

**ROOT-STIMULATING ACTIVITY
FROM VARIOUS GELLING AGENTS
USED IN TISSUE CULTURE**

By

Georgina Dede Arthur

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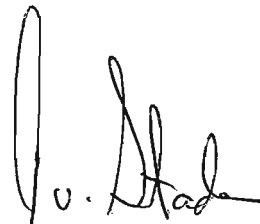
DECLARATION

I hereby declare that this thesis submitted for the degree of Doctor of Philosophy in the Research Centre for Plant Growth and Development, School of Botany and Zoology, Faculty of Science Agriculture, University of Natal Pietermaritzburg, is the result of my own investigation except where work of others is acknowledged.



GEORGINA DEEDE ARTHUR

We declare that the above statement is correct



PROF. J. VAN STADEN
(SUPERVISOR)



DR W.A. STIRK
(CO-SUPERVISOR)

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ABSTRACT

Extracts of gelling agents have been shown to stimulate rooting and this study was initiated to investigate the presence of root stimulating substances in gelling agents. After screening a number of gelling agents, four were selected, namely; Agar Bacteriological, Agar Commercial Gel, Difco Bacto Agar and Gelrite were selected and examined for the presence of root-stimulating substances using mungbean bioassay.

Water extracts of Agar Bacteriological, Agar Commercial Gel and Difco Bactol Agar stimulated rooting of mungbean cuttings. Addition of Charcoal neither reduced nor increased rooting produced by the water extract of the first two agars but when added in conjunction with Difco Bacto Agar rooting was reduced. Autoclaving, however reduced rooting in extracts of the gelling agents. The possibility that root-stimulating substances may not be the same in all the gelling agents can not be excluded. Extraction of Gelrite with water was problematical and was therefore excluded.

IBA solution and water extracts of the gelling agents separately promoted good rooting in mungbeans cuttings. Rooting in extracts of autoclaved frozen-thawed gelling agents was poor, however, IBA + gelling agents gave high rooting at the 100% concentration and this could possibly be due to an additive effect of the IBA. Addition of charcoal reduced rooting significantly in extracts of IBA + gelling agents. Using 80% acidic methanol, reasonable levels of rooting substances were obtained from the residue extract of this complex (IBA + gelling agent+charcoal) of all the gelling agents except Gelrite indicating that root-promoting substances were adsorbed by charcoal. The low rooting in the presence of the Gelrite extract was attributed to the matrix of the polymer of the Gelrite.

Ethyl acetate fractionated extracts (EA-pH 8.0; EA-pH 3.0; and Aqueous) obtained from the four gelling agents stimulated rooting indicating the presence of numerous root promoting substances. Gelrite gave good rooting with both the 50 and 100% concentrations of all the fractions.

Purified water and ethanol extracts of the gelling agents exhibited auxin-like activity when separated by paper chromatography and compared with IBA and IAA standards. Using HPLC, IAA was quantified in all the gelling agents with Difco Bacto Agar and Agar Commercial Gel having the highest IAA concentration and Gelrite the lowest IAA concentration. IAA concentration in Agar Bacteriological was a third of the level detected in Difco Bacto Agar.

The information from this work may enable researchers to consider gelling agents as sources of auxin-like compounds and other plant hormones as well as support media for use in tissue culture procedures and also increase the enthuse for further research into the nutrient types and levels in gelling agents.

PAPERS IN PREPARATION

G.D. Arthur, W.A.Stirk and J. van Staden 2003. Screening of gelling agents (Agar and Gelrite) for auxin-like activity.

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G.D Arthur, W.A.Stirk and J. van Staden 2003. Separation, purification and quantification of root stimulating substances in gelling agents.

CONFERENCE CONTRIBUTION FROM THIS THESIS

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
A _F C	Autoclaved-frozen extract control
A _F Cha	Autoclaved-frozen extract with charcoal
A _F IBA	Autoclaved-frozen extract with IBA
A _F Cha + IBA	Autoclaved-frozen extract with charcoal and IBA
A _F Cha+ IBAr	Residue from the autoclaved-frozen extracts with charcoal and IBA
C	Control extract
CA	Control autoclaved
Cha	Charcoal-added
ChaA	Charcoal-added autoclaved
DHZ	Dihydrozeatin
ELIZA	Enzyme-linked immunosorbent assays
GA	Gibberellins
GA ₃	Gibberellic acid
GLC	Gas liquid chromatography
GC-MS	Gas liquid chromatography mass spectrometry
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IAld	Indole -3-aldehyde
IAM	Indole-3-acetamide
IBA	Indole-3-butyric acid
IBA-A	IBA autoclaved
IBA-Cha	IBA Charcoal added
IBA-ChaA	IBA Charcoal added and autoclaved
IBA-S	IBA solution
ICA	Indole-3-carboxylic acid
IEt	Indole-3-ethanol
ILA	Indole-3-lactic acid
IM	Indole-3-methanol
IPA	Indole-3-pyruvic acid

iPA	isopentenyladenosine
iP	isopentenyladenine
NAA	Naphthalene acetic acid
NMR	Nuclear magnetic resonance
PAA	<i>p</i> -Phenylacetic acid
PC	Paper chromatography
PGR	Plant growth regulator
TLC	Thin layer chromatography
TRP	Tryptophan
tZ	<i>trans</i> -Zeatin
tZR	<i>trans</i> -Zeatin riboside
Z	Zeatin
ZR	Zeatin riboside

CHAPTER 1

INTRODUCTION

1.1. GELLING AGENTS

1.1.1. STIMULATION OF ROOTING BY AGAR EXTRACTS

Substances related to plant polysaccharides have some biological activity that stimulates shoot regeneration and/or root growth. ICHIMURA & ODA (1998) indicated that water extracts of agar stimulated root growth of vegetables such as Japanese radish, tomatoes, spinach and Welsh onion and confirmed that several root growth-stimulating substances are found in agar. These substances are of low molecular weight and are highly hydrophilic. Compounds such as caulerpil from the green alga *Caulerpa toxifolia* (RAUB, CARDILLINA & SCHWEDE, 1987) and 3-(hydroxyl) indole (BERNART & GERWICK, 1990) have also been reported to promote root growth of lettuce. Since the latter compounds are not highly hydrophilic, it was suggested that they are not the root stimulating compounds found in agar extracts tested by ICHIMURA & ODA (1998). The root growth-stimulating substances in agar which are highly hydrophilic also seem to be different from known phytohormones such as indole acetic acid (IAA), gibberellic acid (GA_3), zeatin (Z) and abscisic acid (ABA). The latter three have little or no effect on root growth of lettuce (ICHIMURA & ODA, 1998).

Agar contains compounds that affect differentiation and development of cultured tissue. ICHIMURA, UCHIUMI, TSUJI, ODA & NAGAOKA (1995) reported that more adventitious shoots from tomato tissues were regenerated when supports derived from plant tissue, such as wood pulp, were used compared to supports made of polyester and ceramics. They attributed this to compounds extracted from these plant raw products. Therefore it was speculated that plant polysaccharides contain common unidentified biologically active compounds that stimulate shoot regeneration and/or root growth.

1.1.2. NATURE OF AGAR

Agar is the common name of gelling polysaccharides or colloidal products that are produced from seaweeds such as *Gelidium*, *Gracilaria* and *Pterocladia*. Agar is widely used in the preparation of food, cosmetics, pharmaceutical and dental products and in biological and medical research. Approximately 60% of the world's present production of agar is derived from *Gracilaria* species (NELSON, YANG, WANG & CHIANG, 1983). Agar quality depends on the species used (CRAIGIE, WEN & VAN DER MEER, 1984; LIGNELL & PEDERSEN, 1989), the life cycle generation (WHYTE, ENGLAR, SAUNDERS & LINDSAY, 1981), season (OZA, 1978; BIRD & HINSON, 1992; PONDEVIDA & HURTADO-PONCE, 1996) and growing environment (WHYTE, ENGLAR, SAUNDERS & LINDSAY, 1981). Environmental changes during the seasonal shifts from dry to wet periods affect yield. Agar composition also varies with nutrient and culture conditions used for seaweed growth (BIRD, HANISAK & RYTHER, 1981; CRAIGIE, WEN, VAN DER MEER, 1984; CRAIGIE & WEN, 1984). Generally, the gel strength is higher in agar from plants grown under nitrogen enrichment and good light conditions. Agar yield is however higher from plants grown under sub-optimal nutrient and light conditions (LIGNELL & PEDERSEN, 1989). The extraction method employed has significant effects on yield and gel strength (BIRD & HINSON, 1992) and possibly the nutrients present.

Four agarophyte species, namely ; *Gracilaria tikvahiae*, *G. blodgettii*, *Gelidium pusillum* and *Gracilariopsis lemaniformis* were examined for agar yield and quality on a quarterly basis (November, March, June and August) (BIRD & HINSON, 1992). The first three species showed slight seasonal variability in agar yield of both native agar (water extraction) and alkaline modified agar with *Gracilaria tikvahiae* giving the lowest yield. All the species showed strong seasonal variations in gel strength. This was species specific rather than related to some common environmental factor. *Gracilariopsis lemaniformis* showed strong seasonal variation in agar yield and produced an unusual native agar which had no detectable gel strength when harvested in March and June but produced high gel strengths after alkali modification. Apart from *Gracilaria tikvahiae*, all

the other species mentioned above produced agar of commercial quality (BIRD & HINSON, 1992).

In another trial, yield, gel strength and chemical composition of agar extracts were analyzed from several species of *Gracilaria* collected from Guam (Micronesia) and Saipan (Taiwan) (NELSON, YANG, WANG & CHIANG, 1983). The highest agar yield was obtained from the Guam harvested *Gracilaria edulis* with mild alkaline pretreatment (1% NaOH for 30 min). The highest gel strengths were obtained from agar extracts of *G. lichnoides* from Saipan after a relatively strong alkaline pretreatment, (5% NaOH for 90 min). Sulfate, pyruvate and 3,6-anhydrogalactose content of the agars ranged from 2.5-5.3%, 0.07-0.16% and 26.5-44.0% respectively. Specific viscosities of the agars ranged from 2.0-4.3 and agar gelling temperature from 25-44°C (NELSON, YANG, WANG & CHIANG, 1983).

Commercial supplies of agar can be derived from mixtures of different seaweed species in various quantities, some of which can be undesirable red and brown algae that do not contribute useful agar. The basic structure of agar is agarobiose. This is a linear polymer of an alternating sequence of 3-linked α -D-galactose and 4-linked α -L-galactose units. The agarobiose molecule is often substituted to a greater or lesser degree with sulfate, O-methyl-ether and/or a pyruvic acid ketal group (ANDERSON, DOLAN & REES, 1965; ARAKI, 1966). Agar can thus be regarded as a family of galactans differing in variety, extent and relative proportions of substituted galactose units (DUCKWORTH & YAPHE, 1971). The characteristics and commercial usefulness of agar is highly dependent on the amount and location of these substituted groups or their structural composition (CRAIGIE, WEN & VAN DER MEER, 1984). Galactans are important for practical use due to their well-known gel-forming ability. This property is highly dependent on the polysaccharide structure. The gel-strength is changed considerably by small modifications to the polymer structure (REES, 1969).

Structural studies of polysaccharides are necessary in order to correlate them with their biological and physical properties. This knowledge helps to identify strains with superior agar qualities and yield, and in the search for other sources for new gelling substances.

Chemical modification of a given polysaccharide to improve its gelling properties is also possible (USOV, 1984). The elucidation of the polysaccharide structure should involve qualitative and quantitative analysis of monosaccharide components, non-carbohydrate substitutes, types of linkages between them, size of monosaccharide rings, anomeric configuration and the sequence of monomers and macromolecular configuration. Extensive studies of the structure and properties of agar from some *Gracilaria* species have been done using chemical, enzymatic, pyrolytic and spectroscopic methods (IZUMI, 1972; SANTOS & DOTY, 1983; USOV, 1984; JI, LAHAYE & YAPHE, 1985). Recently several new physico-chemical methods, such as ^1H ^{13}C NMR spectroscopy, have become powerful tools in structural analysis, permitting rapid and reliable identification of known structures or discovery of new structural features without splitting the polymeric molecules. Some of the investigations on the polysaccharides include the elucidation of the primary structure of sulfated galactans and neutral xylans, as well as configuration studies of galactan molecules in solution and gels (USOV, 1984).

1.1.3. EXTRACTION OF AGAR FROM ALGAE

Prior to agar extraction, the seaweed is air dried at 26°C without washing for approximately 48 h. The dried seaweed is then pulverized in a blender. There are several ways to extract the agar. These include washing and bleaching of the dried seaweed, extraction with hot water at a mildly acidic pH, gelation of the extract and denaturing of the gel by freezing, thawing or pressing and drying of the resulting agar product. These methods are discussed below in more detail as processing may have important consequences for growth regulator content.

1.1.3.1. Water extraction method (Native Agar)

Dried seaweed is soaked in distilled water overnight. The pH is adjusted (if necessary) to 6.0-6.5 and the product then autoclaved for 6 h at 121°C at a pressure of 1.1 kg cm². The extract is then filtered under pressure with hot water through cheesecloth and the residue washed once with hot water. Samples are then pressure filtered with nitrogen gas through celite using a 10 µm filter. The filtrate is cooled and frozen whereafter the sample is thawed in a plastic container perforated at the base to allow the thawed water

to drip from the container. The freeze-thaw process is repeated. The resulting wet agar is subsequently dried at 40°C to a constant weight. The final product is called Native Agar (BIRD & HINSON, 1992).

1.1.3.2. Akali pretreatment

Dried seaweed samples are treated with 1.0 N NaOH at 80°C for 1 h in a water bath and are then removed and filtered through cheesecloth. The algal material is washed copiously with tap water to remove the NaOH residue and returned into distilled water. The pH is adjusted to 6-7 and the sample left overnight at room temperature. The pH of the sample is readjusted to 6.0-6.5 prior to water extraction as described for the Native Agar.

NAIRN, FURNEAUX & STEVENSON (1995) noted that separation of the gelling agar component from water-soluble salts, small organic molecules, non-gelling polysaccharides and other polymers, which are present in commercial agar in varying amounts, is not completely achieved during the extraction processes. Thus these compounds could be present in various amounts in the agar and may affect agar purity.

1.1.4. NATURE OF GELRITE

The supply of agar producing seaweeds occasionally becomes limiting, causing agar prices to rise sharply. Seaweed cultivation is beginning to provide a more constant supply of these raw resources, but the current knowledge of desirable species and their associated agar characteristics limits the number of algal species used. However, it is thought that comparative studies of agar from different seaweeds may help to increase the variety of cultivated species that produce high quality agar (BIRD & HINSON, 1992). In this context, microbes, which produce reasonable amounts of good quality gelling agents, have been considered as an additional sources of gelling agents.

Gelrite (gellan gum) formally known as PS-60 or S-60 is a naturally derived, highly purified agar-like polysaccharide obtained from *Pseudomonas* species. Gelrite is a novel bacterial polysaccharide compared to gel-forming polysaccharides produced by other

bacteria. *Pseudomonas* species produce copious amounts of an exocellular heteropolysaccharide after incubation for 3 days at 30°C in medium containing 3% glucose as a carbon source (MOORHOUSE, COLEGROVE, STANDFORD, BAIRD & KANG, 1981). The polysaccharide is composed of approximately 46% glucose, 30% rhamnose, 21% uronic acid and 3% O-acetyl. The gel is a linear polymer comprising of glucuronic acid, glucose and rhamnose with glucose and rhamnose residues linked 1,4 (MOORHOUSE, COLEGROVE, STANDFORD, BAIRD & KANG, 1981). It is noted that the majority of glucose is β -linked. Upon deacetylation by a mild alkaline treatment it produces a brittle and optically dense, but clear gel which is thermoreversible. The gel exhibits excellent heat stability that withstands autoclaving (i.e. 121°C for 15 min) for several cycles. It displays several interesting properties including self-gelling, agar-like rigidity, compatibility with nutrient additives, thermal stability and optical clarity (KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982). The percentage decrease in gel strength is similar to that of seaweed derived agar during an initial autoclave cycle. This is significant as most media undergo one such sterilization cycle. Like agar it is also resistant to enzymatic degradation. It differs from agar in that a gel strength equivalent to that of agar can be obtained at half the agar concentration, which makes Gelrite more economical. As with Gelrite, gelation requires the presence of mostly monovalent or divalent cations (KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982).

