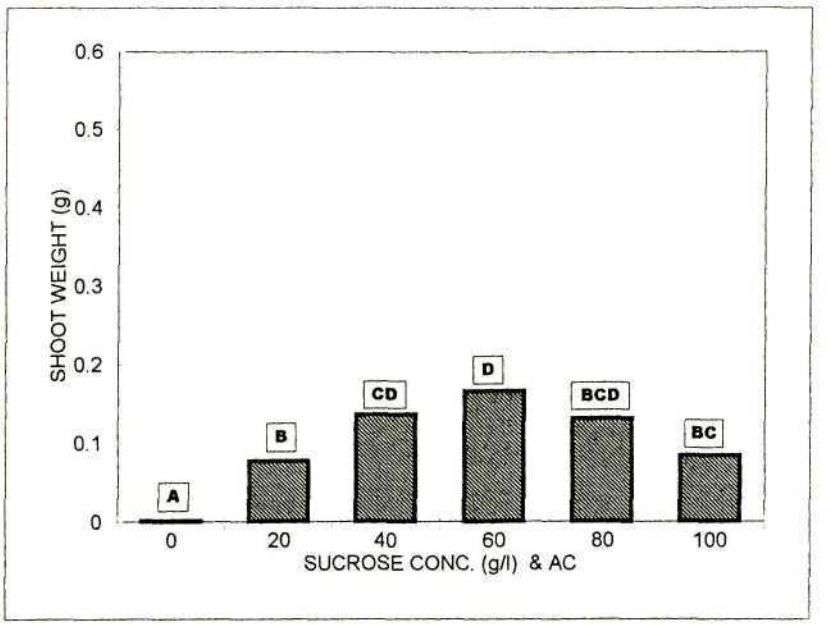


Fig. 32: The weight (FW) of shoots of *Scilla natalensis* Form A after 8 weeks on media containing various concentrations of sucrose in combination with AC. Bars with the same letters are not significantly different at P=0.05.

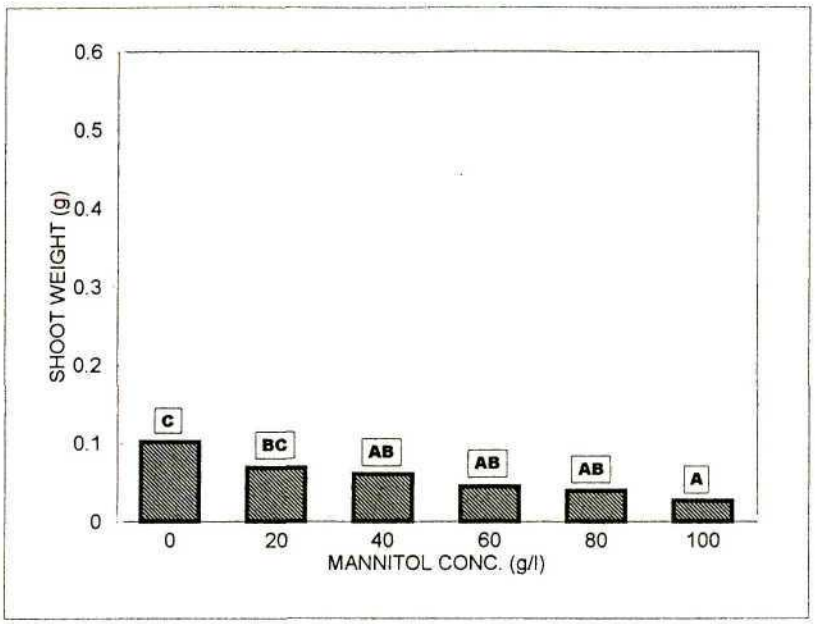


Fig. 33: The weight (FW) of shoots of *Scilla natalensis* Form A after 8 weeks on media containing various concentrations of mannitol. Bars with the same letters are not significantly different at P=0.05.

3.4.2. EFFECT OF CARBOHYDRATES, ACTIVATED CHARCOAL AND GELLING AGENTS ON THE PHYSICAL PROPERTIES OF MEDIA.

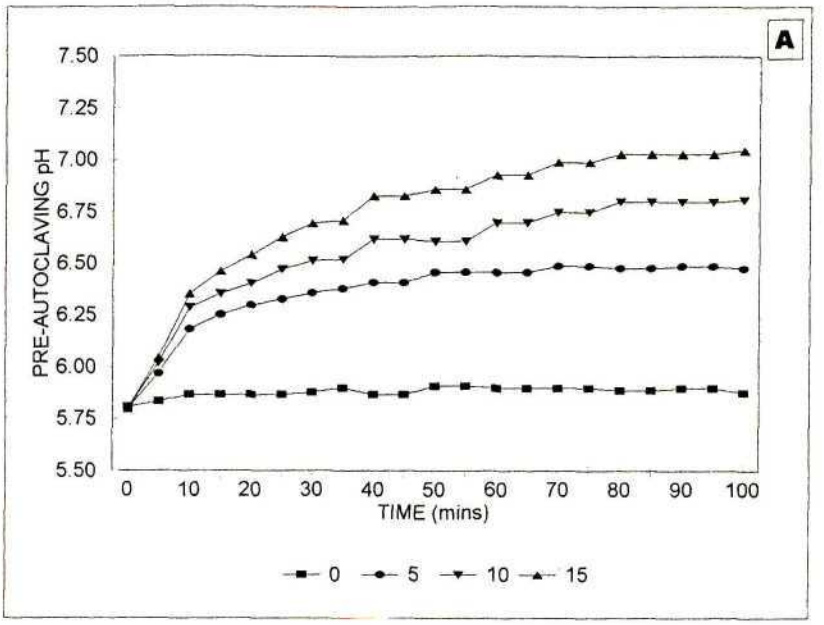
3.4.2.1. Effect of activated charcoal on the pre-autoclaving pH and adsorption/desorption of ions from media.

The pH of control media (no activated charcoal (AC)) remained constant, while the pH of media containing AC increased after the pH had been adjusted to 5.8 (Figs. 34a & b). The pH increased rapidly initially and then levelled-off after about 30 minutes. This was influenced by the brand of AC. The pH of the alkaline BDH AC increased more than that of the acid-washed Sigma AC. Thus, the pH of media containing 15 $g.l^{-1}$ BDH AC increased by 1.3 units from 5.8 to 7.1, while the pH of media containing 15 $g.l^{-1}$ Sigma AC increased by only 0.5 units from 5.8 to 6.3 after 100 minutes. This also was influenced by the concentration of AC increasing with increasing AC concentration. The preparation procedure of media containing AC significantly influences the pH (WANN, VEAZEY & KAPHAMMER, 1997). Thus, in subsequent experiments, the media were shaken/ equilibrated for 30 minutes before the pH was adjusted to 5.8.

Neither BDH nor Sigma AC adsorbed or released significant amounts of calcium, magnesium or potassium into the media (Figs. 35a, b, c & d). Both BDH and Sigma AC, however, released significant amounts of sodium into the media. This was confirmed by the positive (no AC) and negative controls (dH_2O and AC). In contrast, some workers reported that AC releases significant amounts of aluminium, copper and nickel into the media (WEATHERHEAD, BURDON & HENSHAW, 1979), while others have reported that AC releases significant amounts of potassium, nitrate and to some extent, nitrite, sulfate and sodium into the media (JOHANSSON, CALLEBERG & GEDIN, 1990). These differences may be due to the brand of AC and the different buffering capacities of the media (MS versus Nitsch)(WANN, VEAZEY & KAPHAMMER, 1997).

Thus, a preliminary comparison of BDH and Sigma AC revealed different properties, which are influenced by the density, purity and pH of the AC. This may influence cultures directly by the adsorption of inhibitory compounds and plant growth regulators, and/or the release of naturally present- or previously adsorbed-compounds into the medium (PAN & VAN STADEN, 1998). Some compounds, which are adsorbed by AC, include FeEDTA, pyroxidin, folic acid, thiamine and nicotinic acid (JOHANSSON,

CALLEBERG & GEDIN, 1990). These different properties also may influence cultures indirectly by the modification of the physical properties of media particularly pH, gel-strength and osmotic potential (DRUART & DE WULF, 1993). It is, therefore, possible that negative growth responses are incorrectly attributed to the addition of AC *per se* rather than the brand of AC and its effect on the physical properties of media. Thus, workers should specify the brand and batch of AC used in media.



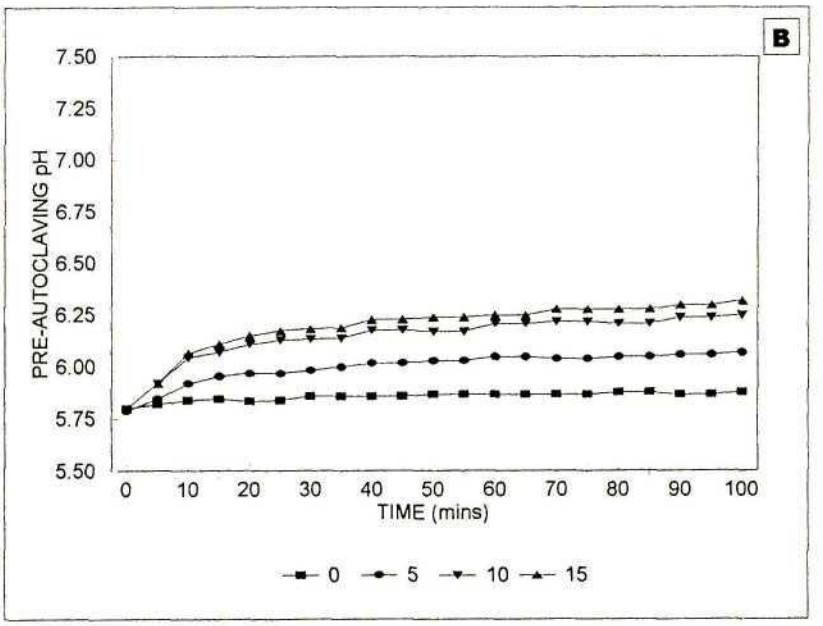
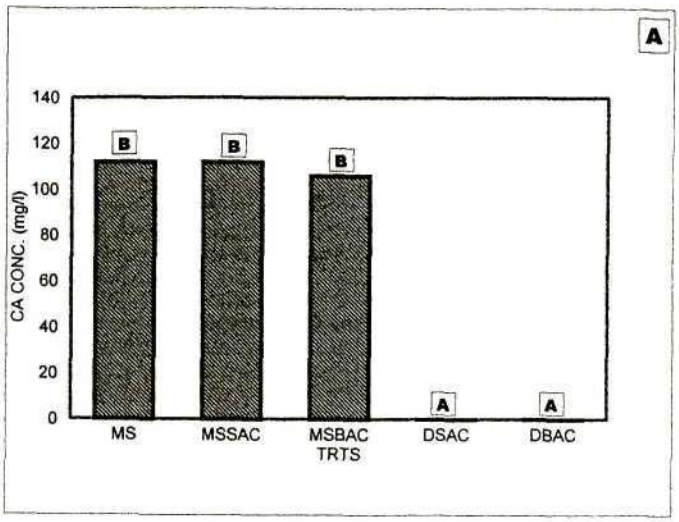
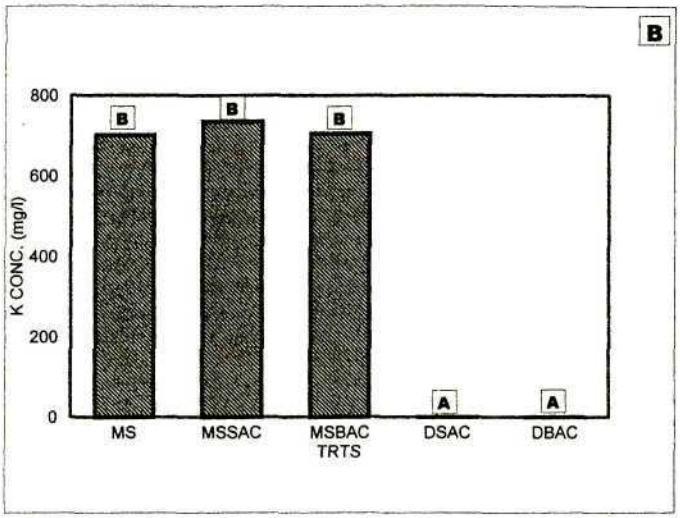
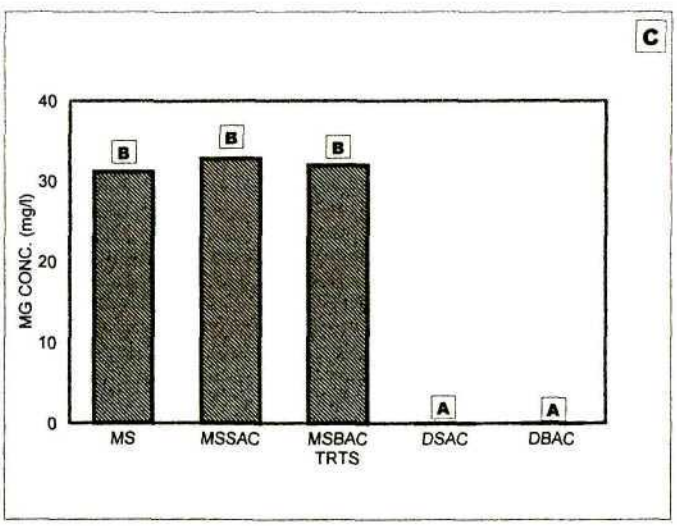


Fig. 34: The change in pre-autoclaving pH of media containing 0, 5, 10 or 15 g. l^{-1} of A: BDH AC; and B: Sigma AC over time.







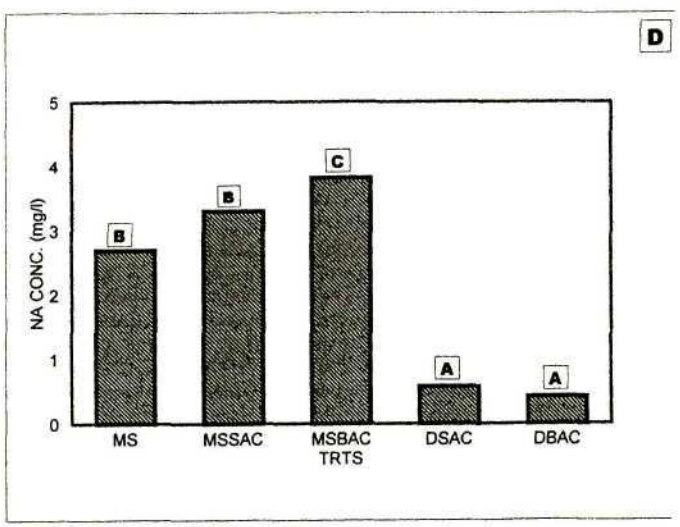


Fig. 35: The adsorption/desorption of A: calcium; B: potassium; C: magnesium; and D: sodium by different brands of AC. MS = control; MSSAC = MS and Sigma AC; MSBAC = MS and BDH AC; DSAC = distilled water and Sigma AC; and DBAC = distilled water and BDH AC.

3.4.2.2. Effect of sterilization method on the breakdown products of carbohydrates.

The sterilization method of media influenced the pH (Table 12). Thus, the pH of filter-sterilized media increased slightly by 0.1 unit. The pH of autoclaved media, however, decreased considerably by 0.4 to 1.5 units. This also was influenced by the carbohydrate source. Since the hydrolysis of various carbohydrates such as fructose and sucrose are pH-dependent (RÉDEI, 1974; GEORGE & SHERRINGTON, 1993), the method of sterilization could be important.

Using TLC, it was shown that medium containing filter-sterilized sucrose yielded only one large dark green spot at R_f 0.34, which co-chromatographed with the sucrose standard. In contrast, medium containing autoclaved sucrose yielded two spots at R_f 0.34 and R_f 0.43 respectively. These spots co-chromatographed with the sucrose standard and the double standard (combination of glucose and fructose) respectively. The medium containing combinations of sucrose and AC also yielded two spots, co-chromatographing with medium containing autoclaved sucrose. The colour, intensity and size of these spots, however, were influenced by the pH, yielding a large dark green spot (R_f 0.34) and a small faint spot (R_f 0.43) at pH 5.8 and *vice versa* at pH 3.8. The medium containing autoclaved glucose yielded one large light green spot at R_f 0.42, co-chromatographing with the glucose standard. The medium containing autoclaved fructose also yielded only one large dark green spot at R_f 0.44, co-chromatographing with the fructose standard. Thus, the breakdown products of glucose and fructose were not detected. This may have been due to: the low concentration and/or lability of these compounds; the inadequate separation of these compounds in the solvent system; or the inadequate development of compounds during flooding with reagent and subsequent heating (RÉDEI, 1974).

Table 12: The effect of sterilization viz. autoclaving (A) or filter-sterilizing (F/S) on the post-sterilization pH of media.

MEDIA	POST-STERILIZATION pH
GLUCOSE (F/S)	5.91
GLUCOSE (A)	5.13
FRUCTOSE (F/S)	5.90
FRUCTOSE (A)	4.27
SUCROSE (F/S)	5.86
SUCROSE (A)	5.36
SUCROSE & AC (A)(pH 5.8)	4.26
SUCROSE & AC (A)(pH 3.8)	3.36

3.4.2.3. Effect of carbohydrates and activated charcoal on the physical properties of media.

The pH decreased slightly with increasing glucose concentration (Fig. 36a). This may be due to the partial breakdown of glucose to 5-(hydroxymethyl)-2-furaldehyde (HMF) and phenolics (SCHENK, HSIAO & BORNMAN, 1991). These compounds would occur at proportionally higher concentrations in media containing high concentrations of glucose. The colour of the media changed from clear to light yellow with increasing glucose concentration. This may be due to the formation of HMF, which causes browning in dehydrated citrus juice (SHAW, TATUM & BERRY, 1967). This also may be attributed to the conversion of up to a third of the glucose into fructose (Lobry de Bruyn - Alberda van Ekenstein transformation)(RÉDEI, 1974). The pH decreased significantly with increasing fructose concentration when fructose was used alone (Fig. 37a)(R^2 =0.798; Y=317.885-61.536X) or in combination with glucose (Fig. 38a). This is probably due to the breakdown of fructose into HMF, hydroxy-acetal furan, levulinic acid and various other minor compounds (RÉDEI, 1974). Since the spectrum and quantity of these minor compounds are pH-dependent (SHAW, TATUM & BERRY, 1967 & 1968), the method of sterilization, that is, autoclaving or filter-sterilization, may be important. The colour of the media also changed from clear to bright yellow with increasing fructose concentration. The pH remained fairly constant irrespective of sucrose concentration (Fig. 39a). Sucrose hydrolyses partially during autoclaving resulting in equimolar concentrations of glucose and fructose (BALL, 1953; GEORGE & SHERRINGTON, 1993), although the degree of hydrolysis is pH-dependent increasing with decreasing pH (GEORGE & SHERRINGTON, 1993). The degree of hydrolysis is also higher when sucrose is autoclaved with other media components particularly FeNa-EDTA, which precipitates when autoclaved with $CaCl_2$ and various microelements. These problems can be circumvented by autoclaving FeNa-EDTA separately from the other media components (SCHENK, HSIAO & BORNMAN, 1991). The pH of combinations of sucrose and Sigma AC decreased significantly with increasing sucrose concentrations (Fig. 40a)(R^2 =0.863; Y=688.872-129.484X). The projected percentage sucrose hydrolysis, which was calculated using the equation, % Sucrose Hydrolysis = $1.9 + 2.9 \times 10^5 (10^{-pH})$(WANN, VEAZEY & KAPHAMMER, 1997)(Table 13), increased with increasing sucrose concentrations. The pH also was influenced by the brand and concentration of AC. This decreased significantly with

increasing AC concentration for media containing Sigma AC (Fig. 41A)(R^2 =0.688; Y=107.474-20.478X), but increased moderately with increasing AC concentration for media containing BDH AC (Fig. 41a). This influenced the projected percentage sucrose hydrolysis, which increased linearly with increasing AC for media containing Sigma AC, but remained fairly constant for media containing BDH AC (Table 14). The pH remained fairly constant irrespective of mannitol concentration (Fig. 42a).

The EC decreased significantly with increasing fructose concentration when fructose was used alone (Fig. 37a)(R^2=0.773; Y=275.508-47.807X) but remained fairly constant irrespective of glucose concentration when glucose was used alone (Fig. 36a) or in combination with fructose (Fig. 38a). The EC also remained fairly constant irrespective of the sucrose concentration (Fig. 39a) but decreased with increasing sucrose concentration when used in combination with AC (Fig. 40a). This was influenced by the brand and concentration of AC. The EC of media containing Sigma AC increased slightly and then decreased (Fig. 41a), while the EC of media containing BDH AC increased significantly with increasing AC concentration (Fig. 41a)(R^2 =0.885; Y=-135.488+23.878X). The increase in EC may be due to the release of sodium into the media by the AC (Fig. 35d) and/or the addition of significant amounts of HCl when the pH of the medium was adjusted (particularly at high BDH AC concentrations. The media containing AC often comprised a thin clear layer above a thicker black layer. The EC decreased significantly with increasing mannitol concentration (Fig. 42a)(R^2=0.659; Y= 467.303-75.530X).

The gel-strength remained fairly constant irrespective of glucose (Fig. 36b) or mannitol concentration (Fig. 42b). The gel-strength decreased with increasing fructose concentration when used alone (Fig. 37b) or in combination with glucose (Fig. 38b). The gel-strength of media increased with increasing sucrose concentration (Fig. 39b). The addition of Sigma AC, however, significantly decreased the gel-strength of media, which decreased with increasing sucrose concentration (Fig. 40b)(R^2 =0.878; Y=97.257-6.804X). A positive linear relationship existed between pH and gel-strength (R^2=0.910; Y=-83.398+18.310X) and EC and gel-strength (R^2 =0.881; Y=-47.456+9.937X). The brand and concentration of AC also influenced gel-strength. The gel-strength increased slightly for media containing up to 15 g l^{-1} Sigma AC and then decreased (Fig. 41b), while the gel-strength of media remained fairly constant irrespective of BDH AC

concentration (Fig. 41b).

Table 13: The projected percentage sucrose hydrolysis for medium containing 5 g l^{-1} Sigma AC and various sucrose concentrations based on the equation: % Sucrose Hydrolysis = 1.9 + 2.9 x 10^5 (10^{-pH})(WANN, VEAZEY & KAPHAMMER, 1997).

SUCROSE CONC. (g l^{-1})	SUCROSE HYDROLYSIS (%)
0	0.0
20	4.1
40	4.6
60	6.7
80	9.2
100	7.1

Table 14: The projected percentage sucrose hydrolysis for medium containing 20 g l^{-1} sucrose and various concentrations of AC based on the equation: % Sucrose Hydrolysis = 1.9 + 2.9 x 10^5 (10^{-pH})(WANN, VEAZEY & KAPHAMMER, 1997).