The gel strength, melting point and setting point of Gelrite are controlled primarily by cation concentration. Monovalent and divalent cations promote gelation of the gel but divalent ions such as magnesium and calcium do so at much lower concentrations than monovalent cations. At calcium concentrations above 0.1%, gel strength begins to decline and the gel is no longer thermoreversible. Tests involving various culture media and biological test media indicate that it is an excellent alternative gelling agent to agar. Since there are a wide variety of microorganisms, it is expected that a wide variety of polysaccharides could be utilized as gelling agents (KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982; SHUNGU, VALIANT, TUTLANE, WEINBERG, WEISSBERGER, KOUPAL, GADEBUSCH & STAPLEY, 1983).

1.1.5. EXTRACTION OF GELRITE FROM *PSEUDOMONAS*

Manufacturing of Gelrite involves the production of potassium or sodium fermentation liquor of bacteria, which is maintained at pH 6.5 (preparation as specified by KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982). Three types of Gelrite can be produced from the liquor, namely native, deacetylated and clarified or purified.

1.1.5.1. Native Gelrite

The Native Gelrite is prepared by heating the fermentation liquor to 95°C and precipitating with two volumes of 99% iso-propanol.

1.1.5.2. Deacetylated Gelrite

With deacetylated Gelrite, the liquor is heated to 95°C, cooled to 80°C and the pH increased to 10. This condition is maintained for 10 min followed by neutralizing with sulphuric acid and precipitating with two volumes of 99% iso-propanol.

1.1.5.3. Clarified Gelrite

Clarified Gelrite is obtained by filtering the hot alkaline-treated fermentation liquor using a Pall Cartridge filter or Gelman membrane filter and then precipitating with 99% iso-propanol. Alternatively, the native Gelrite can be reconstituted to 1% concentration in deionized water followed by heating deacetylation, filtration and precipitation with 99% iso-propanol. A fibrous precipitate is recovered with sieves and dried in a forced-air tray drier at 55°C for 1h. One of the most noticeable differences between native and deacetylation Gelrite is the gel texture. Native Gelrite forms an elastic or a soft gel in the presence of cations whereas both deacetylation and clarified or purified Gelrite produces a firm nonelastic or brittle gel (KANG, VEEDER, MIRRASOUL, KANEKO & COTTRELL, 1982).

1.1.6. EFFECT OF EXTRACTION METHODS ON PLANT GROWTH REGULATORS IN RAW MATERIAL (ALGAE AND *PSEUDOMONAS*) DURING PREPARATION OF GELLING AGENTS

Many indoles including IAA, are fragile compounds that undergo non-enzymatic oxidation in the presence of light, oxygen or peroxides. Indole-3-methanol (IM) breaks down rapidly below pH 6.5 (SUNBERG, SANDBERG & JENSEN, 1985) as do indole-3-acetaldehyde (IAAld) and indole-3-pyruvic acid (PA) yielding a number of products including IAA (ATSUMI, KURASHI & HAYASHI, 1976; SWEETSER & SWARTZFAGER, 1978; HEMBERG & TILLBERG 1980; ALLEN, RIVIER & PILET, 1982). EPSTEIN, COHEN & BANDURSKI (1980) reported extensive enzymatic conversion of tryptophan (TRP) to an IAA-like substance. SANDBERG, CROZIER & ERNSTSEN (1987) stated that such processes could clearly affect the accuracy of IAA determinations. Low temperatures and the use of an antioxidant in the extraction medium however, significantly reduce the decomposition of IPA (IINO, YU & CARR, 1980). The use of an antioxidant, the avoidance of high temperatures and high light intensities reduces the formation of IAA from other indoles (ERNSTSEN, SANDBERG & CROZIER, 1986) and there is also a reduction in the breakdown of IAA to IM (ATSUMI, KURASHI & HAYASHI, 1976). This does not exclude the possibility of degradation of other substances. The rate of conversion of IM to a less polar unidentified substance was enhanced as acidity increased (SANDBERG, 1984; SUNBERG, SANDBERG & JENSEN, 1985). It is clear from the above that the high temperatures and pH conditions involved in the preparation of gelling agents will certainly cause the breakdown of those plant growth regulators in the raw materials which are unstable under such conditions. It is therefore unlikely that these compounds will be detected in gelling agents even though they may occur in the raw materials.

1.1.7. IMPORTANCE OF GELLING AGENTS IN TISSUE CULTURE

In tissue culture a solidified medium is almost indispensable for good callus formation and regeneration (HENDERSON & KINNERSLEY, 1988). Tissue culture media are usually solidified with agar in order to support explant and shoot clusters. Other explant

supports such as filter paper, cotton wool, polyester fleece, glass beads, silica gels, gelatin acylamide gels, starch co-polymers and alginate are used (MBANASO & ROSCOE, 1982). The results show that agar will not be completely replaced in the near future. Agar is widely used due to its convenient properties such as stability and resistance to metabolism during use. Agar may contain compounds that affect differentiation and development of cultured tissue (ANAGNOSTAKIS, 1974), hyperhydricity, and can cause toxic symptoms. Other gelling agents are either toxic or incapable of hydric control (NAIRN, FURNEAUX & STEVENSON, 1995).

The types and concentrations of gelling agents affect the growth of tissue cultures in various ways. For example somatic embryogenesis in cucumber (*Cucumis sativus* L. cv. Clinton) (LADYMAN & GIRARD, 1992), rooting of sweetgum (*Liquidamber styraciflua* L.) and shoot tip cultures (LEE, WETZSTEIN & SOMMER, 1986) are all affected by agar types and concentration. SELBY, LEE & HARVEY (1989) found enhanced organogenesis and elongation of shoots of Sitka spruce, when grown on relatively soft gels compared to more rigid ones. The softer gel allowed the cultures to have better contact with the medium resulting in improved uptake of water, growth regulators and nutrients from the culture medium. When radiolabelled benzyladenine [¹⁴C]BA was incorporated into the gelling medium, the uptake of the cytokinin by the explant was affected by the rigidity of the gel, irrespective of whether the gelling agent was of agar or non-agar origin (DEBERGH, 1983; BORNMAN & VOLGELMANN, 1984).

The major criteria in selecting the brand and the concentration of agar seems to be its availability and price. Often the use of agar as a solidifying agent in tissue culture media is taken for granted. DEBERGH (1983) showed a large range of solidity of media with increasing concentrations, not only within the type of agar, but also for the same concentration within different brands. In the case of the commonly used concentration of 10 g l⁻¹ agar, the work varied by as much as 42 joules between Difco Bacto, Difco Noble, Gibco Phytagar and Merck Agar (DEBERGH, 1983). This emphasizes the importance of specifying the brand and the type of agar used for a trial (DEBERGH, 1983).

Highly purified agar types from the same manufacturer gave better yields. Difco Noble

Agar proved to be extremely good and only a slight yield in embryo from anther culture of *Nicotiana tabacum* was observed when activated carbon was added to it indicating its purity (KOHLENBACH & WERNICKE, 1978). MACRAE & VAN STADEN (1990) reported that unless agar is highly purified or charcoal added to the medium, there is a potential danger of growth retardation or inhibition. Dialyzed agar or highly purified agar at low concentrations increased anther response in *Nicotiana tabacum* (WERNICKE & KOHLENBACH, 1977, KOHLENBACH & WERNICKE, 1978). This confirms the presence of inhibitors in commercial preparations of agar. Another consideration is that non-toxic compounds in agar may break down during autoclaving and become toxic (WEATHERHEAD, BURDON & HENSHAW, 1978).

1.1.8. INFLUENCE OF pH ON AGAR MEDIUM

Medium pH has a pronounced effect on the growth of tissue *in vitro* and influences some plant developmental processes (OWEN, WENGERD & MILLER, 1991). Thus, the selectivity of the culture media is pH dependent. Most agars have a pH between 5.5 and 6.0 (SARMA, MAESATO, HARA & SONODA, 1990). A pH range of 5.7-5.8 is suitable for maintaining all the salts in soluble form, even with relatively high phosphate levels, and permits rapid growth and differentiation of tissue (MURASHIGE & SKOOG, 1962). The pH of both liquid and semi-solid media decreases by a few pH units after sterilization by autoclaving (COUSSON & TRAN THANH VAN, 1981). Murashige and Skoog medium adjusted to various pH values decreased by 0.6-1.3 pH units after autoclaving. Post-autoclave pH drop was prevented by adding different buffers (PIERIK, 1987; AMINO & TAZAWA, 1988). However, some of these buffers were toxic to the plants, particularly those capable of being assimilated by plants (PIERIK, 1987).

Variation of post-autoclave pH depends on the method of agar addition, which influences the gel strength of the medium. The post-autoclave pH will also depend on the rate of cooling and heating, culture medium composition and sterilization temperature (OWEN, WENGERD & MILLER, 1991). These physical changes of the medium affect nutrient uptake and therefore may influence morphogenic responses (SARMA, MAESATO, HARA & SONODA, 1990). It has been noted that increasing agar

concentration improves the media setting properties. An agar concentration of 5 g l⁻¹ produced weak gels but these became more rigid with increasing pH. High agar concentrations and high pHs produce gels too hard for measurement of spreading radii. Sitka spruce [*Picea sitchensis* (Bong.) Carr.] shoot growth was greatest on weaker gels and inhibited on the more rigid gels (SELBY, LEE & HARVEY, 1989). Culture medium pH had little effect on adventitious shoot growth when 5 g l⁻¹ agars were used, whilst at higher liquid concentrations, increasing pH reduced shoot elongation. On the most rigid gels, the adventitious buds remained stunted and failed to develop into normal shoots. This effect was particularly pronounced at 10 and 12.5 g l⁻¹ agar and at pHs above 6.0 (SELBY, LEE & HARVEY, 1989).

1.1.9. EFFECT OF AUTOCLAVING ON AGAR COMPOSITION

Heat sterilization can induce profound changes in the culture medium components including decomposition of heat-labile nutrients, reactions between sugars and amino acids, pH changes and the formation of toxic compounds. During autoclaving of sucrose, 5-hydroxymethylfurfural was produced which inhibited the growth of *Nicotiana tabacum* cultures proving that inhibition can arise after autoclaving (WEATHERHEAD, BURDON & HENSHAW, 1978). This confirms the findings of TANAKA (1981) that 'drastic changes' in the state of a gel can be brought about by small changes in the external conditions of temperature and pH. However, ICHIMURA & ODA (1995) showed that the active substance in the extracted agar which stimulated shoot regeneration of tomato is a thermostable, highly hydrophilic compound. However, they were uncertain whether the hydrophilic substance of low molecular weight that stimulated rooting in lettuce ICHIMURA & ODA (1998) were identical to shoot regeneration substances present in the extracted agar.

1.1.10. AGAR RELATED PROBLEMS IN TISSUE CULTURE

Undesirable effects associated with agar have been noted, among which are impurities, batch-batch variability, phosphate binding and flocculation, inhibition of growth and impartment and impairment of hyperhydricity and cytokinin uptake. Certain types of

growth inhibitors are present in agar (ROMBERGER & TABOR, 1971; WERNICKE & KOHLENBACH, 1976; KOHLENBACH & WERNICKE, 1978; HU & WANG, 1983). Impurities introduced into agar are responsible for significant differences in the concentration of an element in comparable media with different levels of agar (DEBERGH, 1983). Cold-water soluble components in commercial agar have been reported to be responsible for toxicity and hydric control. Rooting has been promoted with or without lower agar concentrations in Norway spruce (VON ARNOLD & ERIKSSON, 1984) and apple (WERNER & BOE, 1980).

Hyperhydric transformation (where air between cells is replaced by water and the tissue has a glassy appearance) increases with lower concentrations of agar and limits the use of liquid culture in some species. Frequency of hyperhydricity in culture of *Prunus* and *Malus* spp. was reduced or lowered by 3-4 weeks storage at 3-4°C. Globe artichock gave a negative result under this cold treatment and the only way to overcome hyperhydricity in tissue-cultured artichoke was to increase the agar content of the medium. Though the main method of hyperhydricity prevention is increasing agar concentration, the occurrence of culture hyperhydricity varies from place to place irrespective of the fact that the same *in vitro* technique of culture and the same plants are used (DEBERGH, 1983). The above indicates that the prevention of hyperhydricity varies, and could also depend on the plant type and possibly the plant species.

1.2. CHEMICAL COMPOSITION OF SEAWEED

1.2.1. EVIDENCE OF PHYTOHORMONES IN ALGAE

There are numerous reports in the literature that microalgal cultures and seaweed extracts enhance plant growth. It is claimed that the efficacy is due to the presence of plant growth regulators (PGRs) produced by the algae. The application of seaweed extracts in terms of solid content and the active compound(s) shows that it is effective at very low concentrations (BRAIN, CHALOPIN, TURNER, BLUNDEN & WILDGOOSE, 1973; CROUCH, SMITH, VAN STADEN, LEWIS & HOAD, 1992). Organic compounds known to elicit strong physiological responses in low doses are plant hormones. Though

the major beneficial effect of seaweed extracts is attributed to cytokinins, the wide range of physiological responses elicited indicate the presence of other groups of plant growth regulators namely, auxins, GA, ABA and ethylene (FEATONBY-SMITH & VAN STADEN, 1983; NELSON & VAN STADEN, 1984; FINNIE & VAN STADEN, 1985; METTING, ZIMMERMAN, CROUCH & VAN STADEN, 1991; CROUCH & VAN STADEN, 1993). Initially, the presence of these hormones in seaweed extracts was demonstrated using paper chromatography (PC). Later confirmations were made using gas chromatography mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and enzyme-linked immunosorbent assays (ELISA) (BERNART & GERWICK, 1990; CROUCH, SMITH, VAN STADEN, LEWIS & HOAD, 1992; LUDWIG-MULLER & EPSTEIN, 1994).

1.2.1.1. Auxins

Algae applied to crops improve yield and plant vigour. This has been attributed to the auxin and cytokinin content of seaweed. Added IAA stimulated rhizoid formation in *Chara* (SIEVERS & SCHROTER, 1971). One of the significant growth responses elicited by seaweed concentrate application is the increase in root initiation. It is well established that both endogenous and synthetic auxins stimulate rooting (JACKSON & HARNEY, 1970; HARTMANN & KESTER, 1975) and that cytokinins inhibit rooting (VAN STADEN & HARTY, 1988; CROUCH, SMITH, VAN STADEN, LEWIS & HOAD, 1992).

Several auxin or auxin-like compounds have been detected in marine algae using bioassays, even though some of them are not supported by any confirmatory tests. MOWAT (1964a & b) reported the presence of IAA and indole-3-glycolic acid in unicellular algae and seaweed, based on paper chromatography. KINGMAN & MOORE (1982) detected IAA in an extract of *Ascophyllum nodosum* using Gas Liquid Chromatography (GLC) techniques. This was confirmed by SANDERSON, JAMESON & ZABKIEWICZ (1987) using GC-MS. The presence of indoles, such as IAA, indole-3-carboxylic acid (ICA), N, N-dimethyltryptamine, indole-3-aldehyde (IAld), iso-indole-1,3-dione (N-hydroxyethylphthalimide), detected in the active fractions of *Ecklonia maxima* (Osbeck) Papenfuss was confirmed by HPLC and GC-MS (CROUCH, SMITH, VAN STADEN, LEWIS & HOAD, 1992). IAA has been detected by GC-MS in *Sargassum*

heterophyllum and other algae (JACOBS, FALKENSTEIN & HAMILTON, 1985). In *Prionitis lanceolata*, 3-(hydroxyacetyl) indole was detected using NMR (BERNART & GERWICK, 1990). Other examples of endogenous auxins in algae are shown in Table 1.1. AUGIER (1976) reported IAA-like activity in algal extracts by bioassay, though the amount detected was usually very low compared to the amounts reported for higher plants. In *Caulerpa paspaloids*, IAA was detected by HPLC with an electrochemical detector and by capillary GC-MS. The amount of IAA detected was in the range reported for angiosperms (JACOBS, FALKENSTEIN & HAMILTON, 1985).