CHARCOAL TYPE & CONC. (g l^{-1})	SUCROSE HYDROLYSIS (%)
SIGMA (5)	7.5
SIGMA (10)	9.1
SIGMA (15)	8.9
SIGMA (20)	10.4
BDH (5)	2.7
BDH (10)	1.9
BDH (15)	2.3
BDH (20)	2.6

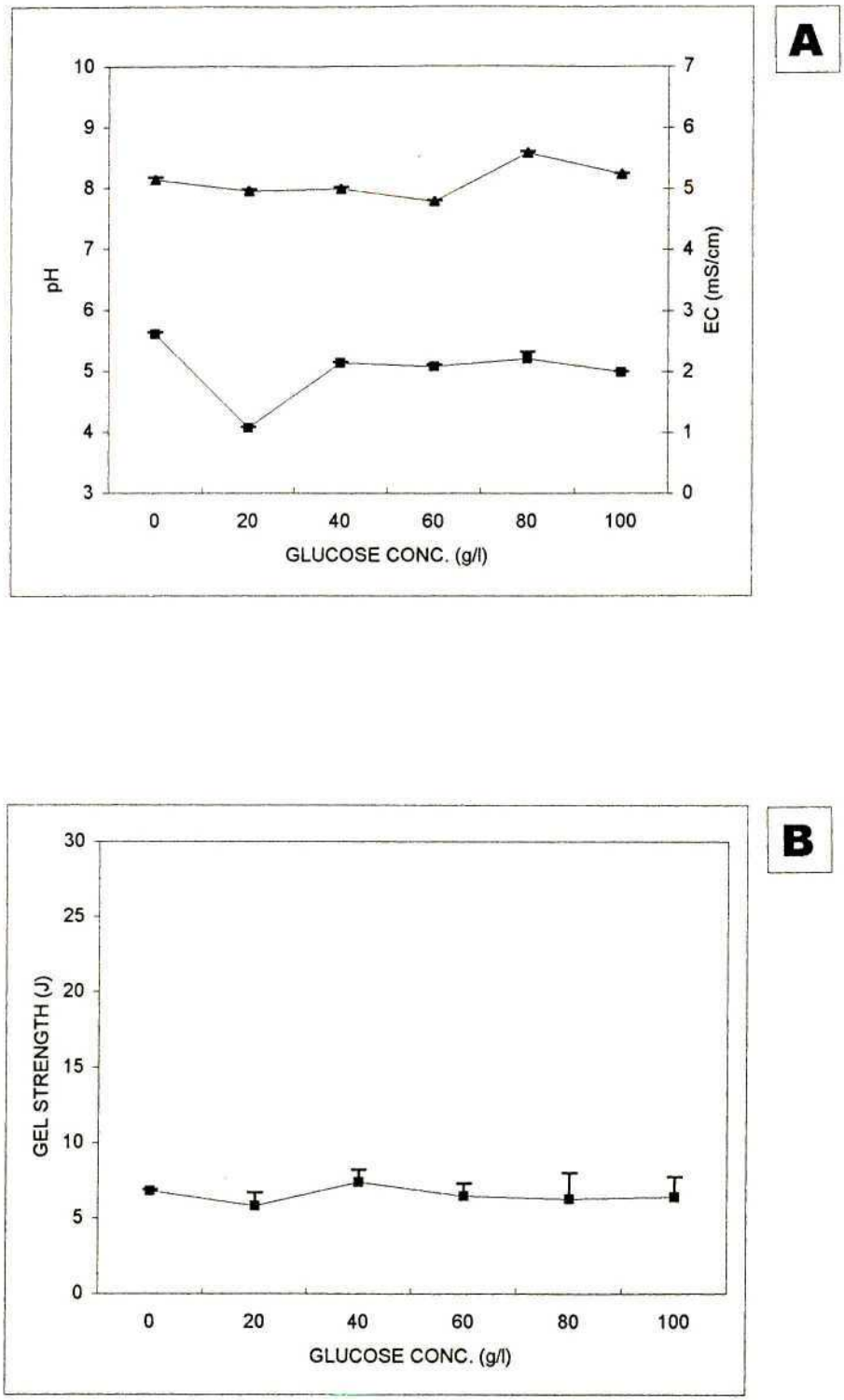
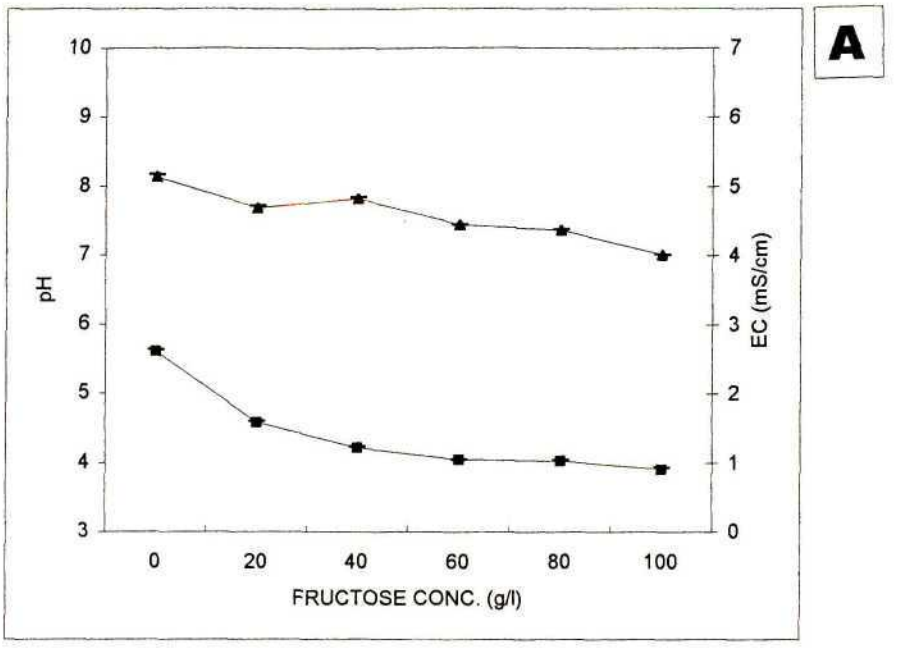


Fig. 36A: The pH (squares) and EC (triangles) of media containing various concentrations of glucose; and B: the gel-strength of media containing various concentrations of glucose.



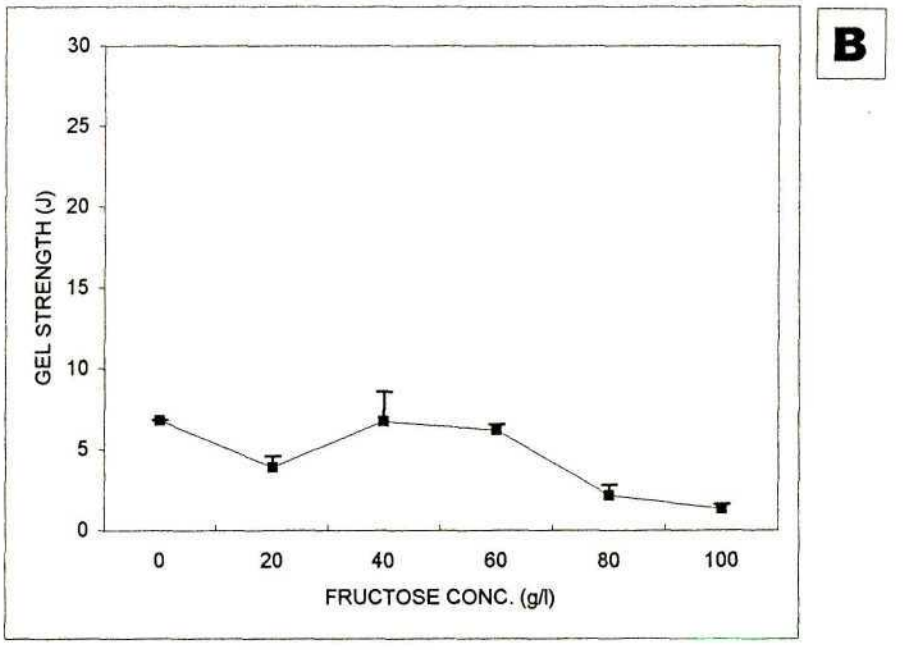


Fig. 37A: The pH (squares) and EC (triangles) of media containing various concentrations of fructose; and B: the gel-strength of media containing various concentrations of fructose.

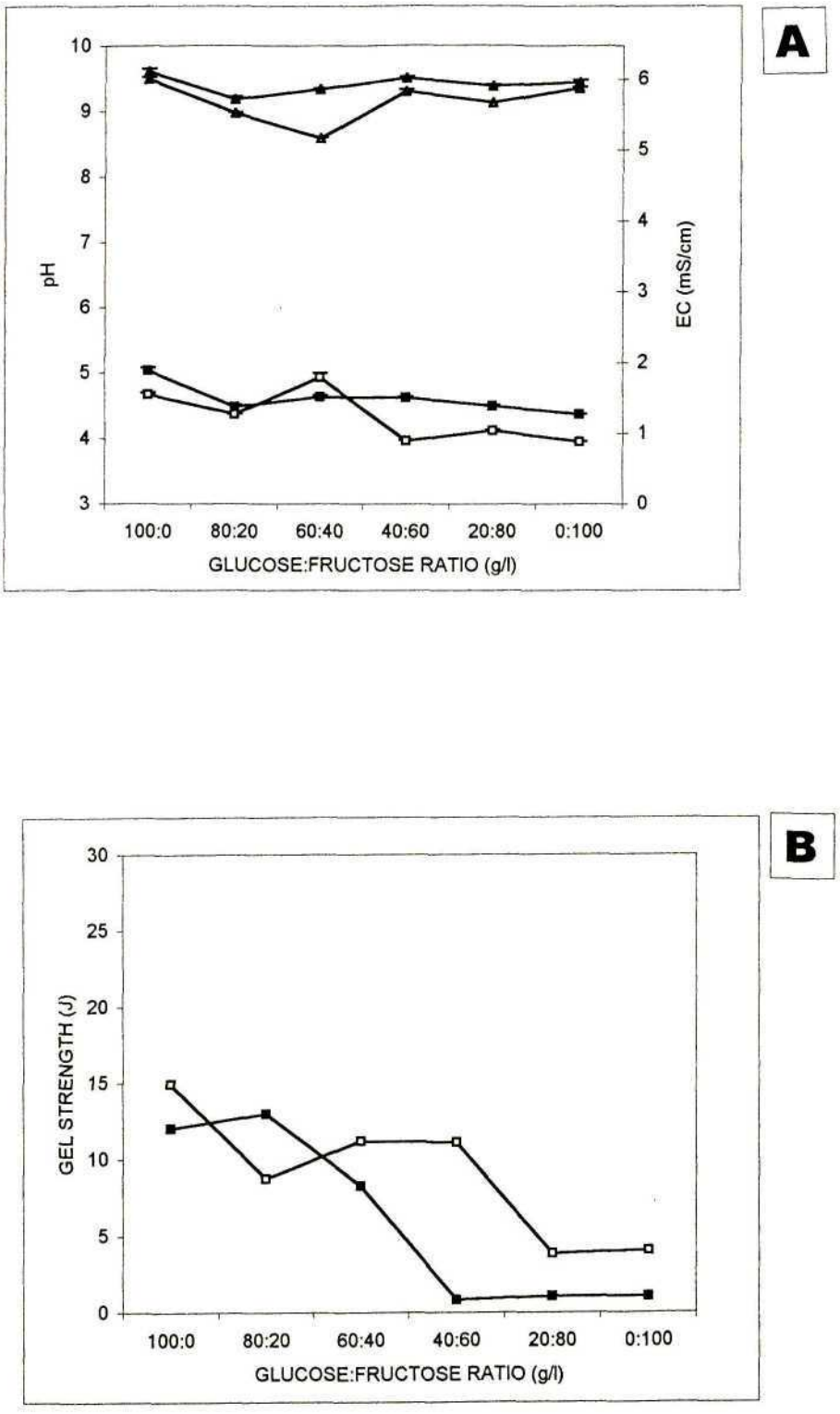


Fig. 38A: The pH (squares) and EC (triangles) of media containing various ratios of glucose and fructose with final carbohydrate concentrations of 20 (full symbols) and 40 g l^{-1} (empty symbols) respectively ; and B: the gel-strength of media containing various ratios of glucose and fructose with final carbohydrate concentrations of 20 (full symbols) and 40 g l^{-1} (empty symbols) respectively.