Table 1.1. Examples of algae in which auxins have been detected

Alga	Auxin detected	Method of detection	Reference
<i>Ascophyllum nodosum</i>	Auxin-like	PC	MOWAT (1964a & b)
<i>Ascophyllum nodosum</i>	IAA	GC-MS	SANDERSON, JAMESON & ZABKIEWICZ (1987)
<i>Caulerpa paspaloides</i>	IAA	GC-MS	JACOBS, FALKENSTEIN & HAMILTON (1985)
<i>Chlorella pyrenoidosa</i>	IAA, auxin-like	PC	MOWAT (1964a & b)
<i>Eckonia maxima</i>	IAA, ICA, IAId, <i>N,N</i> -dimethyltryptamine, <i>N</i> -hydroxyethyl phthalimide	HPLC, GC-MS	CROUCH, SMITH, VAN STADEN, LEWIS & HOAD (1992)
<i>Fucus vesiculosus</i>	Auxin-like	TLC	BUGGELIN & CRAIGIE (1971)
<i>Oscillatoria</i> sp.	Auxin-like	PC	MOWAT (1964a & b)
<i>Prionitis lanceolata</i>	Auxin-like	NMR	BERNART & GERWICK (1990)

However, several other reports have raised doubts as to whether IAA is an endogenous auxin of algae. BUGGELIN & CRAIGIE (1971) looked for bound IAA in 10 species of algae but found no evidence for it in their extracts using thin layer chromatography (TLC) and the Ehrlich colour test. BUGGELIN (1976) confirmed that auxins are not endogenous regulators of algae as synthetic auxins did not induce any clear extra

elongation with non-auxin analogues when tested on the growth of *Alaria*.

Root stimulating substances in the green algae compared with IAA, IPA and indole-3-acrylic (IACA) indicated that the dimer IACA behaves much like the indole which inhibits growth at high concentrations and promotes growth at low concentrations (NITSCH & NITSCH, 1956; RAUB CARDELLINA & SCHWEDE, 1987). There is no doubt that agar extracted from algae do contain other mineral nutrients and hormones, particularly auxins which this study is solely concerned with. These may be in the free form as they occur in the algae or be in a conjugated form, and the amount expected to be present in agar will always be lower than that found in the algae.

1.2.1.2. Cytokinins

The growth responses of many crops as a result of the application of seaweed and commercial seaweed preparations have been attributed to cytokinins, which are known to occur at relatively high levels in these organisms (PEDERSEN, 1973; BLUNDEN & WILDGOOSE, 1977; FEATONBY-SMITH & VAN STADEN, 1983; TAY, MACLEOD, PALNI & LETHAM, 1985). Cytokinins seem to play a significant role in the growth and development of unicellular and multicellular algae and several findings prove their occurrence in seaweed (MOONEY & VAN STADEN, 1986). *Trans*-zeatin (*tZ*), *trans*-zeatin riboside (*tZR*) and isopentenyladenine (iP) have been tentatively identified in the brown alga *Sargassum heterophyllum* (Turn) J. Ag using paper chromatography, HPLC and the soybean callus bioassay (MOONEY & VAN STADEN, 1987). The same cytokinins were detected in a commercial seaweed preparation of *Durvillea potatorum* (Labillardierre Areschoug) (TAY, MACLEOD, PALNI & LETHAM, 1985). Unequivocal identification of isopentenyladenosine (iPA) from *Sargassum muticum*, *Porphyra perforata* and *Chara globularis* using GC-MS (ZHANG, YAMANE, TAKAHASHI, CHAPMAN & PHINNEY, 1989; ZHANG, CHAPMAN, PHINNEY, SPRAY, YAMANE & TAKAHASHI, 1991) and Z, *cis*-ZR, dihydrozeatin (DHZ), iP, 2-hydroxy-6-methylaminopurine and 2-hydroxyl-methylzeatin by NMR (FAROOQI, SHUKLA, SHUKLA & BHAKUNI, 1990) confirmed the occurrence of cytokinins in seaweeds. Mass spectrometric stable isotope dilution methods were used to identify and quantify cytokinins such as *tZ*, *tZR* and their dihydro derivatives, iP, iPA and cytokinin glucosides in the seaweed extract Seasol (TAY, MACLEOD, PALNI & LETHAM, 1985; TAY, PALNI

& MACLEOD, 1987).

1.2.1.3. Gibberellins

Gibberellin-like compounds are also found in seaweeds (KATO, PURVES PHINNEY, 1962; MOWAT, 1963; HUSSAIN & BONEY, 1973). Gibberellin-like activity in *Enteromorpha prolifera* and *Ecklonia radiata* was detected using paper chromatography and the dwarf maize mutant seedling bioassay (JENNINGS, 1968). Using the lettuce hypocotyl bioassay, it was found that the amount of activity in three freshly made seaweed preparations (Maxicrop, Algifert and SM3) varied from 0.03 to 18.4 mg l⁻¹ (WILLIAMS, BRAIN, BLUNDEN, WILDGOOSE & JEWERS, 1976).

1.2.1.4. Ethylene

Using TLC and GC techniques, 1-amino-cyclo-propane-1-carboxylic acid was detected in seaweed concentrate prepared from the brown Kelp *Ecklonia maxima* (Osbeck) Papenfuss. The level of this ethylene-releasing compound was estimated as 9.29 nmol ml⁻¹ (NELSON & VAN STADEN, 1985).

1.2.1.5. Abscisic acid

Water-soluble compounds from *Laminaria digitate* and *Ascophyllum nodosum* detected using lettuce hypocotyl growth, and GLC showed properties similar to ABA (HUSSAIN & BONEY, 1973). Using GC-MS, ABA was identified in *Ascophyllum nodosum* (BOYER & DOUGHERTY, 1988).

1.2.2. MINERAL NUTRIENTS IN ALGAE

Marine algae contain all major and minor plant nutrients (STEPHENSON, 1968) and trace elements (YAMAMOTO & ISHIBASHI, 1972; YAMAMOTO, OTSUKA, OKAZAKI & OKAMOTO, 1979). The mineral element supplement and other ingredients in the seaweed concentrate improve the crops yield. Application of seaweed concentrate to lettuce plants, receiving an adequate supply of nutrients, enhanced the uptake of calcium, potassium and magnesium but had little effect on nutrient stressed plants (CROUCH, BECKETT & VAN STADEN, 1990). Application of seaweed to plants increased the availability of nitrogen (BECKETT & VAN STADEN, 1989), phosphorus

(DE VILLIERS KOTZE & JOUBERT, 1983), potassium (BECKETT & VAN STADEN, 1989), calcium (SENN & KINGMAN, 1978; DE VILLIERS KOTZE & JOUBERT, 1983), manganese (BLUNDEN, 1972), magnesium (SENN & KINGMAN, 1978) and zinc (BECKETT & VAN STADEN, 1990).

1.2.3. AMINO ACIDS IN ALGAE

All common amino acids occur in seaweeds (PELLEGRINI, 1969; MUNDA & GUBENSEK, 1975). Investigations on proteins, peptides and free amino acid content of marine algae have been based on the analysis of species collected at all stages of their growth. Free amino acids such as alanine, aspartic acid, glutamic acid, glycine and cystine-cysteine are present in *Laurencia papillosa* (Rhodophyta), *Cystoseira berbeta* (Phaeophyta) and *Enteromorpha intestinalis* (Chlorophyta) in roughly proportionate amounts (MOHSEN, KHARBOUSH, KHALEAFA, METWALLI & AZAB, 1975). However, there were considerable differences between the percentages of leucine, methionine, proline and glutamine in these species (MOHSEN, KHARBOUSH, KHALEAFA, METWALLI & AZAB, 1975)

1.3. IMPORTANCE OF PHYTOHORMONES IN ALGAE

Endogenously produced growth substances regulate the growth and development of higher plants and the same effect could be expected in algae. If the plant regulators have beneficial effects on algae, then some response might be seen when the putative substances are exogenously applied to algae. The evidence which is quite extensive, is both conflicting and equivocal (LANG, 1965; EVANS, 1984). Exogenous application of phytohormones to algal explants or whole plants indicate that these hormones play a role in algal growth and development, including processes of cell division and elongation, organogenesis, apical dominance, respiration and photosynthesis (MOONEY & VAN STADEN, 1986).

Auxin and kinetin stimulate growth of algae (JENNINGS, 1969, DAWES, 1971). However, BUGGELN (1976) could not observe any effect with the application of

exogenous auxin. Cytokinins promoted branching in *Fucus vesiculosus* and growth in some other marine algae but had no effect on other species. GA promoted cell division in *Ulothrix* and *Ulva* (EVANS, 1984). The auxins, p-hydroxy-phenylacetic acid (OH-PAA) and p-phenylacetic acid (PAA) induced branching and expansion of fronds in *Fucus spiralis* and increased the growth and weight of plantlets compared to the control. The same observation was made when this treatment was given to *Porphyra tenera* (FRIES, 1977; FRIES & IWASAKI, 1976). *In vitro* growth of *Ascophyllum nodosum* increased when IAA, iPA or Z were applied (FRIES, 1988). In axenic culture, *Enteromorpha compressa* formed tubular sporelings which developed into small bulbous thalli. Application of PAA and OH-PAA in concentrations of 10^{-7} - 10^{-5} M induced the thalli to stretch into real tubes. Kinetin ($100 \mu\text{g l}^{-1}$) had a similar effect, which was enhanced when applied in combination with PAA (FRIES & ABERG, 1978).

1.4. MICROBIAL BIOSYNTHESIS OF AUXIN

A wide variety of soil microorganisms are capable of producing physiological active auxin that may have a pronounced effect on plant growth and development. Microbes isolated from the rhizosphere of various crops appear to have great potential to synthesize and release auxins because of the rich supply found in the substrate (BROWN, 1972; BAREA, NAVARRO & MONTOYA, 1976; STRZELCZYK & POKOJSKA-BURDZIE, 1984). BAREA, NAVARRO & MONTOYA (1976) found that 86% of the bacteria isolated from the rhizosphere of various plants produced auxins in addition to other PGRs. Numerous pathogens are also producers of auxins and are known to cause hyperauxiny in infected plants (MAHADEVAN, 1984). Production of auxins by microbial isolates varies greatly amongst different species and strains of the same organisms and this is also influenced by culture conditions, growth stage and availability of the substrate (FRANKENBERGER & ARSHAD, 1995).

In vitro studies demonstrated that some microbial cultures could produce small amounts of IAA in the absence of a physiological precursor. SIMALY & BERSHOVA (1957) stated that under natural conditions it is possible that IAA is synthesized in greater quantities than in pure culture (DVORNIKOVA, SKRYABIN & SUVOROV, 1968).

1.4.1. EPIPHYTIC MICROORGANISMS

Plants are colonized by numerous epiphytic microflora which produce auxins and may contribute to the endogenous auxin pool of that plant. CHANDRAMOHAN & MAHADEVAN (1968) reported that epiphytic bacteria, fungi and actinomycetes produce IAA from TRP. Various bacteria have been demonstrated to produce IAA (WICHNER & LIBBERT, 1968a & b; LIBBERT & SILHENGST, 1970). Non-sterile pea, cucumber and maize contained more auxins than sterile plants. Reinfection with an IAA-producing bacterial strain restored the auxin content of the sterile plant (LIBBERT, KAISER & KUNERT, 1969).

1.4.2. *PSEUDOMONAS SYRINGAE* PV. *SAVASATANOI*

Pseudomonas species are the main sources of Gelrite. Early investigation demonstrated the ability of *P. syringae* pv. *savasatanoi* to produce IAA from TRP (HUTZINGER & KOSUGE, 1967). Later, molecular studies confirmed that bacteria contain two plasmid-borne genes *iaaM* and *iaaH*, with the enzymes TRP-2-monooxygenase (TOM) and IAM hydrolase respectively, that act in concert to produce IAM from TRP (COMAI & KOSUGE, 1980; COMAI, SURICO & KOSUGE, 1982; WHITE & ZIEGLER, 1991). The genes *iaaM* and *iaaH* maintain a substantial homology with their counterpart *tms1* and *tms2* from *Agrobacterium tumefaciens* (YAMADA, PALM, BROOKS & KOSUGE, 1985) and synthesize IAA from TRP by the IAM pathway (COMAI & KOSUGE, 1980; COMAI, SURICO & KOSUGE, 1982).

Cytokinin production by *P. syringae* pv. *savasatanoi* has also been reported (SURICO, SPARAPANO, LERARIO, DURBIN & LACOBELLIS, 1975; SURICO, EVIDENTE, LACOBELLIS & RANDAZZO, 1985; MACDONALD, POWELL, REGIER, GLASS, ROBERTO, KOSUGE & MORRIS, 1986; MORRIS, POWELL, BEATY, DURLEY, HOMMES, LICA & MACDONALD, 1986). In contrast with *A. tumefaciens*, *P. syringae* pv. *savasatanoi* produces galls without transformation of the host cells. Rather than DNA transfer, it exerts its pathogenic effects by virtue of close association with the host cells, at least in part, by secreting high amounts of phytohormones (COMAI, SURICO & KOSUGE, 1982; MORRIS, 1986 & 1987). SILVERSTONE, BOSTOCK, GILCHRIST

& KOSUGE (1990) suggested that IAA production enhances survival of *P. syringae* pv. *savasatanoi* in infected tissue. The pIAA (the plasmid that carries the IAA production gene) encodes an additional function that contributes to ecological fitness. In a trial using olive, oleander, ash, privet, phillyrea and jasmine plants most of the 131 strains of *P. syringae* pv. *savasatanoi* used (except from those isolated from ash) produced auxins and harbored the *iaa* gene. It was concluded that the cause and effect relationship responsible for pathogenesis by *P. syringae* pv. *savasatanoi* varies from host to host (GARDEN, DAVID, MOREL, GLICKMANN, ABU-GHORRAH, PETIT & DESSAUX, 1992). Table 2.1 gives examples of auxin producing *Pseudomonas* species.

Table 1. 2. Examples of *Pseudomonas* species in which auxins have been detected

Species	Auxins	Method of detection	Reference
<i>Pseudomonas</i> spp.	IAA	PC	BAREA, NAVARRO & MONTOYA (1976)
<i>Pseudomonas</i> spp.	IAA	PC	BROWN (1972)
<i>P. caryophylli</i>	IAA, IAId, ILA	TLC, HPLC, GC-MS	FETT, OSMAN & DUNN (1987)
<i>P. fluorescens</i>	IAA	Colourimetry	ASTRÖM, GUSTAFSSON & GERHARDSON (1993)
<i>P. syringae</i> pv. <i>glycinea</i>	IAA, IAId, ILA	TLC, HPLC,, GC-MS	FETT, OSMAN & DUNN (1987)
<i>P. syringae</i> pv. <i>phaseolicola</i>	IAA	TLC, HPLC, GC-MS	FETT, OSMAN & DUNN (1987)
<i>P. syringae</i> pv. <i>syringae</i>	IAA, IAId, ILA, IAM	TLC, HPLC, GC-MS	FETT, OSMAN & DUNN (1987)

1.5. AUXIN AS A ROOT STIMULATING FACTOR

IAA was the first phytohormone to be isolated and identified. It is naturally present in all plants. IAA is active in a range of bioassays at submicrogram concentrations and any compound that exhibits IAA-like activity is referred to as an auxin. It has been implicated in many processes of plant growth and development including stem elongation, root initiation, ethylene biosynthesis and tissue vascularization (DAVIES, 1995). Indoles other than IAA are thought to represent an alternative source of precursors for IAA and are only active when converted to IAA. However, it is thought that a number of indole compounds may be active compounds, biosynthetic intermediate degradative products, storage or transport forms and detoxification products or artifacts. IAA can exist in plant tissue either as free or bound forms. The readily extractable auxins are referred to as free auxins. The bound forms are those liberated from tissues when subjected to enzymolysis, hydrolysis or autolysis. IAA being a free auxin, is readily utilized during growth. Bound auxins are usually considered as storage forms from which IAA or detoxification products are formed as a result of relatively high levels of IAA (MOORE, 1979).