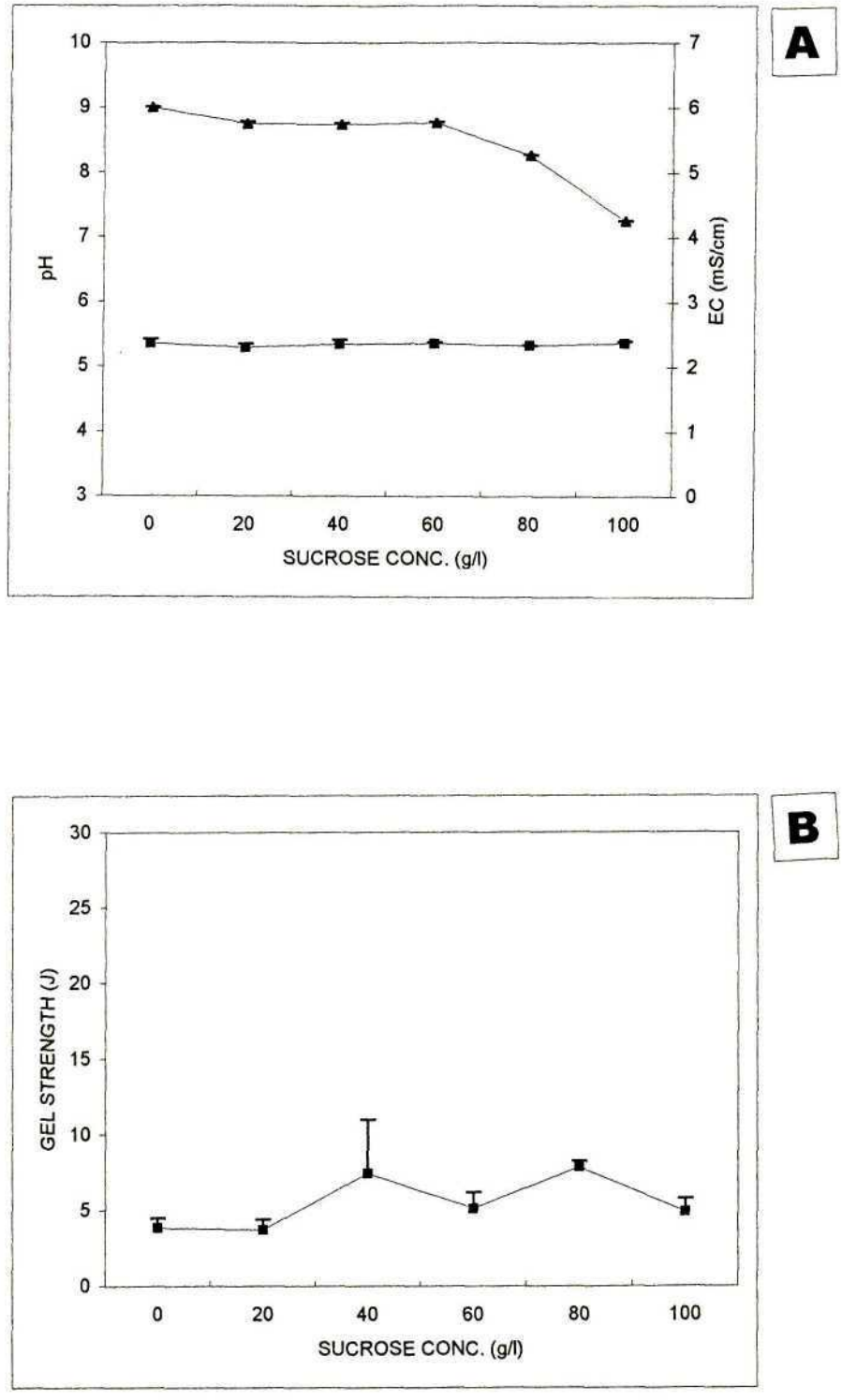
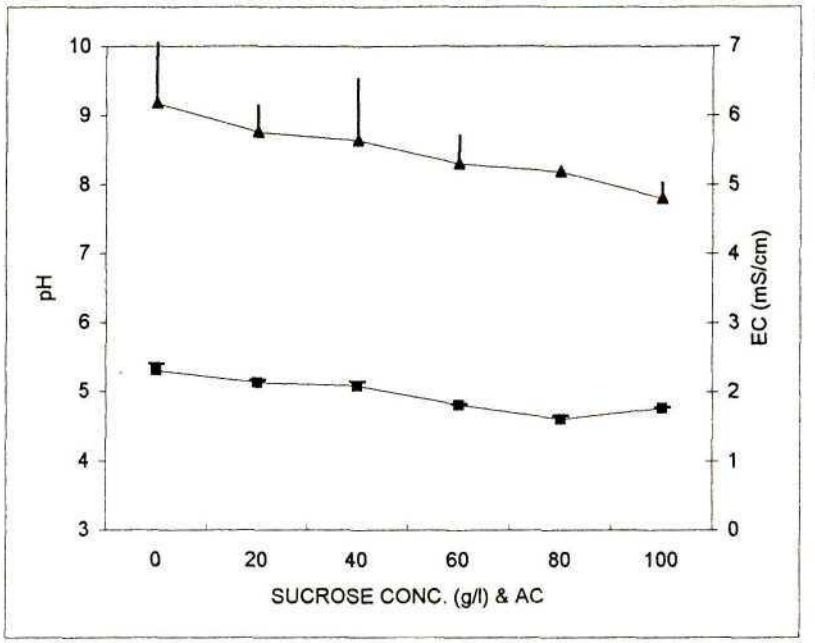


Fig. 39A: The pH (squares) and EC (triangles) of media containing various concentrations of sucrose; and B: the gel-strength of media containing various concentrations of sucrose.



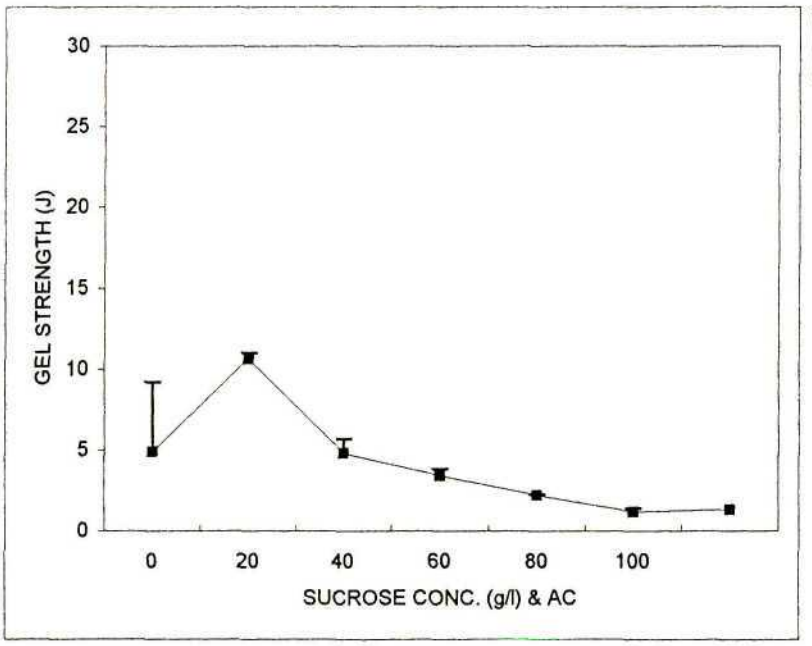


Fig. 40A: The pH (squares) and EC (triangles) of media containing various concentrations of sucrose in combination with Sigma AC; and B: the gel-strength of media containing various concentrations of sucrose in combination with Sigma AC.

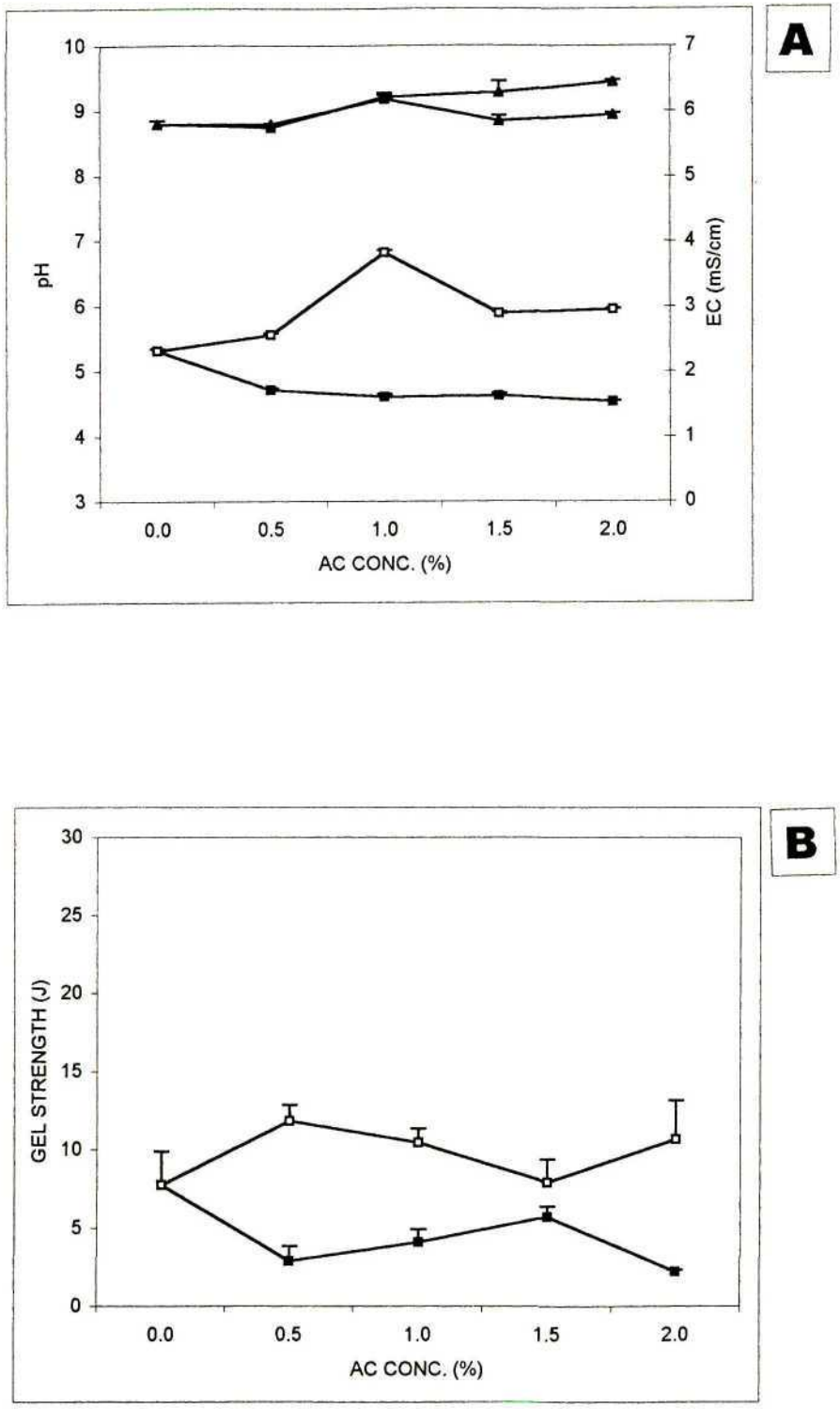


Fig. 41A: The pH (squares) and EC (triangles) of media containing various concentrations of BDH AC (empty symbols) and Sigma AC (full symbols); and B: the gel-strength of media containing various concentrations of BDH AC (empty symbols) and Sigma AC (full symbols).