It is well established that both endogenous and synthetic auxins stimulate rooting (JACKSON & HARNEY, 1970; HARTMANN & KESTER, 1975). A number of indole compounds and phenylacetic derivatives have been reported with auxin activity. Indole butyric acid (IBA) and naphthalene acetic acid (NAA) are used commercially for initiation of rooting especially in certain difficult-to-root cuttings (FRANKENBERGER & ARSHAD, 1995).

Rooting of stem cuttings was one of the first uses of auxins and the most commonly used auxin is IBA. It is considered to be the strongest root promoter while NAA and 2,4-dichlorophenoxy acetic acid (2,4-D) are also known to promote root development. However, 5,6-dichloroindole-3-acetic acid methyl ester (5,6-Cl₂-IAA-Me) and 3-((benzyl)senenieryl) acetic acid (BSAA) are more effective than IBA in stimulating adventitious root formation of mungbean hypocotyls. IBA is particularly effective at lower concentrations (PAN & TIAN, 1999). Endogenous rooting factors other than auxins, are

also produced by leaves or buds. Such factors are believed to occur in easy-to-root plants (RICHARD, 1964). Many difficult-to-root-plants fail to respond to auxins and other root promoting substances or a combination of them (TRUELSEN, 1961).

1.6. EFFECT OF AUXINS IN THE PRESENCE OF OTHER COMPOUNDS

Synergism exists between auxins and different phenolic compounds. Phenol does not show any root promoting effects, but when they are combined with compounds with an indole nucleus synergism appears. The rooting factor detected in the basal sections of pear hard-wood cuttings was believed to be a condensation product between exogenous IBA, and a phenolic compound produced by physiologically activated old Home pear buds (FADL & HARTMANN, 1967).

1.7. BIOSYNTHESIS OF IAA

IAA is active in a range of bioassays at submicrogram concentrations and it is believed to be an important endogenous regulator controlling such processes as cell enlargement and cell division. The primary IAA precursor is TRP, with concentrations, of an order of magnitude greater than IAA (SCHNEIDER & WIGHTMAN, 1978). Using the *Avena* coleoptile bioassay, a hypothetical pathway for IAA biosynthesis was initially proposed that indicated that tryptamine and not tryptophan was involved because the latter was inactive (WINTER, 1966). However, using *Avena sativa* as well as *Zea mays* and *Pisum sativum*, it was shown that indole cannot be converted to IAA without tryptophan as an intermediary (ERDMANN & SCHIEWER, 1971; HEERKLOSS & LIBBERT, 1976). The proposed pathways are indicated in Figures 1.1 and 1.2.

1.7.1. TRYPTAMINE PATHWAY

The main pathway of IAA biogenesis proceeds from TRP via IAl_d. Two different intermediaries between TRP and IAl_d are tryptamine and IPA. The tryptamine pathway involves decarboxylation of TRP followed by oxidative deamination of IAl_d. TRP

decarboxylation appears unknown in intact plants but through *in vitro* studies it has been detected in cell-free preparations from tomato and barley shoots (GIBSON, BARRETT & WIGHTMAN, 1972; GIBSON, SCHNEIDER & WIGHTMAN, 1972). In walnut, tryptamine is involved in the formation of 5-hydroxytryptamine (serotonine) (GROSSE & KLAPHECK, 1979).

1.7.2. INDOLE-3-PYRUVIC ACID PATHWAY

The IPA pathway is probably the most predominant route for IAA production in higher plants and requires deamination of TRP to IPA followed by decarboxylation (SCHNEIDER & WIGHTMAN, 1978). IPA is very unstable and can break down during sample extraction and purification to yield a number of products including IAA. Therefore, the conversion of IPA to IAA in plant tissue might possibly be attributed to this conversion rather than enzymatic conversion. Extensive non-enzymatic conversion of TRP to an IAA-like substance has also been reported (EPSTEIN, COHEN & BANDURSKI, 1980). Such a process could affect the accuracy of IAA determination. McDOUGALL & HILLMAN (1978) stated that exogenous IAA affects the pattern of a large number of developmental phenomena and this is taken as an indication that IAA could act in concert with other substances known to influence growth.

1.7.3. TRYPTOPHAN-INDEPENDENT PATHWAY

In some plants the importance of TRP as an IAA precursor is minor (BALDI, MAHER, SLOVIN & COHEN, 1991). These plants that cannot make TRP are able to synthesize IAA *de novo* (WRIGHT, SAMPSON, NEUFFER, MICHALCZUK, SLOVIN & COHEN, 1991). In a study of IAA biosynthesis in *Lemna*, the incorporation of label [¹⁵N]-anthranilate and [²N₃]-TRP into IAA and TRP was simultaneously measured. Labelled anthranilate rapidly goes into IAA and TRP. This conversion to the IAA pool by [¹⁵N]-anthranilate slightly precedes that to the TRP pool. This confirms that more than one route to IAA exist in these plants (RAPPARINI, COHEN & SLOVIN, 1999). Orange pericarp (*orp*) is a seedling lethal mutant of maize, caused by mutations in the duplication of unlinked recessive gene *orp1* and *orp2* (WRIGHT, MOEHLENKAMP,

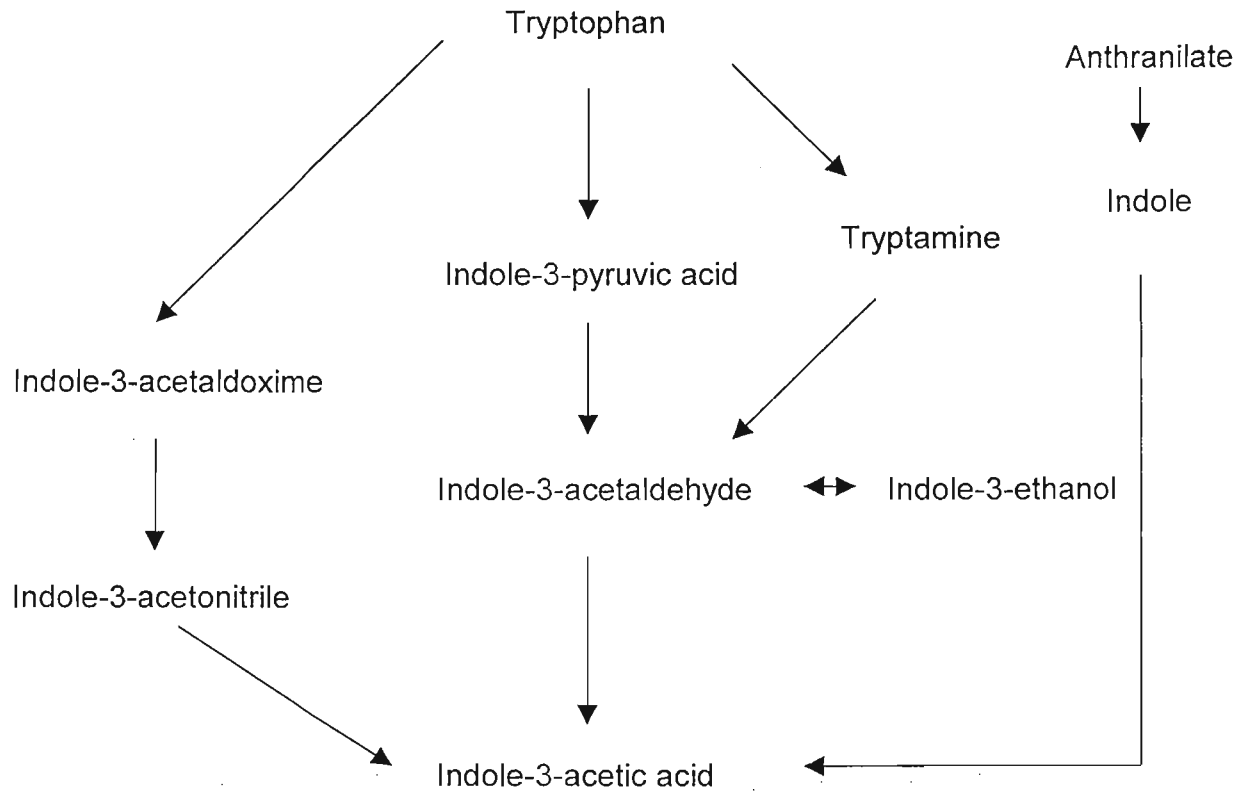


Figure 1. 1. Tryptophan and non-tryptophan pathways in plants thought to be involved in the biosynthesis of IAA. Adapted from SLOVIN, BANDURSKI & COHEN (1999); LUDWIG-MULLER & COHEN (2002).

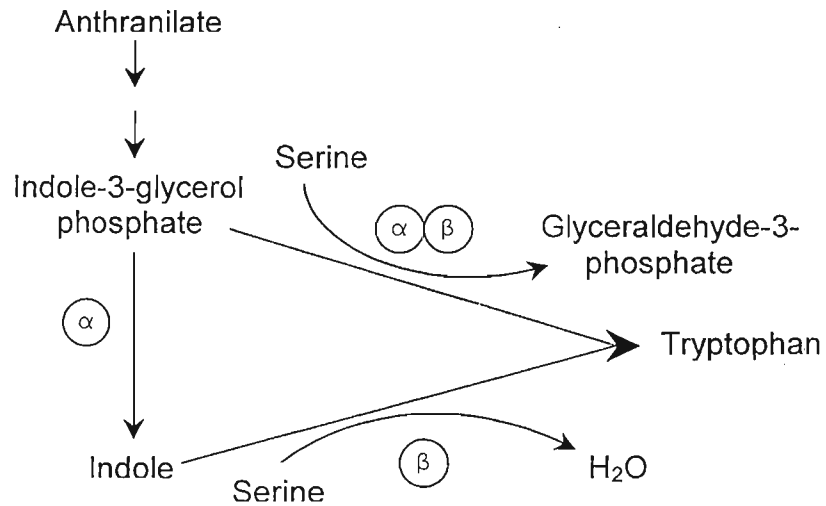


Figure 1.2. Tryptophan biosynthesis from anthranilate. The three enzymatic steps at the end of the pathways are catalyzed by different forms of tryptophan synthase. The subunits α and β of tryptophan synthase are indicated. Adapted from WRIGHT, MOEHLENKAMP, PERROT, NEUFFER & CONE (1992).

PERROT, NEUFFER & CONE, 1992). Studies have shown the *orp* to be defective in TRP synthase β activity which is necessary to convert indoles to TRP (WRIGHT, MOEHLENKAMP, PERROT, NEUFFER & CONE, 1992). Orange pericarp produces IAA *de novo* and accumulates up to 50 times the level of IAA found in wild type seedlings. Labelling studies established that the *orp* mutants are able to convert some ^{15}N -anthranilate to IAA but not to TRP. Neither *orp* seedlings nor control seedlings convert TRP to IAA in significant amounts, even when the *orp* seedlings are fed levels of labelled TRP high enough to reverse the lethal effects of the mutation (WRIGHT, SAMPSON, NEUFFER, MICHALCZUK, SLOVIN & COHEN, 1991). Strong additional evidence for IAA production via a TRP-independent pathway was observed in an *in vitro* experiment using maize seedlings where the conversion of [^{14}C] indole to IAA was not inhibited by unlabelled TRP, [^{14}C] TRP or [^{14}C] serine substituted for [^{14}C] indole (ÖSTIN, LLIĆ & COHEN, 1999).

1.8. THE NEED TO ISOLATE GROWTH STIMULATING COMPOUNDS FROM GELLING AGENTS

The role of gelling agents as neutral supporting material for plants in tissue culture is highly emphasized, but the influence on plant growth in terms of nutrients and plant growth regulator supply need to be considered. A knowledge of the types and amounts present and a detailed procedure for isolation, identification and quantification of nutrient and growth stimulatory compounds in any particular batch of gelling agent will be valuable to micro-propagators in the choice, type and amount of nutrients and plant growth regulators that must be added to any particular medium. The beneficial aspects of this is that it will alleviate inhibition and growth retardation from lethal media resulting from the addition of nutrients and growth regulators that already exist in sufficient quantities in the gelling agents to give optimum growth of explants. In some cases supplementation of the medium with plant growth regulators will enhance normal growth, while at other times it may not be necessary. Though levels of growth stimulatory compounds in algae are low compared to angiosperms, it is possible that this low amount present in the gelling agents will be exceedingly soluble in water and active enough to stimulate growth. Our interest therefore, was to determine the presence,

identity and quantity of root stimulatory substance(s) in some commonly used commercial gelling agents.

1.9. OBJECTIVES

The aims of the study were:

1. To determine the different types and concentrations of gelling agents which stimulate rooting in mung beans; and
2. To isolate and identify the root stimulatory compounds from these gelling agents.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. GELLING AGENTS FOR INITIAL SCREENING

The gelling agents listed in Table 2.1 were used in the initial screening. They were chosen as they are routinely used in tissue culture.

Table. 2.1. Gelling agents for initial screening

Agar type	Batch number	Manufacturer	Country
Agar-Agar Powder	Batch No. 9465826	Associated Chemical Enterprise	Republic of South Africa
Agar Bacteriological No.1	Lot D 7039-9-2	Oxoid Ltd	Basingstoke, Hampshire, England
Agar Bacteriological	Batch No.1016116	Biolab Diagnostic (PTY) Ltd	Fedsure, Midrand, Republic of South Africa
Agar Commercial Gel	Lot 87125	Biolab Diagnostic (PTY) Ltd	Fedlife, Midrand, Republic of South Africa
Agar Technical No.3	Lot 35313145	Oxoid Ltd	Basingstoke, Hampshire, England
Difco Bacto-Agar	Batch No. 0140-01	Difco Laboratories	Detroit, Michigan, USA
Difco Bitek™-Agar	Batch No. 0140-01	Difco Laboratories	Detroit, Michigan, USA
Gelrite	BK/CC Reg No: CC86/11094/23	Labretoria, Waterkloof	Pretoria, Republic of South Africa
Malt Extract Agar	Lot 40	Biolab Diagnostic (PTY) Ltd	Fedsure, Midrand, Republic of South Africa
Malt Extract Broth	Lot/ CH-B= No. 15556308	Unipath Ltd	Basingstoke, Hampshire, England
Mueller-Hinton Agar	Batch No. 10007760	Biolab Diagnostic (PTY) Ltd	Fedsure, Midrand, Republic of South Africa
Nutrient Broth Oxoid	Batch No. 34332304	Oxoid Ltd	Basingstoke, Hants, England
Nutrient Agar	Lot No. 24538389	Oxoid Ltd	Basingstoke, Hants, England

2.2. EXTRACTION OF AGAR WITH WATER

Twenty grams of gelling agent were suspended in 500 ml distilled water and stirred for 1 h at room temperature using a magnetic stirrer. The gelling agent extracts were filtered through Whatman No.1 filter paper and then filtered using a 0.45µm millipore filter. The residues were further extracted with 200 ml distilled water, filtered as above and the two gelling agent extracts combined. The pH was recorded. The filtrate was then dried *in vacuo* at 45°C using a rotary evaporator and the residue air dried. The residue was resuspended in 200 ml distilled water.

2.3. MUNGBEAN BIOASSAY

2.3.1. GROWING OF MUNGBEANS

Mungbeans (*Vigna mungo* L.) seeds were surfaced decontaminated by soaking in 3.5% (m/v) sodium hypochlorite for 20 min and then rinsed well in running tap water. Seeds were soaked in tap water to cover only half their surfaces for 6 h at room temperature to ensure adequate aeration. Seeds were then planted on moist vermiculite in trays (26.5 X 17.8 X 6.6 cm) and allowed to germinate in a Conviron (growth chamber) at 26°C with a 16 h light and 8 h dark cycle and a light intensity of 160 µmol m⁻² s⁻¹. After 10 days, uniform hypocotyl cuttings of approximately 12 cm in length with two primary leaves were prepared from the seedlings and used in the mungbean bioassay.

2.3.2. PREPARATION OF TEST SOLUTIONS

2.3.2.1. Gelling agents

Full strength solution (100%) of each gelling agent extract was made by resuspending each air-dried sample in 200 ml distilled water from which ten concentrations were prepared: 100%, 50%, 20%, 10%, 5%, 2%, 1%, 0.5%, and 0.25% respectively. Twenty ml of each solution were measured into test tubes (diameter 2.5 cm, 10 cm long). Each concentration was replicated four times.

2.3.2.2. IBA standard

IBA was used as a standard at concentrations of 10^{-7} - 10^{-3} M. Twenty ml of each solution were measured into test tubes (2.5 x 10 cm long). Each concentration was replicated four times and bioassayed. Water was used as control.

2.3.3. TREATMENT WITH TEST SOLUTIONS

Five mungbean cuttings were placed in each test solution for 6 h at 25°C at a light intensity of $160 \mu\text{mol m}^{-2}\text{s}^{-1}$. The total number of cuttings per concentration tested were 20 (4 x 5 cuttings).

2.3.4. ROOTING QUANTIFICATION

After treating the mungbean cuttings with the test solutions for the 6 h, the bases of the cuttings were rinsed with tap water to remove any residual solution and then transferred to a second set of clean test tubes containing 20 ml tap water. These were left in the Conviron for 10 days at 25°C with a 16 h light, 8 h dark cycle, and a light intensity of $160 \mu\text{mol m}^{-2}\text{s}^{-1}$. Tap water was added when necessary to ensure good growth. The number of roots on each cutting was counted after 10 days. The means and standard errors for each treatment were calculated and interaction within treatments determined using Minitab™ Statistical Software, (Release 13.1 (2000), Minitab Inc; PA, USA).

CHAPTER 3

DETECTION OF ROOT STIMULATING SUBSTANCES IN GELLING AGENT EXTRACTS

3.1. INTRODUCTION

3.1.1. GENERAL ROOTING AND THE INFLUENCE OF PHYTOHORMONES

Rooting of stem cuttings of many species is regulated by auxin polarly transported from the growing apex or from growing buds (WENT & THIMANN, 1937). Generally, removal of the growing apex and the buds decreased rooting although there are exceptions to the rule (BIRAN & HALEVY, 1973 a & b; HUSS-DANELLE, ELIASSON & ÖHBERG, 1980).

Adventitious rooting has been considered as a single-phase process in which auxins play a major role. However, evidence accumulated over the years provide arguments leading to a separation of this developmental process into successive, but interdependent, physiological phases with different requirements. These rooting phases are: 1) induction, defined as the time period which precedes any histological event; 2) initiation, when cell divisions initiate the formation of internal root meristems; and 3) expression, where there is internal growth of root-primordia followed by root emergence (KEVERS, HAUSMAN, FAIVRE RAMPANT, EVERS & GASPAR, 1997).

There are changes in endogenous IAA levels in cuttings during these three successive rooting phases. There is always a transient increase in the endogenous level of free IAA during the inductive phase, followed by a decrease to reach a minimum at the initiation phase, and a renewed increase during the expression phase (BELLAMINE, PENEL, GREPPIN & GASPAR, 1998). Root formation is only attributed to the inductive phase

by many researchers and an increase in IAA content has been associated with rooting (GASPAR, KEVERS & HAUSMAN, 1997). However, good rooting in terms of both the quality of the root system and the percentage rooting within a population of shoots depends on the optimal progress of each of the three successive phases. Improvement in rooting has been obtained using different rooting media designed to promote each of the three physiological phases (BERTHON, BEN TAHAR, GASPAR & BOYER, 1990; JAY-ALLEMAND, CAPELLI & CORNU, 1992). However, one hormone may play several roles at different phases of a single physiological process (BELLAMINE, PENEL, GREPPIN & GASPAR, 1998).

Calcium is one of the few mineral nutrients which influence rooting (GASPAR COUMANS, 1987). It has been shown to be fundamentally involved in cell division and root primordial elongation. Calcium is essential in the expression phase of rooting either as a single mineral or as a secondary messenger in the action of auxin (BELLAMINE, PENEL, GREPPIN & GASPER, 1998). Polarly transported substances regulate rooting of stem cuttings of many species. Exogenous application of auxin usually restores the capacity to form roots after detopping and debudding (VAN OVERBEEK, GORDON & GREGORY, 1946). The rooting response of cuttings to exogenous auxins is influenced by auxin concentration as well as the duration of the treatment (SMITH & THORPE, 1975; JAMES 1983). IBA has to be present in the solution for several days in order to stimulate rooting.

Compared to IAA, IBA is more effective in the promotion of rooting in a wide variety of plants and is used commercially to root many plant species world wide (WEAVER, 1972; WIESMAN, EPSTEIN & RIOV, 1988). IBA applied as a rooting solution stimulates rooting and also counteracts decreased rooting caused by unfavourable light conditions. ELIASSON (1980) noted that the effect of IAA was of a complex nature with a decrease in the number of roots formed within a certain concentration range. Adventitious root production by hypocotyl cuttings of mungbean seedlings has been extensively used in the past to detect and measure substances which promote rooting (KAWASE, 1964; PAN & ZHAO, 1994). An important aspect of the mungbean rooting test is its reported insensitivity to IAA except in the presence of one or more groups of

substances generally called rooting cofactors (HESS, 1961). Thus, IBA rather than IAA was used in this trial as a standard.

Research in the area of rooting by gelling agent extracts used routinely in micropropagation is limited. The development of adventitious shoots of *Picea abies* (L) Karst. was affected by the agar concentration in the culture medium. Increasing the agar concentration from 0.5% to 2.0% decreased hyperhydricity but at the same time reduced shoot growth and rooting potential (VON ARNOLD & ERIKSSON, 1984). WENT (1929) observed that the leaves and buds on stem cuttings of *Acalypha wilkesiana* Muell. Arg. promoted rooting and assumed that they were the sources of the originally hypothetical root forming phytohormones (rhizocaline). He established a bioassay using etiolated pea epicotyls. Since then, other bioassays of importance have been developed, these include the *Avena* straight growth test (NITSCH & NITSCH, 1956), lettuce root growth test (RAUB, CARDELLINA & SCHWEDE, 1987), Salkowski's colour test (PAECH & TRACEY, 1955) and the mungbean root initiation bioassay (HESS, 1961). Techniques which have been used in conjunction with the above mentioned bioassays for isolation and characterization of the phytohormones, include GC-MS, HPLC and ELISA. (Section 1.8.2.). The mungbean rooting bioassay is very suitable for detecting physiological activity and monitoring the purification of root stimulating substances (PAN & GUI, 1997; WIESMAN & RIOV, 1994; PAN & TIAN, 1999). It provides a considerable degree of reproducibility of statistically satisfactory results. Thus its routine use in the present study.

FADL & HARTMANN (1967) found that the putative auxin-phenol conjugate (condensation product between exogenous auxin (IBA) and a phenolic compound produced by physiological active Old Home pear buds) promoted rooting in mungbean cuttings. However, WILSON & VAN STADEN (1990) stated that since such a large diversity of compounds have promotory activity in the mungbean bioassay, the physiological significance of this observation is unclear. In addition to phenolics, it was thought that certain promoters act in concert with auxins (WILSON & VAN STADEN, 1990). The bioassay developed by HESS (1964) has been used to infer the presences of rooting promoters and inhibitors in plant extracts. High promotory activity was found

in plant extracts with good rooting ability (GESTO, VAZQUEZ & VIEITEZ, 1977; RAVIV & REUVENI, 1984) while high inhibitory activity was found in plant extracts with poor rooting ability (LIPECKI & DENNIS, 1972; BIRAN & HALEVY, 1973b). Both high promotory and low inhibitory activities were associated with good rooting ability (RICHARD, 1964).

Some gelling agents do contain additives such as salt and peptones which stimulate root and shoot growth. Peptones are protein derivatives that are water soluble. They are not coagulated by heat and do not precipitate on saturation of the solutions with ammonium sulphate. Peptones may provide one or both of the following: nitrogen, amino acids, including tryptophan (EDDLEMAN, 1999).

3.2. AIM

The aim of this study was to investigate whether aqueous extracts of gelling agents stimulate rooting of mungbean cuttings.

3.3. MATERIALS AND METHODS

3.3.1. EXTRACTION OF GELLING AGENTS

The thirteen gelling agents listed in Table 2.1. (Section 2.1) were extracted as described in (Section 2.2) and screened for biological activity using the mungbean bioassay described in Section 2.3.

3.4. RESULTS

Table 3.1 shows physical characteristics of the 13 gelling agents they are grouped according to poor, intermediate and good rooting patterns in the mungbean assays. The three categories of gelling agents were based on the highest average rooting per concentration as follows: 1) poor, rooting below 20, comparable to rooting in water (control) and the lower concentrations of IBA standard at 10^{-6} - 10^{-7} M; 2) intermediate,

rooting at 20-30, comparable to rooting in IBA standard at 10^{-5} - 10^{-4} M; and 3) good, rooting above 30 roots, comparable to rooting in IBA standard at 10^{-3} M. The results are graphically presented in Figures 3.1, 3.2 and 3.3 respectively. The rooting of the IBA standard is shown in Figure 3.1F and the water (control), which is the zero concentration, is indicated in all the graphs.

Gelrite was one of the poorest rooting gelling agents and was the only gelling agent for which extraction was problematical. A similar rooting pattern in mungbean cuttings was noticed in Figures 3.2 and 3.3 where an increase in concentration of gelling agent extract increased rooting, except for Mueller-Hinton Agar (Figure 3.2D) where the optimum concentration for rooting was in the range of 10-20% and was comparable to 10^{-4} M and 10^{-5} M of IBA.

Table 3.1. Characteristics and problems associated with extraction of the gelling agents screened. The gelling agents were grouped into those that gave poor, intermediate and good rooting

Gelling agents		Characteristics and problems associated with extractions
POOR ROOTING		
Agar Technical No.3	-	Used widely in tissue culture
Gelrite	-	Highly purified, widely used in tissue culture. Difficult to extract in water (personal observation)
Nutrient Broth Oxoid	-	May contain blood serum and sugar
Nutrient Agar	-	May contain 10% blood and other biological fluids
Malt Extract Broth	-	Contains malt
INTERMEDIATE ROOTING		
Agar-Agar Powder	-	No information on composition
Agar Bacteriological No.1	-	Low salt level
Agar Commercial Gel	-	Widely used in tissue culture
Mueller-Hinton Agar	-	Meat infusion and casein hydrolysate present
GOOD ROOTING		
Agar Bacteriological	-	Special grade of agar purified for general microbiological use
Difco Bacto Agar	-	Purified agar, with low levels of extraneous matter, pigmented portions and salts
Difco BiTek™ Agar	-	Not much information on composition
Malt Extract Agar	-	Soypeptone present

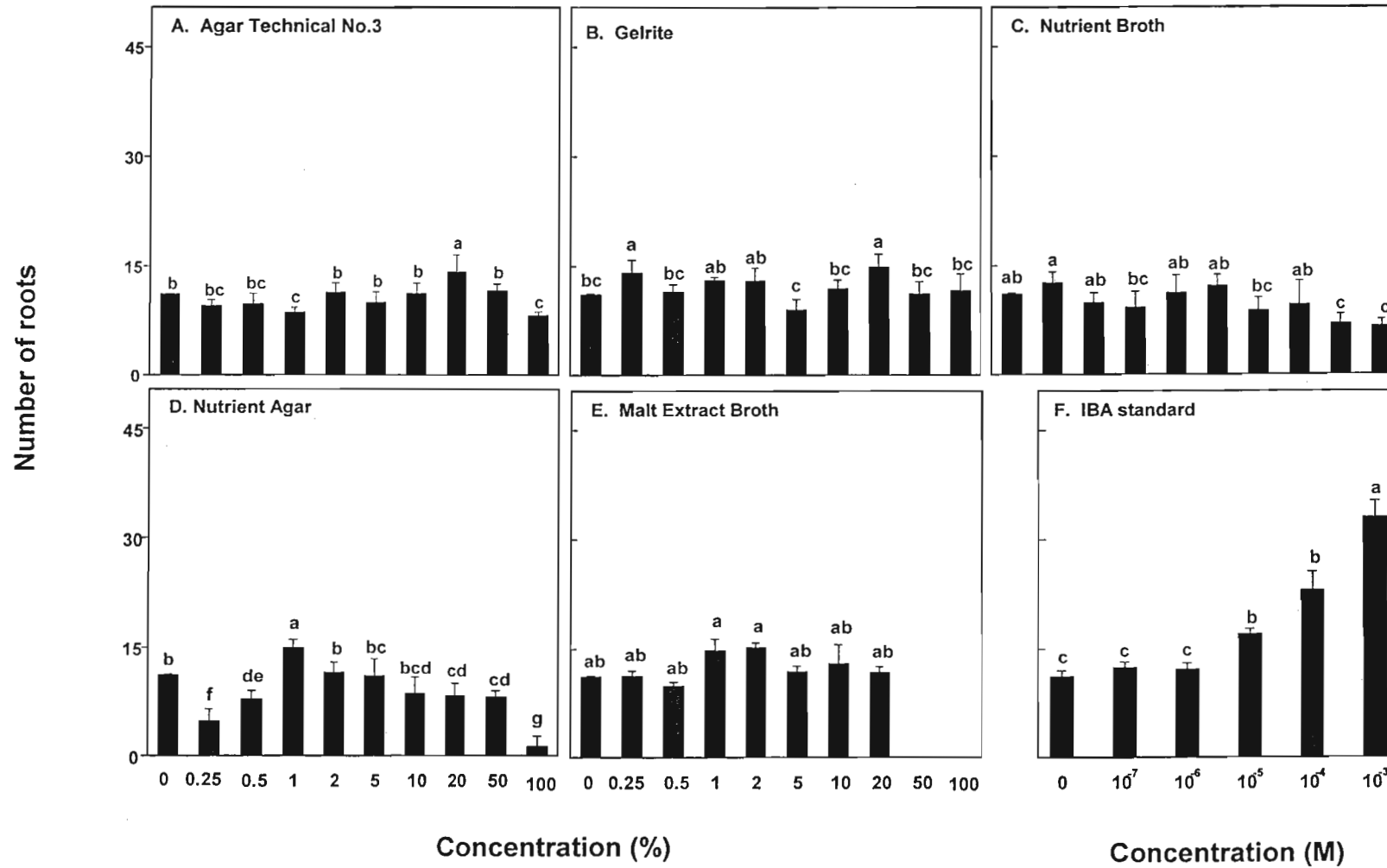


Figure 3.1. Average number of roots produced by mungbean cuttings with various concentrations of gelling agents which gave a poor rooting response. Different letters indicate significant differences ($p=0.05$) within each gelling agent. IBA was used as standard (Figure 3.1F).