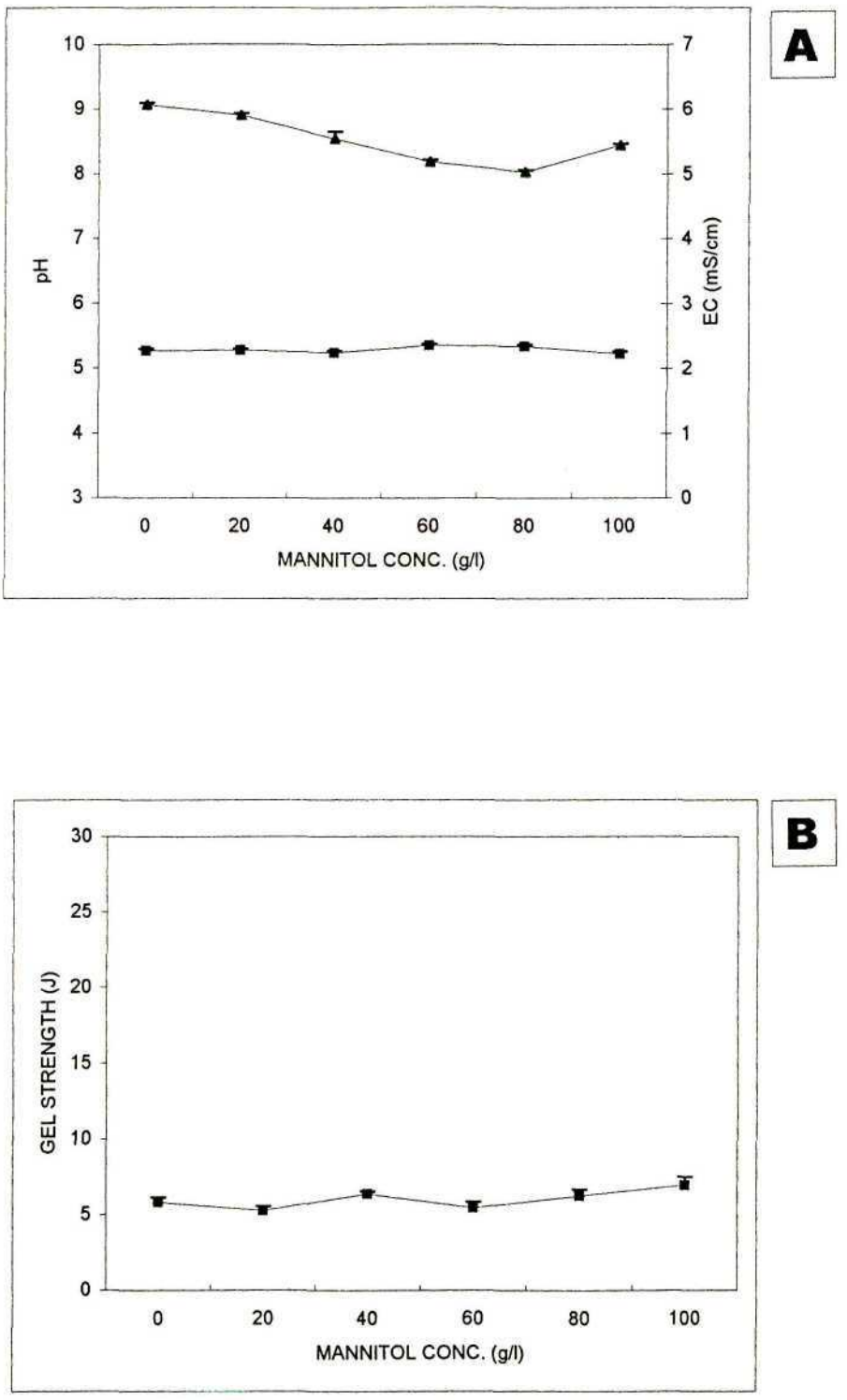


Fig. 42: The pH (squares) and EC (triangles) of media containing various concentrations of mannitol; and B: the gel-strength of media containing various concentrations of mannitol.

3.4.2.3. Effect of pre-autoclaving pH, basal salt concentration and gelling agent concentration on the physical properties of Gelrite®-solidified media.

The pH of media with low pre-autoclaving pH values between 3.8 and 4.4 increased moderately with autoclaving (Fig. 43a). In contrast, the pH of media with higher pre-autoclaving pH values between 5.4 and 9.8 decreased considerably after autoclaving. Thus, the pH of media with a pre-autoclaving pH of 5.8 decreased by only 0.5 units, while the pH of media with a pre-autoclaving pH of 7.8 decreased by 1.2 units. Similar trends have been reported for agar-solidified media (SKIRVIN, CHU, MANN, YOUNG, SULLIVAN & FERMANIAN, 1986; SARMA, MAESATO, HARA & SONODA, 1990; WETZSTEIN, KIM & SOMMER, 1994). The method of agar addition to the medium also influences the decrease in pH (SARMA, MAESATO, HARA & SONODA, 1990), which ranges between 0.2 and 0.5 units (WETZSTEIN, KIM & SOMMER, 1994) and 0.6 and 1.3 units (SARMA, MAESATO, HARA & SONODA, 1990). The pH decreases markedly when the medium is exposed repeatedly to high temperatures (once to dissolve, then to sterilize the medium)(SARMA, MAESATO, HARA & SONODA, 1990). In contrast, the gellan gum PS-60 (=Gelrite®), which is derived from *Pseudomonas elodea*, is thermally-stable and can withstand several cycles of autoclaving (KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982). The pH of Gelrite®-solidified medium remained fairly constant over several autoclaving cycles, while the pH of agar-solidified medium decreased moderately over the same number of cycles (Fig. 44a). The colour of Gelrite- and agar-solidified media changed with increasing number of autoclaving cycles. The Gelrite-solidified medium changed from clear to moderately yellow, while the agar-solidified medium changed from opaque white to yellow-ochre. This may be due to the caramelisation of the sucrose in the media. The pH decreased significantly with increasing basal salt concentration (Fig. 45a)(R^2= 0.737; Y=623.412-103.200X). In contrast, the pH remained fairly constant with increasing Gelrite® concentration (1 to 4 g l^{-1})(Fig. 46a). Other workers, however, showed that the pH increased considerably for media containing more than 5 g l^{-1} Gelrite® (JARAMILLO & SUMMERS, 1990).

The EC increased significantly from 0.7 to 5.7 mS cm^{-1} with increasing BM concentration (Fig. 45a)(R^2= 0.989; Y=15.807+19.593X). The EC also increased moderately from 5.8 to 6.2 mS cm^{-1} with increasing pre-autoclaving pH (Fig. 43a). This may be due to the

increased solubility of certain elements at particular pH values. Several metallic microelements such as copper, iron, manganese and zinc are more soluble at low pH values, while others such as molybdenum are more soluble at higher pH values. Although precipitation of salts occurred at pre-autoclaving pH values between 7.8 and 9.8, the EC was still high due to the addition of significant amounts of KOH when the pH of the medium was adjusted. The EC of Gelrite®-solidified medium increased moderately over several autoclaving cycles, while the EC of agar-solidified medium increased considerably over the same number of cycles (Fig. 44a). Although Gelrite® contains significantly more calcium (3 to 85 times), magnesium (3 to 85 times), potassium (2 to 170 times) and phosphorous (2 to15 times) than various brands of agar (ANONYMOUS, 1992), the EC remained fairly constant irrespective of Gelrite® concentration (Fig. 46a). In contrast, the EC of agar increases with increasing agar concentration (DEBERGH, 1983).

The gelling capacity of PS-60 (= Gelrite®) is dependant upon a certain minimum salt concentration. The type and concentration of salt influences the gel-strength with divalent cations such a magnesium and calcium promoting gelling at lower concentrations than monovalent cations (KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982). Similarly, certain ions such as K^+, SO_4^{2-} and Cl^- promote gelling in agar, whereas others such as NO_3^- retard gelling. In MS media, the high retarding ion: promoting ion ratio may have reduced the gel-strength of media (WETZSEIN, KIM & SOMMER, 1994). The gel-strength increased with low BM concentrations (20 to 40 %), levelled-off with intermediate to high BM concentration (40 and 80 %), then decreased moderately at high BM concentration (100 %)(Fig. 45b). Thus, the gelling capacity of Gelrite® is dependent upon a certain minimum BM concentration, which ranges from approximately 3.0 to 5.7 mS cm^{-1} (40 to 100 % of MS salts). The gel-strength of media with pre-autoclaving pH values between of 3.8 and 4.4 was low (Fig. 43b). The gel-strength, however, increased rapidly with increasing pre-autoclaving pH values between 4.4 and 5.4, levelled-off between 5.4 and 7.8, and then decreased moderately between 7.8 and 9.8. The decrease in gel-strength between 7.8 and 9.8 was due to the precipitation of salts. This resulted in opaque/turbid media. Thus, low (<5.4) and high (>7.8) pre-autoclaving pH values decreased the gel-strength considerably. A normal-curve existed between pH and gel-strength. The gel-strength of media decreased considerably for both Gelrite®- and agar-solidified media with an increasing number of

autoclaving cycles (Fig. 44b). A negative curvilinear relationship existed between gel-strength and the number of autoclaving cycles. Some workers showed that the decrease in gel-strength is similar for agar- and PS-60-solidified media over a single autoclaving cycle. This decrease in gel-strength is more marked for agar-solidified medium than for PS-60-solidified medium with successive autoclaving cycles (KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982). The decrease in gel-strength was similar for agar- (-87 %) and Gelrite®- solidified (-92 %) media over a single autoclaving cycle. The gel-strength increased linearly with increasing Gelrite® concentration (Fig. 46b)(R^2=0.933; Y=0.135+1.12X). Thus, the gel-strength of media containing 4 g l^{-1} Gelrite® is 39 times higher than for media containing 1 g l^{-1} Gelrite®. The availability of water is influenced by the gel-strength. This is largely determined by the matric potential (tenacity with which water is held in the solid phase of the gel) and expressibility (ease with which water is expressed in response to the mechanical deformation of the gel by the explant) of the media (OWENS & WOZNIAK, 1991). The expressibility, which is inversely related to gel-concentration and gel-strength, is markedly different for Gelrite® and various brands of agar. In addition, the matric potential of Gelrite® decreases rapidly for media containing between 1.2 and 4 g l^{-1} Gelrite® (OWEN & WOZNIAK, 1991). The gel-strength also is influenced by storage time increasing by 46 % over 14 (KLIMASZEWSKA & SMITH, 1997). Thus, "drastic changes in the state of the gel can be brought about by small changes in the external conditions, eg. temperature, pH" (TANAKA, 1981 IN DEBERGH, 1983). It is, therefore, possible that negative growth responses are incorrectly attributed to the addition of particular gelling agents *per se* rather than the effect that these gelling agents have on the physical properties of media.