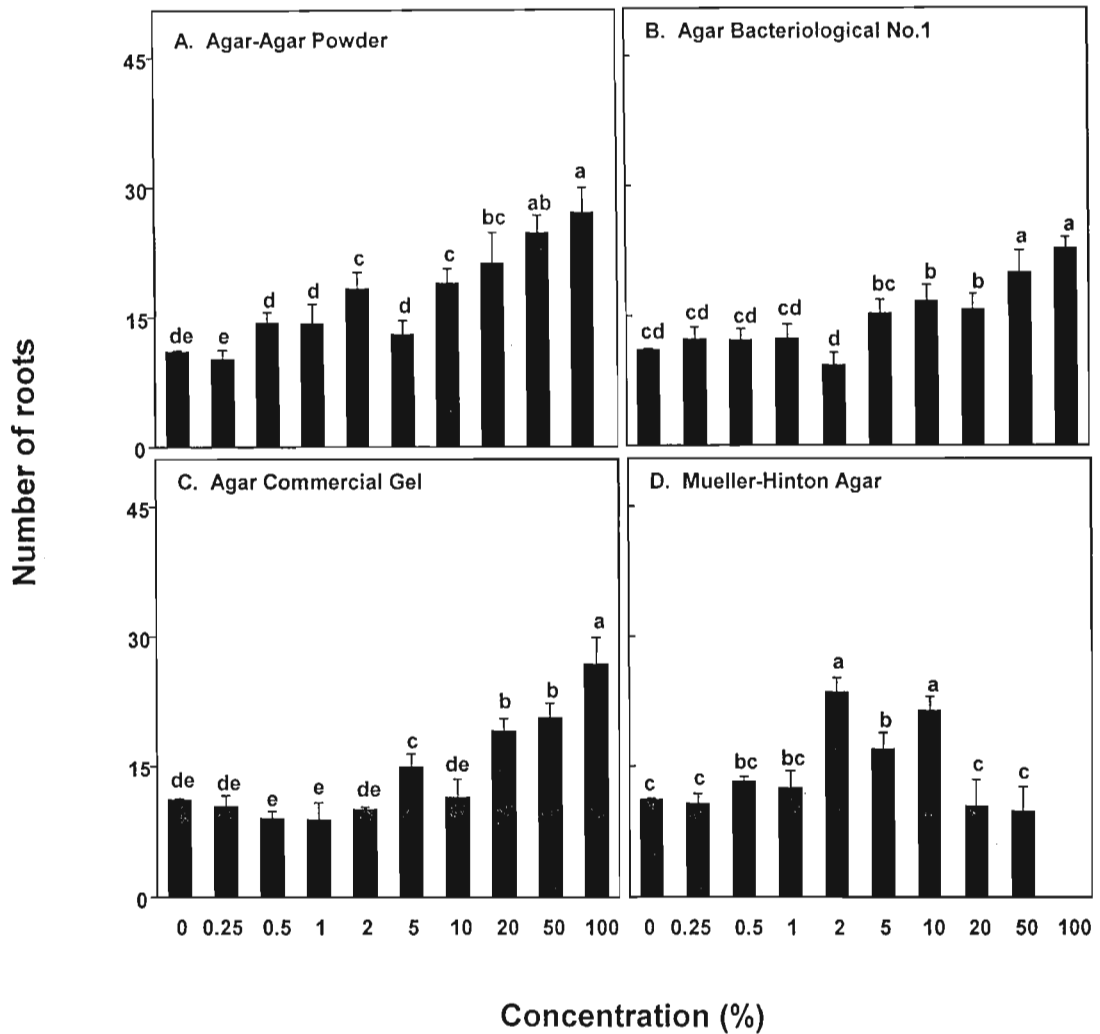


Figure 3.2. Average number of roots produced by mungbean cuttings with various concentrations of gelling agents which gave an intermediate rooting response. Different letters indicate significant differences ($p=0.05$) within each gelling agent. IBA was used as standard (Figure 3.1F).

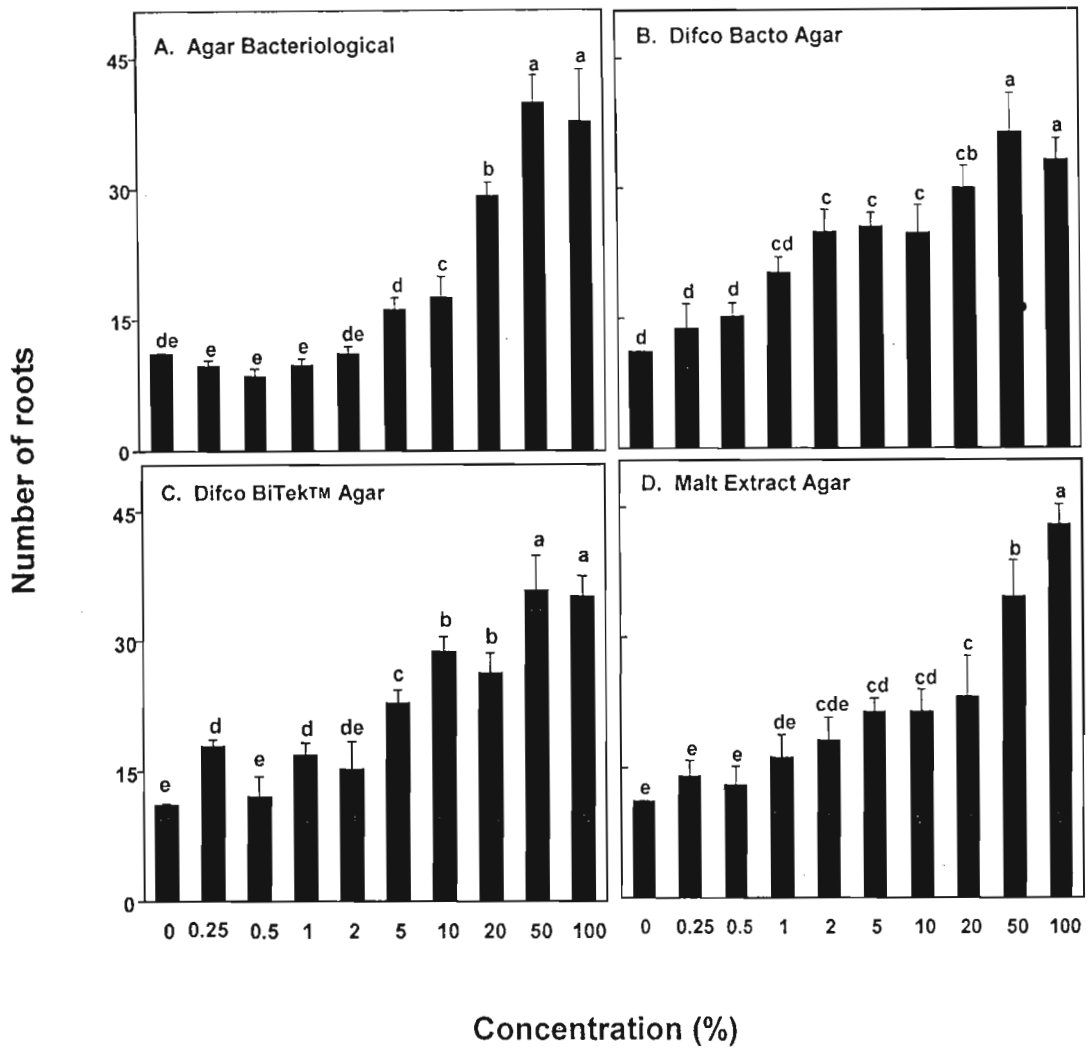


Figure 3.3. Average number of roots produced by mungbean cuttings with various concentrations of gelling agents which gave a good rooting response. Different letters indicate significant differences ($p=0.05$) within each gelling agent. IBA was used as standard (Figure 3.1F).

At the 100% concentration of Mueller-Hinton Agar there was a complete failure of rooting of mungbean cuttings. A similar effect was observed at 50% and 100% concentrations of Malt Extract Broth (Figure 3.1E). Malt Extract Agar (Figure 3.3D) gave the highest rooting for all the gelling agents tested

3.5. DISCUSSION

The low rooting of the gelling agent extract in Figure 3.1 suggests that either certain inhibitors are affecting root development or root stimulating substances are low or absent. The absence of roots with the high concentrations of Malt Extract Broth (50% and 100% concentrations) and Mueller-Hinton Agar (Figure 3.1D & E) indicates toxicity at these concentrations. The effect of peptones was clearly seen in the rooting of Malt Extract Agar (Figure 3.1D) which at 100% concentration gave rise to 30% more roots than the most effective concentration of IBA (10^{-3} M) (Figure 3.1F). It could also be a synergistic effect between root stimulating substances and peptones which was an additive to the agar. It is apparent from this result and other work (ICHIMURA & ODA, 1998) that root stimulating substances are present in some gelling agents. However, from this initial work the nature of the compounds are not known and thus studies were carried out on three of the good gelling agents with respect to root initiation.

The three gelling agents were selected for the identification of root stimulating substances based on the following criteria:

- i) Good rooting -where highest average rooting in mungbean cuttings in the gelling agent extracts at any concentration level from (0.5% to 100%) was comparable to rooting in IBA 10^{-4} M- 10^{-3} M
- ii) Widely used in tissue culture;
- iii) The absence of additives such as mineral salts, other extracts and peptones to ensure that rooting was not due to additives but rather to stimulating substances from the gelling agents only; and
- iv) General information on the composition and preparation of the products clearly

stated on the bottle label.

All gelling agents which had peptones and other additives were avoided.

Three agar preparations Agar Bacteriological, Agar Commercial Gel and Difco Bacto Agar as well as Gelrite were selected for closer scrutiny. Rooting in Agar Bacteriological was high. Difco Bacto Agar and Difco BiTek™ Agar are from the same manufacturer and promoted high rooting. However, the Difco Bacto Agar had composition information on the label and was thus selected instead of the Difco BiTek™ Agar. Agar Commercial Gel was selected instead of Agar Bacteriological No. 1 because it is widely used in tissue culture. Gelrite was included irrespective of its poor rooting, which was suspected to result from problems in extraction, due to its jelly-like nature. The selection of Gelrite was based on the fact that it is widely used in tissue culture and that it is the only gelling agent among those tested that is derived from bacteria. The next step was to assess if any of the subsequent extraction procedures could elute substances that would promote rooting.

CHAPTER 4

EFFECT OF AUTOCLAVING, CHARCOAL AND FREEZE-THAWING ON ROOT STIMULATING SUBSTANCES IN EXTRACTS FROM GELLING AGENTS

4.1. INTRODUCTION

4.1.1. EFFECT OF CHARCOAL AND AUTOCLAVING ON ROOT STIMULATING SUBSTANCES IN EXTRACTS FROM GELLING AGENTS

In culture media activated charcoal may promote or inhibit *in vitro* growth. Activated charcoal adsorbs a variety of organic molecules and chelates heavy metals, which are inhibitors present in plant nutrient media and agar gels (ANAGNOSTAKIS, 1974; TYAGI, RASHID & MAHESHWARI, 1980). Many materials are adsorbed and desorbed from colloidal soil particles and activated charcoal acts similarly (PROSKAUER & BERMAN, 1970). Desorption from charcoal depends on factors such as the grade of the activated charcoal, based on raw materials used, and the process of preparation, temperature, pH of solution and the type of solvent, solubility and chemical composition of the compound subject to adsorption (YAM, ERNST, ARDITT, NAIR & WEATHERHEAD, 1990). However, the adsorbed compound may be less mobile than in soil due to the larger internal surface area of the activated charcoal (PROSKAUER & BERMAN, 1970).

In another culture of *Anemone canadensis* the addition of small amounts of ethanol stimulated embryogenesis in the presence of charcoal. The speculation was that ethanol in some way facilitated the adsorption of inhibitors to charcoal or by being adsorbed

itself prevents the adsorption of some stimulating substances (JOHANSSON, CALLEBERG & GEDIN, 1990). Adsorbed products are generally released by activated charcoal (JOHANSSON & ERIKSSON, 1977; LITZ & CONOVER, 1980; JOHANSSON, CALLEBERG & GEDIN, 1990) and are made available to explants by active uptake (JAISWAL & AMIN, 1987; M'KADA, DORION & BIGOT, 1991). Alteration of medium pH to an optimum level for morphogenesis has been reported as having a beneficial effect on activated charcoal (OWEN, WENGERD, & MILLER, 1991). BONGA (1982) stated that the effectiveness of charcoal might be altered by impurities. In addition to promoting and regulating growth, activated charcoal releases growth-promoting substances already present on the charcoal or which had previously been adsorbed (PAN & VAN STADEN, 1998). Activated charcoal used in the nutrient medium had adsorptive preference for moderately polar rather than polar or highly polar organic compounds.

Plant regulators such as auxins and/or cytokinins in various concentrations or combinations are added to nutrient media to enhance propagation. Activated charcoal is able to adsorb high concentrations of growth regulators such as BA, IAA, NAA and kinetin (WEATHERHEAD, BURDON & HENSHAW, 1978) in both liquid and solid media (NISSEN & SUTTER, 1990). During *in vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinaster*, addition of activated charcoal to rooting medium improved the potential for adventitious rooting and enhanced the number and length of roots (DUMAS & MONTEUUIS, 1995). In anther culture, addition of activated charcoal to agar medium increased the frequency of plant regeneration (ANAGNOSTAKIS, 1974; WERNICKE & KOHLENBACH, 1976; TYAGI, RASHID & MAHESHWARI, 1980).

Yield of embryos from anther culture of *Nicotiana tabacum* in a liquid medium without agar was significantly better than in media solidified with agar (WERNICKE & KOHLENBACH, 1976). There was an improved response when charcoal was added to the agar, although embryo yield was still relatively low. However, with *Datura innoxia* anther culture, adding charcoal to agar-containing medium greatly improved growth compared to that in liquid medium (WERNICKE & KOHLENBACH, 1976). The explanation was that inhibitors are leached from the anthers and that the nature and the quantity of inhibitors vary among plants. Thus, in some cases, as with *N. tabacum*,

charcoal might not be able to remove all the inhibitors resulting in similar growth in liquid medium (TYAGI, RASHID & MAHESHWARI, 1980).

The stability of IAA, IBA and other auxins in nutrient medium is very important. Light affects the degradation of IAA and IBA in both liquid and solid media (NISSEN & SUTTER, 1990). Charcoal provides a degree of darkness which may prevent entry of too much light which would destabilize the plant growth regulators. During culturing excessive production of polyphenols is known to cause browning and eventually death of some tissues. Light exclusion by activated charcoal may have a secondary effect in reducing discolouration of tissues in culture, since light was suggested to increase the activity of enzymes associated with phenol oxidation (LININGTON, 1991).

To avoid build up of phenols, explants are transferred to fresh medium. This might lead to cell mutation and the loss of ability of cells to undergo embryogenesis. Addition of charcoal or polyvinylpyrrolidone (PVP) to cultures can eliminate this problem. Activated charcoal reduced browning of palm explants and culture medium, thus increasing explant survival and organogenesis (SUGANO, IWATA & NISHI, 1975; HABAGUCHI, 1977; TISSERAT, 1979). Activated charcoal also reduced browning and improved growth of *Strelitzia reginae* and *Anemone oronaria* (MENSUALI-SODI, PANIZZA, SERRA & TOGNONI, 1993).

Products of autoclaved sugars such as 5-hydroxymethyl-2-furaldehyde (HMF) (REDEI, 1974) are known to reduce *in vitro* growth of plant tissue. Charcoal was used to eliminate the inhibition of growth of tobacco anther culture by HMF which increased yield by 200-300% in the anther culture (WEATHERHEAD, BURDON & HENSHAW, 1978). However, charcoal had a negative effect on *Prunus silicina* when grown in culture. There was a decrease in the percentage of rooted shoots and chlorosis and severe leaf fall occurred (ROSATI, MARION & SWIERCZWSKI, 1980). Hyperhydricity was observed in *Picea abies* with the addition of charcoal to the media (VON ARNOLD, 1982).

4.1.2. EFFECT OF FREEZE-THAW TECHNIQUE ON PHYTOHORMONES IN GELLING AGENTS AND GELLING AGENTS WITH IBA

Many of the advances in biotechnology would not have been possible without the availability of polysaccharides derived from seaweed. For example, highly-refined agar (agarose) is used for cell and tissue culturing and in DNA fingerprinting.

Agar is insoluble in cold water but soluble in hot water. A hot aqueous 1.5% agar solution is clear but when cooled below 40°C, a firm opaque resilient gel is formed. This melts at temperatures greater than 80°C. This difference between gelling and melting temperatures is known as hysteresis. Originally gelling agents were produced by allowing them to freeze-and-thaw outdoors during the winter months. During these thaw cycles, unwanted soluble salts, nitrogenous material and pigments were leached from the agar. Today gelling agents are made using this basic extraction freeze-thaw process with some modification depending on the manufacturer (FOREMAN & WHYTE, 1997).

ICHIMURA and ODA (1994) found that extracts of woodpulp and commercial filter paper greatly stimulated root growth of several plants. Another report also confirms that substances related to plant polysaccharide do stimulate root regeneration (USUDA & SHIMOGAWARA, 1994). When frozen leaves of 24-day-old maize (*Zea mays* L.) plants were thawed on moist filter paper at 26°C (freeze-thaw treatment) several enzymes, including phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase (RuBPC) were inactivated and degraded. The course of inactivation and degradation were of Pseudo first order and the half times for inactivation of PEPC and RuBPC were 3.2 and 2.4 min respectively. The effect of the freeze-thaw treatment on the inactivation and degradation varied between enzymes (USUDA & SHIMOGAWARA, 1994). Commercial agar and wood pulp stimulated shoot and root growth, thus subjecting them to freeze-thaw treatment could affect the growth substances present in them.

4.2. AIMS

The main objective of this part of the thesis was to determine the effect of autoclaving, the addition of activated charcoal and freezing on the root stimulating substances that could be extracted from agar preparations.

4.3. MATERIALS AND METHODS

4.3.1. EXTRACTION OF GELLING AGENTS WITH WATER

Three gelling agents, Agar Bacteriological, Agar Commercial Gel and Difco Bacto Agar were chosen for further investigation as a result of the good rooting response of mungbeans with extracts obtained from them (Chapter 3). Even though the extraction of Gelrite with distilled water was a problem due to its jelly-like nature, the effect of the extract on rooting was deemed important and it was therefore included for extraction. The general extraction method was used as described in Chapter 2 (Section 2.2). Various additional methods for extracting the rooting agents are described below. The pH was measured for each extract. All extracts were tested for biological activity using the mungbean bioassay as described in Chapter 2 (Section 2.3).

4.3.2. METHODS OF EXTRACTION

Water extracts of the three gelling agents were prepared in two ways: A) ordinary extraction as described in Section 2.2 and B) autoclaved, frozen extraction described below. The extraction from Gelrite was done using the latter method only since the former method did not work.

A) Ordinary Extraction

i) *Control extract (C)*

Twenty grams of gelling agent were suspended in 500 ml distilled water and stirred for 1 h at room temperature using a magnetic stirrer. The gelling agent extracts were filtered as described in Section 2.2 and bioassayed using the mungbean bioassay.

ii) Control autoclaved (CA)

Gelling agent extracts were made as described for the control extracts as above (C) and after filtration, were resuspended in 200 ml distilled water. This was autoclaved at a pressure of 1 bar and temperature of 121°C for 20 min and then bioassayed.

iii) Charcoal added (Cha)

A water extract was made from the gelling agent as described above for the control extract (C). After this gelling agent extract had been filtered, powdered activated charcoal (BDH, Lot No. K25149270.843, pH 8-10) (1.4 g) was added and the mixture stirred for 2 h. This was then filtered through Whatman No.1 filter paper and then filtered with 0.45µm millipore filter. The charcoal treated filtrate was then dried and bioassayed.

iv) Charcoal added and autoclaved (ChaA)

Extraction was performed as for the charcoal added extract (Cha). The air dried sample was resuspended in 200 ml distilled water, and then autoclaved and bioassayed.

v) IBA as a rooting factor

IBA was used as standard at concentrations between 10^{-3} M - 10^{-7} M. The treatments were; 1) IBA solution at the various M concentrations (IBA-S); 2) IBA Autoclaved (IBA-A) at a pressure of 1 bar and temperature of 121°C for 20 min; 3) IBA with charcoal added (IBA-Cha), where 2.8 g l⁻¹ charcoal was added to the IBA solutions, stirred for 2 h and filtered using Whatman No.1 filter paper; and 4) IBA with charcoal added and autoclaved (IBA-ChaA). The controls were distilled water (DW), autoclaved distilled water (ADW), activated Charcoal Extract (ACE) autoclaved activated Charcoal extract (AACE). Both control extracts of activated charcoal (ACE and AACE) were obtained using distilled water. Each concentration of a treatment was replicated four times and bioassayed.

B) Autoclaved-Frozen Extraction

i) Control (A_FC)

Twenty grams of gelling agent were suspended in 500 ml distilled water and autoclaved at a pressure of 1 bar and temperature of 121°C for 20 min and allowed to cool to room

temperature. This was then frozen at $-20\text{ }^{\circ}\text{C}$ for two days after which it was thawed for a further two days at room temperature. The resulting extract was filtered through Whatman No.1 filter paper. Distilled water (200 ml) was used to wash the agar residue. The filtrates were combined and dried down *in vacuo* at $45\text{ }^{\circ}\text{C}$ and bioassayed.

ii) Autoclaved-frozen extract with charcoal ($A_F\text{Cha}$)

Twenty grams of gelling agent with 1.4 g activated charcoal were suspended in 500 ml distilled water. This was autoclaved, then frozen and thawed and bioassayed.

iii) Autoclaved-frozen extract with IBA ($A_F\text{IBA}$)

Twenty grams of gelling agent were suspended in 500 ml distilled water and 0.0406 g (10^{-3} M) IBA added. This was autoclaved, frozen-extracted and bioassayed.

iv) Autoclaved-frozen extract with charcoal and IBA ($A_F\text{Cha} + \text{IBA}$)

Twenty grams of gelling agent with 1.4 g activated charcoal and 10^{-3} M IBA ($A_F\text{Cha} + \text{IBA}$) was suspended in 500 ml distilled water, autoclaved, frozen-extracted and bioassayed.

4.3.3. RELEASE OF ABSORBED METABOLITES FROM THE CHARCOAL

The charcoal residue from the autoclaved-frozen gelling agent extracts with IBA and charcoal ($A_F\text{Cha} + \text{IBA}$) were extracted for 10 min with 200 ml of 80% acidic methanol, adjusted to pH 4.5 using HCl. This was dried *in vacuo* at $40\text{ }^{\circ}\text{C}$, air dried and resuspended in 200 ml distilled water and bioassayed

4.4. RESULTS

4.4.1. IBA AS A ROOTING FACTOR

One feature common to all the treatments of IBA and gelling agents was that rooting at the lower extract concentrations and in water (control) were confined to the basal portion

of the cuttings, whereas rooting at the higher concentrations was distributed over two thirds of the entire hypocotyl section. Roots generally emerged 4 to 5 days after the pulse treatment.

Increasing concentrations of IBA (IBA-S) significantly increased the number of roots compared to the distilled water control (DW) (Figure 4.1A). A similar trend was observed when the solution was autoclaved (IBA-A) (Figure 4.1B). Addition of charcoal (IBA-Cha) (Figure 4.1C) greatly reduced rooting at all concentrations, except at the highest IBA concentration (10^{-3} M IBA). Similarly rooting was reduced when charcoal was added to the medium and autoclaved (Figure 4.1D). Table 4.1 shows significant differences between the treatments at the three highest IBA concentrations tested. At the highest concentration (10^{-3} M IBA) there was a significant difference between IBA-S (highest rooting) and the other treatments, but there was no significant difference between rooting in 10^{-3} M of IBA-S and 10^{-4} M of IBA-A. However, rooting in the latter was not significantly different from that in 10^{-4} M IBA-S, 10^{-3} M IBA-A and 10^{-3} M IBA-Cha treatments (Table 4.1). The autoclaving treatment (IBA-A) (Figure 4.1B) reduced the pH of the medium compared to the IBA-S treatment (Figure 4.1A), from 6.7 to 5.7 respectively. IBA-Cha and IBA-ChaA treatments (Figures 4.1C and D) had pH values of 9.5 and 9.4. This is an indication of a pH increase resulting from the addition of charcoal.

Table 4.1. Statistical analysis of the average number of roots on mungbean cuttings with various concentrations of IBA and between the various treatments. Different letters indicate significant differences (P=0.05)

Concentration (M)	Treatments			
	IBA-S	IBA-A	IBA-Cha	IBA-ChaA
10^{-5}	23.2±2.9 ^{cde}	24.2±1.8 ^{cd}	18.2±2.0 ^{de}	16.4±1.4 ^e
10^{-4}	40.2±2.6 ^b	43.2±2.4 ^{ab}	18.6±2.0 ^{de}	19.8±1.2 ^{de}
10^{-3}	49.6±4.6 ^a	40.6±5.1 ^b	37.9±3.0 ^b	30.0±5.1 ^c

IBA-S - IBA solution; IBA-A - IBA Autoclaved; IBA-Cha - IBA Charcoal added; IBA-ChaA - IBA Charcoal added + Autoclaved

4.4.2. ORDINARY EXTRACTION: EFFECT OF AUTOCLAVING AND CHARCOAL ON THE ABILITY OF THE GELLING AGENT EXTRACT TO PROMOTE ROOTING

4.4.2.1. Agar Bacteriological

The average root number in the mungbean cuttings in the control (C) significantly increased as the concentration of the gelling agent extract increased above 5% (Figure 4.2A). Autoclaving (CA) significantly reduced rooting and there were no significant differences between the extract concentrations (Figure 4.2B). Addition of charcoal (Cha) had no effect compared to the control treatment (Figure 4.2C). The trend was similar to the rooting pattern of the control (C) treatment (Figure 4.2A). When the gelling agent extract containing charcoal was autoclaved, rooting was greatly reduced at all the gelling agent extract concentrations (Figure 4.2D). There was no significant difference between rooting at the highest concentrations (100%) of C (pH 7.3) and Cha (pH 9.7) treatments. Rooting was relatively low at all concentrations of treatments CA (pH 6.4) and ChaA (pH 9.0) and were not significantly different (Table 4.2).

4.4.2.2. Agar Commercial Gel

A similar trend was observed as with the Agar Bacteriological extracts. Rooting of the mungbean cuttings was greatly reduced when autoclaved (CA) (Figure 4.3B), compared to the control (C) extracts (Figure 4.3A). Addition of charcoal (Cha) significantly reduced rooting at the lower gelling agent extract concentrations (0.25%) compared to the water control but at the higher gelling agent extract concentrations, there was a significant increase in rooting (Figure 4.3C). The addition of charcoal combined with autoclaving (ChaA) resulted in low rooting at all gelling agent extract concentrations (Figure 4.3D). There was no significant difference between rooting in 50 and 100 % concentrations of treatments C (pH 6.6) and Cha (pH 8.9). The lowest rooting was in CA (pH 6.1) and ChaA (Figures. 2C and 4.3C) treatments (Table 4.2).

4.4.2.3. Difco Bacto Agar

The increase in mungbean rooting with increasing extract concentration was not as clear as in the control (C) extracts of Agar Bacteriological (Figure 4.2A) and Agar Commercial

Gel (Figure 4.3A), but there was still a significant increase in rooting at 20% gelling agent extract concentrations and higher compared to the water control (Figure 4.4A). Autoclaving of the extract (CA) (pH 6.7) (Figure 4.4B), addition of charcoal (Cha) (pH 8.6) (Figure 4.4C) and the addition of charcoal and autoclaving (ChaA) (pH 7.7) (Figure 4.4D) greatly reduced rooting of the mungbean cuttings. The control (C) (pH 6.5) gave the highest rooting and there was no difference between concentrations of 20, 50, and 100 %. In contrast to the other two agars, rooting in CA and Cha for Difco Bacto Agar was not significantly different (Table 4.2)

Table 4.2. Statistical analysis of the average number of roots on mungbean cuttings with various water extracts of gelling agents (20-100% concentrations) across all treatments. Different letters indicate significant differences (P=0.05)

Agar type	Conc. (%)	Treatments			
		C	CA	Cha	ChaA
Agar Bacteriological	20	29.5±2.1 ^{cd}	14.6±2.7 ^{gh}	26.8±2.5 ^{cde}	20.8±4.5 ^{ef}
	50	34.0±2.0 ^{bc}	19.2±5.0 ^{fd}	29.7±3.0 ^{cd}	18.2±1.8 ^{gh}
	100	37.8±3.0 ^{ab}	12.4±3.0 ^h	41.6±3.4 ^a	14.4±1.5 ^{gh}
Agar Commercial Gel	20	26.7±1.8 ^{bcd}	18.1±2.0 ^{ef}	21.7±1.5 ^{def}	18.4±1.7 ^{ef}
	50	35.1±4.0 ^a	23.7±6.6 ^{bcd}	30.7±2.4 ^{ab}	16.5±3.0 ^f
	100	34.0±2.0 ^a	22.4±3.2 ^{cd}	29.3±3.1 ^{abc}	19.7±2.2 ^{def}
Difco Bacto Agar	20	27.3±5.2 ^a	15.2±5.3 ^{bcd}	14.7±3.0 ^{cde}	9.2±2.3 ^e
	50	21.2±2.7 ^{ab}	16.1±5.5 ^{bcd}	16.6±1.8 ^{bc}	10.1±1.8 ^{de}
	100	24.3±1.6 ^a	13.1±1.1 ^{cd}	13.3±0.7 ^{cde}	11.3±0.7 ^{cde}

C - Control; CA - Control Autoclaved; Cha - Charcoal added; ChaA -Charcoal added + Autoclaved

4.4.3. FREEZE -THAW TECHNIQUE : EFFECT OF AUTOCLAVED FREEZE-THAWED GELLING AGENT EXTRACTS ON ROOTING

4.4.3.1. Agar Bacteriological

There was no increase in rooting of the mungbean cuttings at all gelling agent I extract

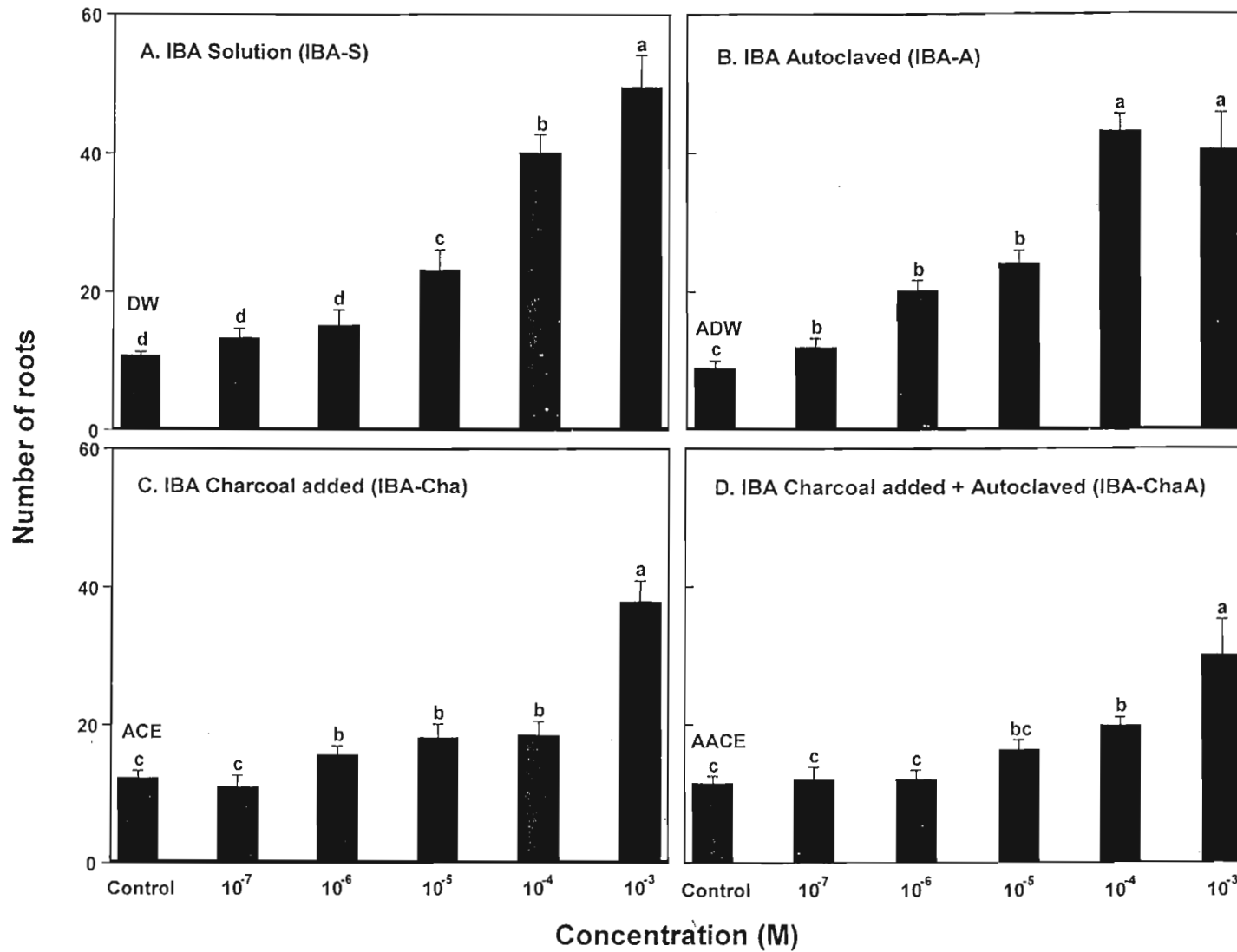


Figure 4.1. Average number of roots produced by mungbean cuttings with various concentrations of IBA. DW-distilled water, ADW-autoclaved distilled water, ACE-activated charcoal extract, and AACE-autoclaved activated charcoal extract. Different letters indicate significant differences ($p=0.05$) within each treatment.