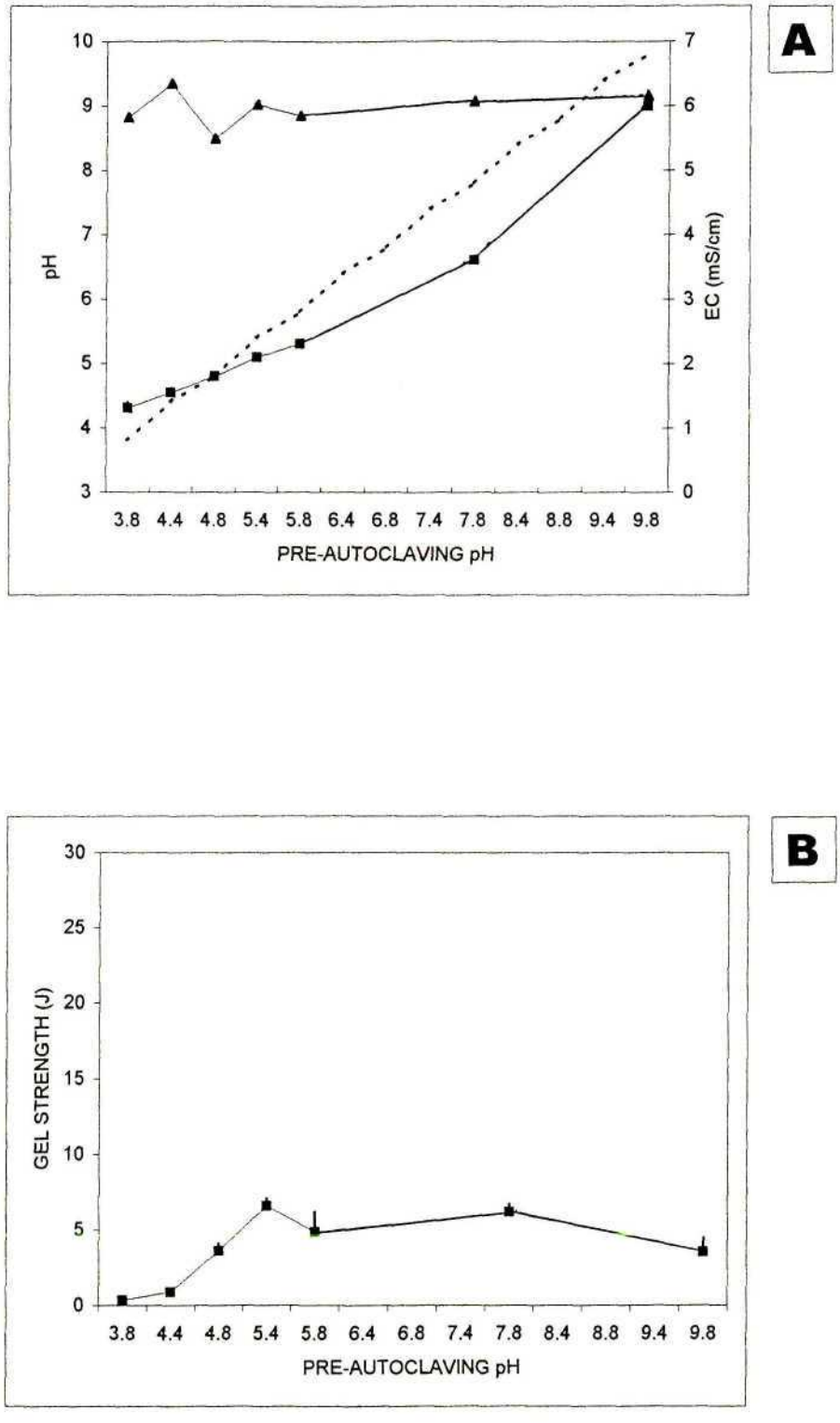


Fig. 43A: The pH (squares) and EC (triangles) of media with various pre-autoclaving pH values (dotted line indicates the pre-autoclaving pH value of media); and B: the gel-strength of media with various pre-autoclaving pH values.

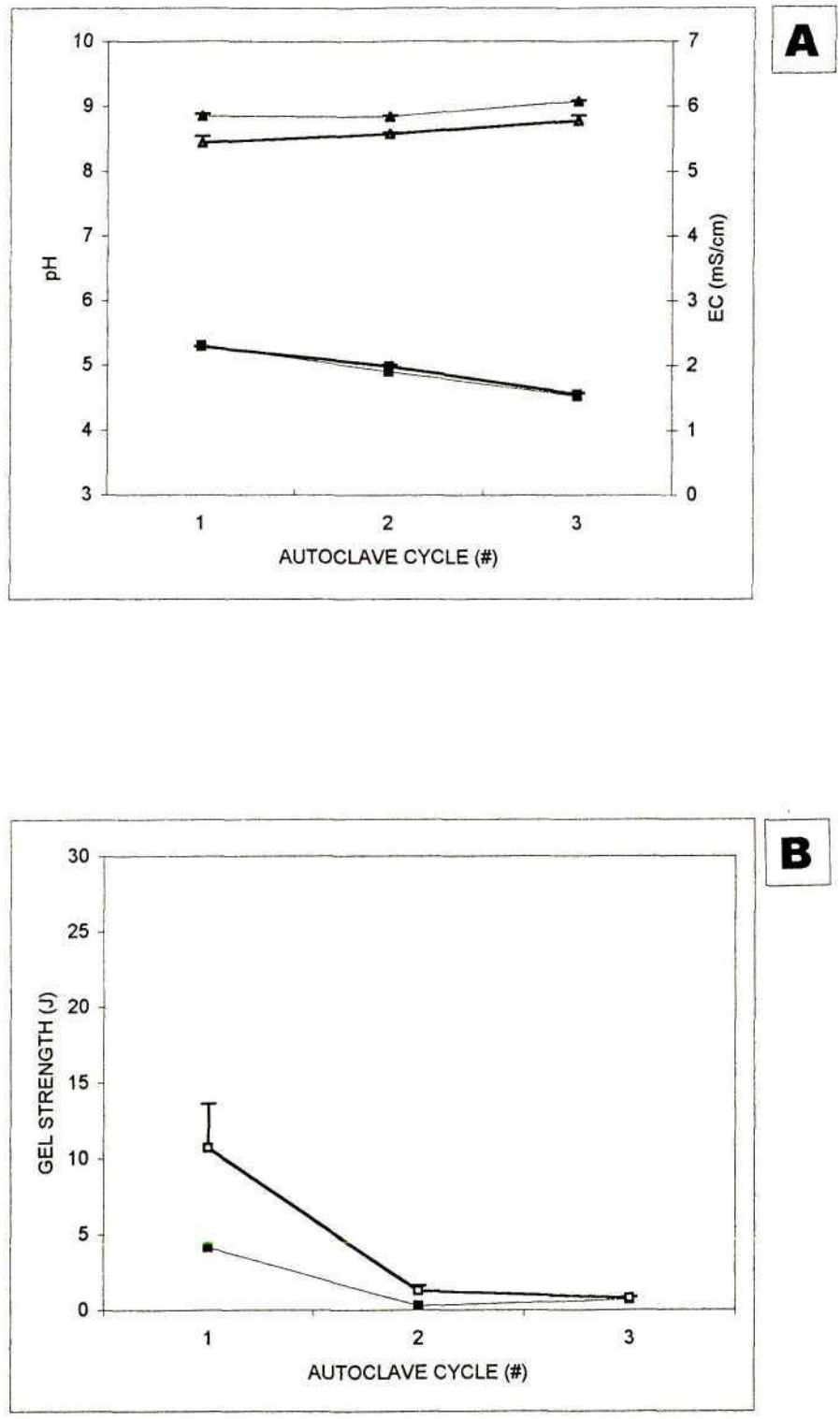


Fig. 44A: The pH (squares) and EC (triangles) of agar-solidified media (empty symbols) and Gelrite®-solidified media (full symbols) after several autoclave cycles; B: the gel-strength of agar-solidified media (empty symbols) and Gelrite®-solidified media (full symbols) after several autoclave cycles.

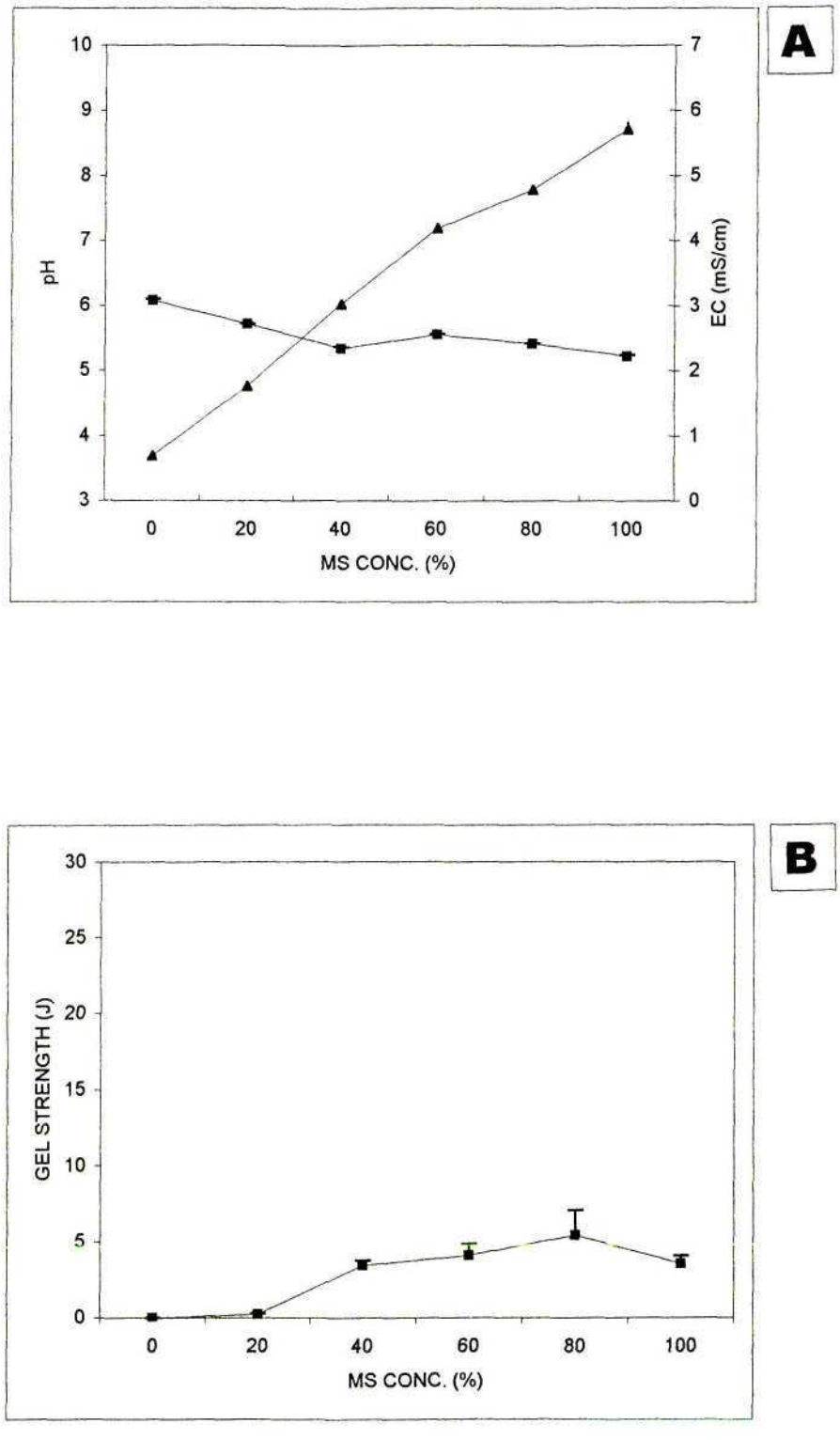


Fig. 45A: The pH (squares) and EC (triangles) of media containing a range of BM concentrations (%); and B: the gel-strength of media containing a range of BM concentrations (%).

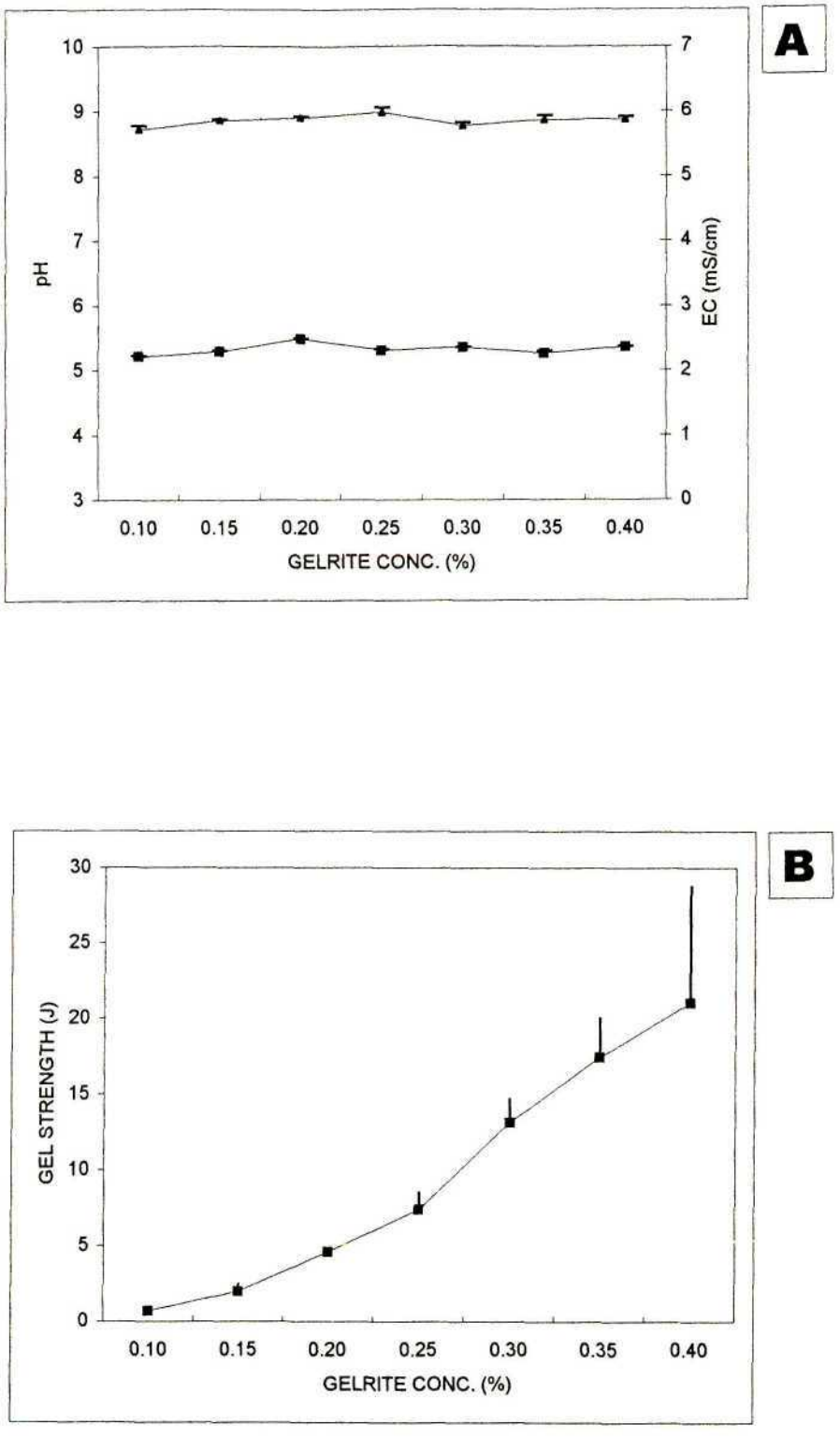


Fig. 46A: The pH (squares) and EC (triangles) of media solidified with various concentrations of Gelrite ®; and B: the gel-strength of media solidified with various concentrations of Gelrite ®.

3.4.2.4. Correlations between growth of shoots and physical properties of media.

The effect of carbohydrate source and activated charcoal on the growth of shoots and the physical properties of the media were determined. The data was pooled and plotted as a matrix plot, which comprised of 15 small graphs showing the relationships between the growth of shoots and the physical properties of media (Fig. 47). The data comprised of a 5 x 6 factorial grid with five carbohydrate sources at six concentrations (Fig. 47-A1). The carbohydrate sources were glucose (black circles), fructose (red squares), sucrose (green diamonds), mannitol (cyan triangles) and sucrose with AC (blue triangles). These carbohydrate sources were supplied at concentrations of 0, 20, 40, 60, 80 and 100 g l^{-1}. The pH of media was influenced by the carbohydrate source (Fig. 47-A3) and the concentration (Fig. 47-B3). The pH of media containing glucose, sucrose or mannitol remained fairly constant irrespective of the concentration (Fig. 47-B3), while the pH of media containing fructose or sucrose with AC decreased with increasing concentration. The EC of media was influenced by the concentration (Fig. 47-B4) and pH (Fig. 47-D4). The EC of media containing fructose, sucrose with or without AC and mannitol decreased linearly with increasing concentration. The gel-strength of media was influenced by the carbohydrate source (Fig. 47-A5) and the concentration (Fig. 47-B5). The gel-strength of media containing mannitol was higher than the gel-strength of media containing glucose or sucrose remaining fairly constant irrespective of concentration. The gel-strength also was influenced by the pH (Fig. 47-D5) and the EC (Fig. 47-E5) increasing with increasing pH or EC irrespective of the carbohydrate. The shoot weight (FW) was influenced by the carbohydrate source (Fig. 47-A6) and the concentration (Fig. 47-B6). The shoot weight was higher for media containing glucose and sucrose than for media containing fructose, mannitol or sucrose with AC. The shoot weight of shoots on media containing glucose, sucrose and mannitol decreased with increasing concentration (Fig. 47-D6), while the weight of shoots on media containing fructose or sucrose with AC increased up to a certain concentration and then decreased. The shoot weight was not influenced by the pH (Fig. 47-D6), the EC (Fig. 47-E6) or the gel-strength (Fig. 47-F6).

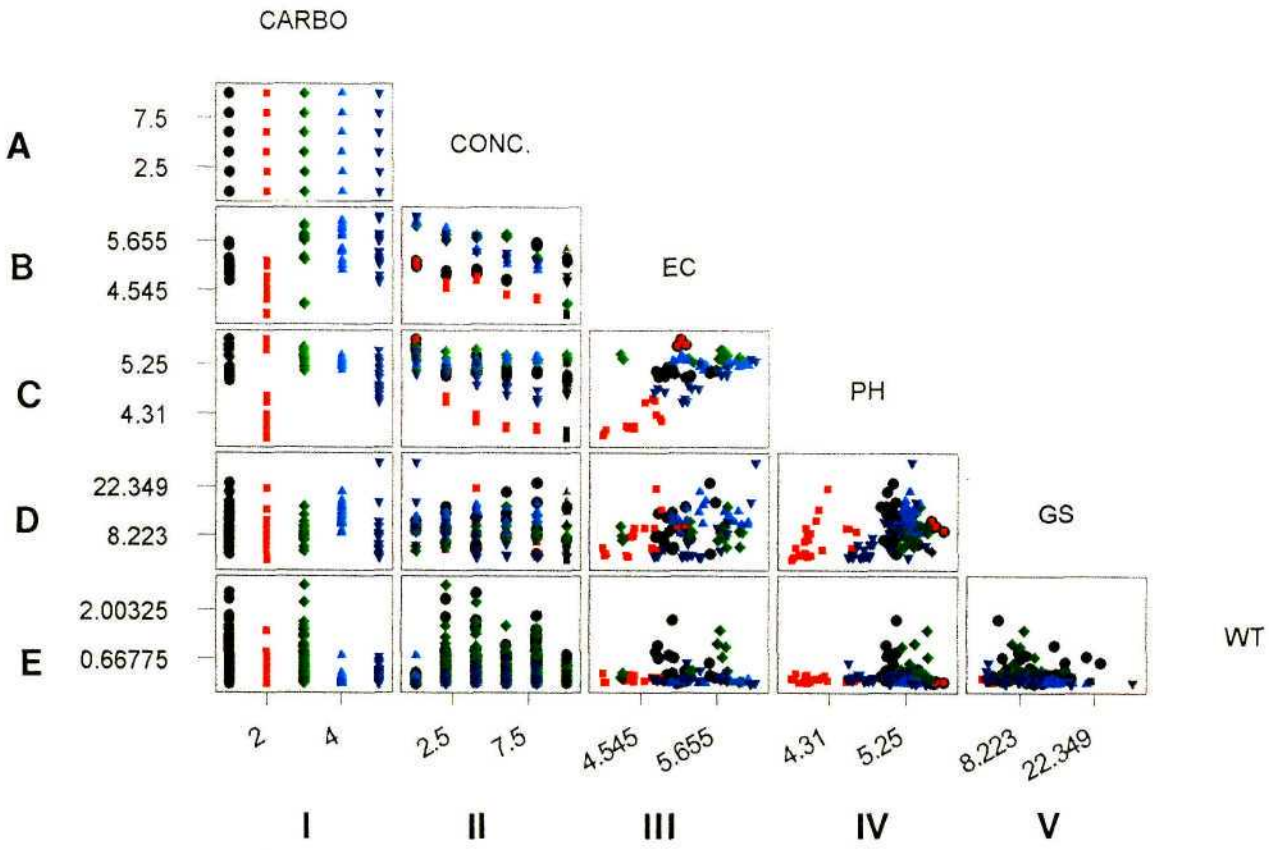


Fig. 47: Matrix plot showing correlations between the physical properties of media and the growth of shoots of *Scilla natalensis* Form A.

3.5. CONCLUSIONS.

Shoots of *S. natalensis* syn. *sensu stricto* (Form A) were subcultured onto media containing various carbohydrates to determine the effect of carbohydrate source and concentration on growth and development.

- The carbohydrate source and concentration significantly influenced the growth and development of shoots of *S. natalensis* syn. *sensu stricto* (Form A). This may be due to the ability of the shoot to utilize the different carbohydrate sources. In the absence of carbohydrates, the shoots were short with spindly leaves and short roots. These shoots may have been unable to fully meet their total carbon requirements by photosynthesis. This may have been influenced by environmental factors such as gas composition within the containers and light quality.
- When media were supplemented with fructose, the shoots were long with broad leaves, small bulbs, and few short to medium length roots at low concentrations. At higher fructose concentrations, the shoots were robust and short with narrow, sometimes deformed leaves, large bulbs, and few stunted, brown roots. This may be due to the breakdown of fructose into HMF, hydroxy-acetal furan, levulinic acid and various other minor compounds. These compounds have been implicated in the "fructose effect or syndrome" of plantlets.
- When sucrose was substituted for fructose, the shoots were robust and long with narrow and often red-pigmented leaves, large bulbs, and many long, thick roots. The large bulb size may have been due to increased respiration and starch synthesis, which increases with increasing sucrose concentration. The red-pigmented leaves, which increased in frequency with increasing sucrose concentration, may be due to the increased availability of substrate for the glycolysis pathway, and the subsequent increased production of erythrose-4-phosphate, which is essential for anthocyanin production. Optimal shoot growth and development occurred on media containing glucose or sucrose (40 to 60 g l^{-1}).
- When AC was supplied in combination with sucrose, the shoots were robust and short with few, and occasionally red-pigmented leaves, small to medium bulbs,

and few, severely stunted roots. This may be due to the release of naturally present compounds into the medium such as aluminium, copper and nickel into the media, which may become toxic at certain pH values. The adsorption of certain essential compounds such as FeEDTA, pyridoxine, folic acid, thiamine and nicotinic acid, the hydrolysis of sucrose and subsequent decrease in the sucrose concentration, or the decrease in the pH of the media may also be contributing factors.

- The carbohydrate source and concentration also significantly influenced the physical properties of media particularly pH, EC and gel-strength. The sterilization method of media viz. filter-sterilization or autoclaving influenced the pH. Whilst the pH of filter-sterilized media increased moderately, the pH of autoclaved media decreased considerably. This was also influenced by the carbohydrate source. Since the hydrolysis of various carbohydrates such as fructose and sucrose are pH-dependent, the method of sterilization could be important.
- The pH decreased slightly with increasing glucose concentration, which may be due to the partial breakdown of glucose to 5-(hydroxymethyl)-2-furaldehyde (HMF) and phenolics. These compounds would occur at proportionally higher concentrations in media containing high concentrations of glucose. The pH decreased significantly with increasing fructose concentration when fructose was used alone or in combination with glucose. This is probably due to the breakdown of fructose into HMF, hydroxy-acetal furan, levulinic acid and various other minor compounds. The spectrum and quantity of these minor compounds are pH-dependent. When sucrose was used in combination with Sigma AC, the pH decreased significantly with increasing sucrose concentrations. The pH also was influenced by the brand and concentration of AC.
- The EC decreased significantly with increasing fructose concentration when fructose was used alone but remained fairly constant irrespective of glucose concentration when glucose was used alone or in combination with fructose. The EC also remained fairly constant irrespective of the sucrose concentration but decreased with increasing sucrose concentration when used in combination with AC. The EC also was influenced by the brand and concentration of AC.
- The gel-strength remained fairly constant irrespective of glucose. The gel-strength decreased with increasing fructose concentration when used alone or

in combination with glucose. The gel-strength of media increased with increasing sucrose concentration although the addition of Sigma AC significantly decreased the gel-strength of media, which decreased with increasing sucrose concentration The brand and concentration of AC also influenced gel-strength.

- A comparison of BDH and Sigma AC revealed different properties, which are influenced by the density, purity and pH of the AC. It is, therefore, possible that negative growth responses are incorrectly attributed to the addition of AC *per se* rather than the brand of AC and its effect on the physical properties of media.
- The matrix plot confirmed that carbohydrates influenced pH, EC and gel-strength of media. This was influenced by the carbohydrate source and concentration, which suggested that the effect of carbohydrate source and concentration on the growth of shoots may be largely due to the indirect effects of these physical properties such as hydrolysis of carbohydrates, the spectrum and quantity of the breakdown products and the availability of nutrients, plant growth regulators and water rather than the direct effects of pH, EC and gel-strength *per se.*

Thus, the growth and development of these shoots can be manipulated by supplementing the media with sub- or supra-optimal concentrations of carbohydrates. This may have commercial applications in acclimatization or germplasm storage of bulbous plants.

CHAPTER FOUR
GENERAL CONCLUSIONS.

In South Africa, large quantities of *Scilla natalensis* are harvested from wild populations and sold as traditional medicine. This is reducing the density, distribution and genetic diversity of wild populations. The enforcement of existing legislation, however, has proved ineffective with plants being traded locally and internationally. It has therefore, been suggested that *ex situ* conservation through cultivation may alleviate pressures on natural resources, whilst meeting the demand for these plants. Conventional propagation of these plants, however, is usually fairly slow. *In vitro* propagation, however, provides a rapid means of propagating selected chemotypes or cultivars.

In the first part of the study, continuous culture systems were established for the three forms of *Scilla natalensis, S. natalensis sensu stricto* (Form A), *S. natalensis* syn. *S. kraussii* (Form B) and *S. natalensis* syn. *S. dracomontana* (Form C). The efficiency of the systems was strongly influenced by genetic factors, viz the form and epigenetic factors, viz the explant type, carbohydrate source, plant growth regulators and gelling agents.

The form, Form A, Form B or Form C respectively, influenced shoot initiation with the larger forms generally producing more shoots than the smaller forms (Form A > Form B > Form C). The data confirmed that the three forms are significantly different in terms of their physiological response to carbohydrates, plant growth regulators and gelling agents *in vitro.* The effect of form could not be compensated for by the addition of either carbohydrates, plant growth regulators or gelling agents. This may provide some support for the reinstatement of these forms as three species, *Scilla natalensis* Planch., *S. kraussii* Bak. and *S. dracomontana* Hilliard & Burtt.

The explant type, that is bulb or leaf explants respectively, significantly influenced shoot initiation. Leaf explants generally produced more shoots than bulb explants. This may have been influenced by other factors such as the age, orientation (apolar or polar), position (distal or proximal), polarity, shape and size of the explants.

The carbohydrate source significantly influenced shoot initiation. The explants generally produced more shoots when cultured on media containing glucose or sucrose than on media containing fructose, lactose, maltose and particularly mannitol. This may have been influenced by the endogenous levels of carbohydrates and/or the ability of the explants to utilize the carbohydrate source. Shoot initiation also may have been influenced by the hydrolysis of various carbohydrates during autoclaving and the subsequent effect on the physical properties of the media.

The cytokinin, auxin and interaction between the cytokinin and auxin significantly influenced shoot initiation. The addition of kinetin or TDZ alone increased shoot initiation although the addition of IAA and particularly NAA alone reduced shoot initiation. Shoot initiation was higher for combinations of kinetin: IAA than for combinations of kinetin: NAA or TDZ: NAA The interaction between kinetin: IAA considerably increased shoot initiation while the interaction between the kinetin: NAA or TDZ: NAA significantly reduced shoot initiation. Optimal shoot initiation for Form A, Form B and Form C occurred on media containing 1 to 2 mg l^{-1} kinetin and 1 to 2 mg l^{-1} IAA.

The gelling agent also influenced shoot initiation with media solidified with Gelrite® producing more shoots than media solidified with Oxoid or Unilab agar. Shoot initiation, however, was not significantly influenced by the interaction between the form and gelling agent or the interaction between explant type and gelling agent.

Shoots were successfully rooted on media containing IAA, IBA or NAA. This was influenced by the form, Form A, Form B or Form C respectively. The plantlets were then acclimatised. These continuous culture systems can be used to produce large quantities of plantlets. *In vitro* propagation, therefore provides a rapid means of propagating selected chemotypes or cultivars, serving both conservation and commercial interests. This may alleviate pressures on natural resources and provide an alternative source of high quality plants for the burgeoning medicinal plant market.

In the second part of the study, the effect of carbohydrate source and concentration on growth and development of shoots of *S. natalensis* syn. *sensu stricto* (Form A) were determined. This has applications for the acclimatisation and germplasm storage of bulbous plants.

The carbohydrate source and concentration significantly influenced the growth and development of shoots. This may be due to the ability of the shoot to utilize the different carbohydrate sources. In the absence of carbohydrates, the shoots were short with spindly leaves and short roots. When media were supplemented with fructose, the shoots were long with broad leaves, small bulbs, and few short to medium length roots at low concentrations. At higher fructose concentrations, however, the shoots were robust and short with narrow, sometimes deformed leaves, large bulbs, and few stunted, brown roots. When sucrose was substituted for fructose, the shoots were robust and long with narrow and often red-pigmented leaves, large bulbs, and many long, thick roots. When AC was supplied in combination with sucrose, however, the shoots were robust and short with few, and occasionally red-pigmented leaves, small to medium bulbs, and few, severely stunted roots. Optimal shoot growth and development in terms of shoot weight (FW) and quality occurred on media containing glucose or sucrose (40 to 60 g l^{-1}).

The carbohydrate source and concentration also significantly influenced the physical properties of media particularly pH, EC and gel-strength. The sterilization method of media viz. filter-sterilization or autoclaving influenced the pH. This was also influenced by the carbohydrate source. The method of sterilization, therefore, could be important since the hydrolysis of various carbohydrates are pH-dependent. The pH decreased slightly with increasing glucose concentration, which may be due to the partial breakdown of glucose to 5-(hydroxymethyl)-2-furaldehyde (HMF) and phenolics. The pH decreased significantly with increasing fructose concentration when fructose was used alone or in combination with glucose. This is probably due to the breakdown of fructose into HMF, hydroxy-acetal furan, levulinic acid and various other minor compounds. When sucrose was used in combination with Sigma AC, the pH decreased significantly with increasing sucrose concentrations. The pH also was influenced by the brand and concentration of AC.

The EC decreased significantly with increasing fructose concentration when fructose was used alone but remained fairly constant irrespective of glucose concentration when glucose was used alone or in combination with fructose. The EC also remained fairly constant irrespective of the sucrose concentration but decreased with increasing sucrose concentration when used in combination with AC. The EC also was influenced by the brand and concentration of AC.

The gel-strength remained fairly constant irrespective of glucose. The gel-strength decreased with increasing fructose concentration when used alone or in combination with glucose. The gel-strength of media increased with increasing sucrose concentration although the addition of Sigma AC significantly decreased the gel-strength of media, which decreased with increasing sucrose concentration The brand and concentration of AC also influenced gel-strength.

Thus, the pH, EC and gel-strength of media were influenced by the carbohydrate source and concentration. The matrix plot, however, suggested that the effect of carbohydrate source and concentration on the growth of shoots may be largely due to the indirect effects of these physical properties such as hydrolysis of carbohydrates, the spectrum and quantity of the breakdown products and the availability of nutrients, plant growth regulators and water rather than the direct effects of pH, EC and gel-strength *per se.*

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5. APPENDIX 1.

ATOMIC ABSORPTION WORKING CONDITIONS (ANONYMOUS, 1979)

ELEMENT	LAMP CURRENT (mA)	FUEL	WAVELENGTH (nm)	SPECTRAL BAND PASS (nm)
CALCIUM	3.5	ACETYLENE	422.7	0.5
MAGNESIUM	3.5	ACETYLENE	285.2	0.5
POTASSIUM	5	ACETYLENE	769.9	1.0
SODIUM	5	ACETYLENE	589.6	1.0