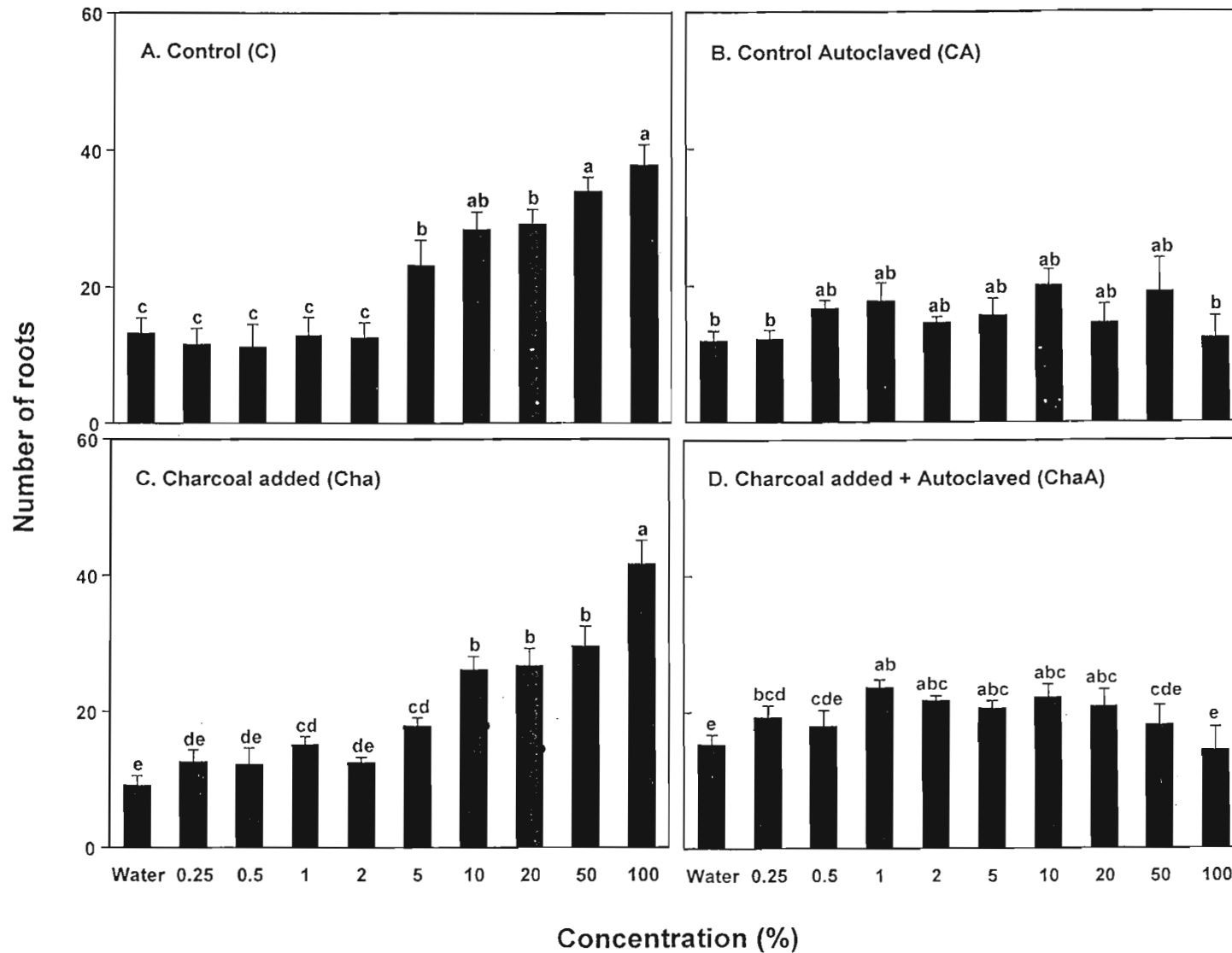


Figure 4.2. Average number of roots produced by mungbean cuttings with various concentrations of Agar Bacteriological water extracts in different treatment combinations. Different letters indicate significant differences ($p=0.05$) within each treatment.

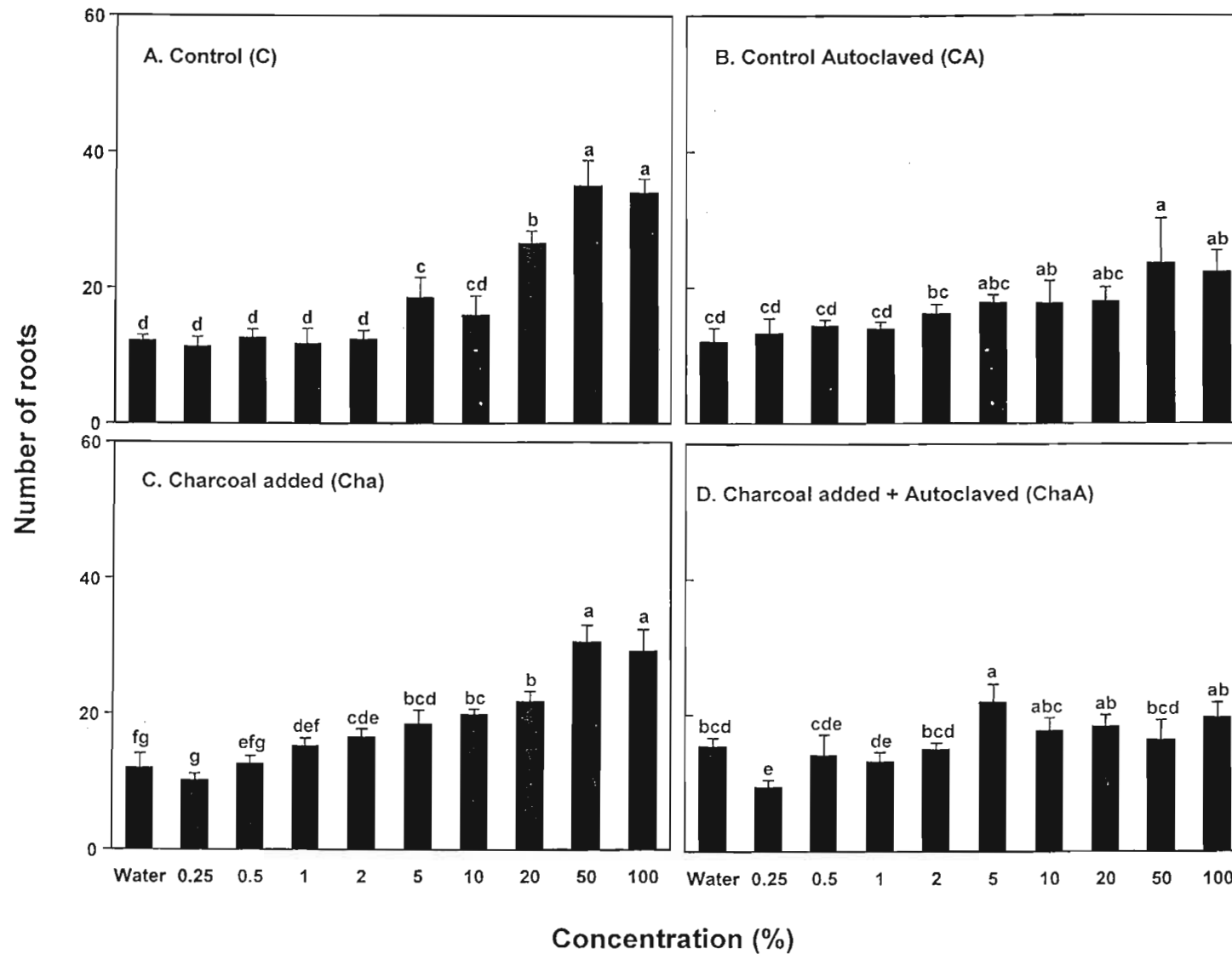


Figure 4.3. Average number of roots produced by mungbean cuttings with various concentrations of Agar Commercial Gel water extracts in different treatment combinations. Different letters indicate significant differences (p=0.05) within each treatment.

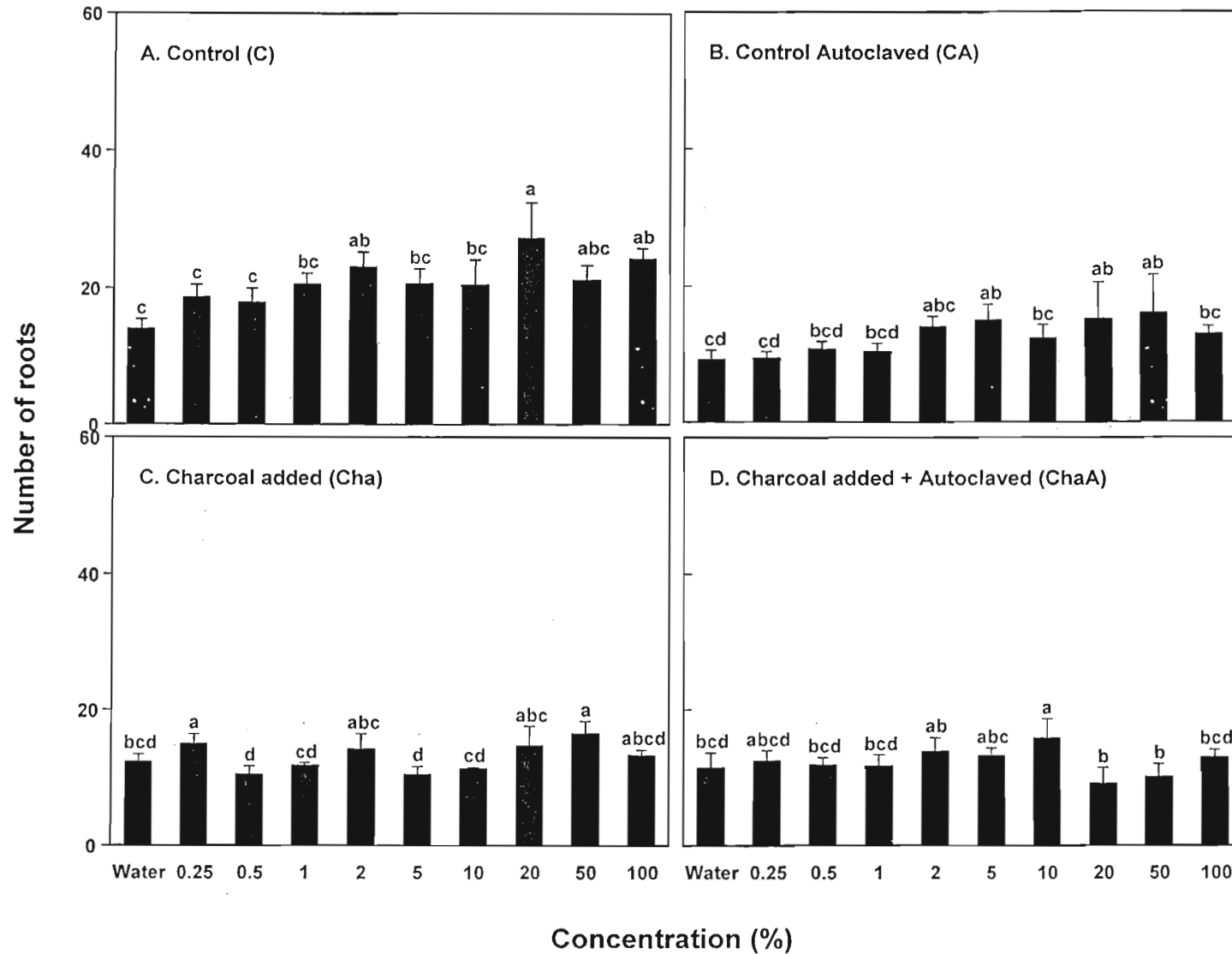


Figure 4.4. Average number of roots produced by mungbean cuttings with various concentrations of Difco Bacto Agar water extracts in different treatment combinations. Different letters indicate significant differences ($p=0.05$) within each treatment.

concentrations in the autoclaved frozen control (Figure 4.5A). Rooting was also low in the charcoal added treatment (A_F Cha) (pH 6.1), although there was a significant increase in rooting at 50 and 100% concentrations (Figure 4.5B). When IBA was added (A_F IBA) (pH 6.4) there was a significant increase in rooting especially at the higher extract concentrations. There was no significant difference in rooting at concentrations of 10% and lower compared to the water control (Figure 4.5C). In charcoal + IBA treatment (A_F Cha+IBA) (pH 8.9) there was a slight but significant increase in rooting at higher concentrations (Figure 4.5D).

4.4.3.2. Agar Commercial Gel

For the control treatment (A_F C) (pH 6.4) rooting was low, although there was a significant increase in rooting as extract concentrations increased above 5% (Figure 4.6A). Rooting in the charcoal added treatment (A_F Cha) (pH 7.2) showed no trend with respect to poor rooting over the entire gelling agent extract concentration range (Figure 4.6B). The reason for this inconsistency is unknown. The IBA added treatment (A_F IBA) (pH 5.5) initiated significantly more roots as the gelling agent extract concentration increased (Figure 4.6C). Rooting in the charcoal + IBA treatment (A_F Cha+IBA) (pH 7.7) was greatly reduced at all concentrations (Figure 4.6D) (Table.4.3)

4.4.3.3. Difco Bacto Agar

In both the control (A_F C) (pH 6.8) (Figure. 4.7A) and charcoal added (A_F Cha) (pH 8.1) treatments (Figure 4.7B), the average number of roots was low. Like the other agars, for the IBA added (A_F IBA) (pH 6.9) treatment, mungbean rooting increased as the gelling agent extract concentrations increased (Figure 4.7C). In the charcoal + IBA treatment (A_F Cha + IBA) (pH 8.2), rooting was low for all the gelling agent extract concentrations (Figure 4.7D).

4.4.3.4. Gelrite

Rooting of the mungbean cuttings in the control (A_F C) (pH 6.3) (Figure 4.8A) and charcoal added (A_F Cha) (pH 7.1) treatments (Figure 4.8B) was similar to rooting with the distilled water control. The addition of IBA (A_F IBA) (pH 6.1) (Figure 4.8C) significantly increased the average root number. Rooting in charcoal + IBA (A_F Cha + IBA) (pH 7.1) (Figure 4.8D) was decreased with increasing gelling agent extract concentrations.

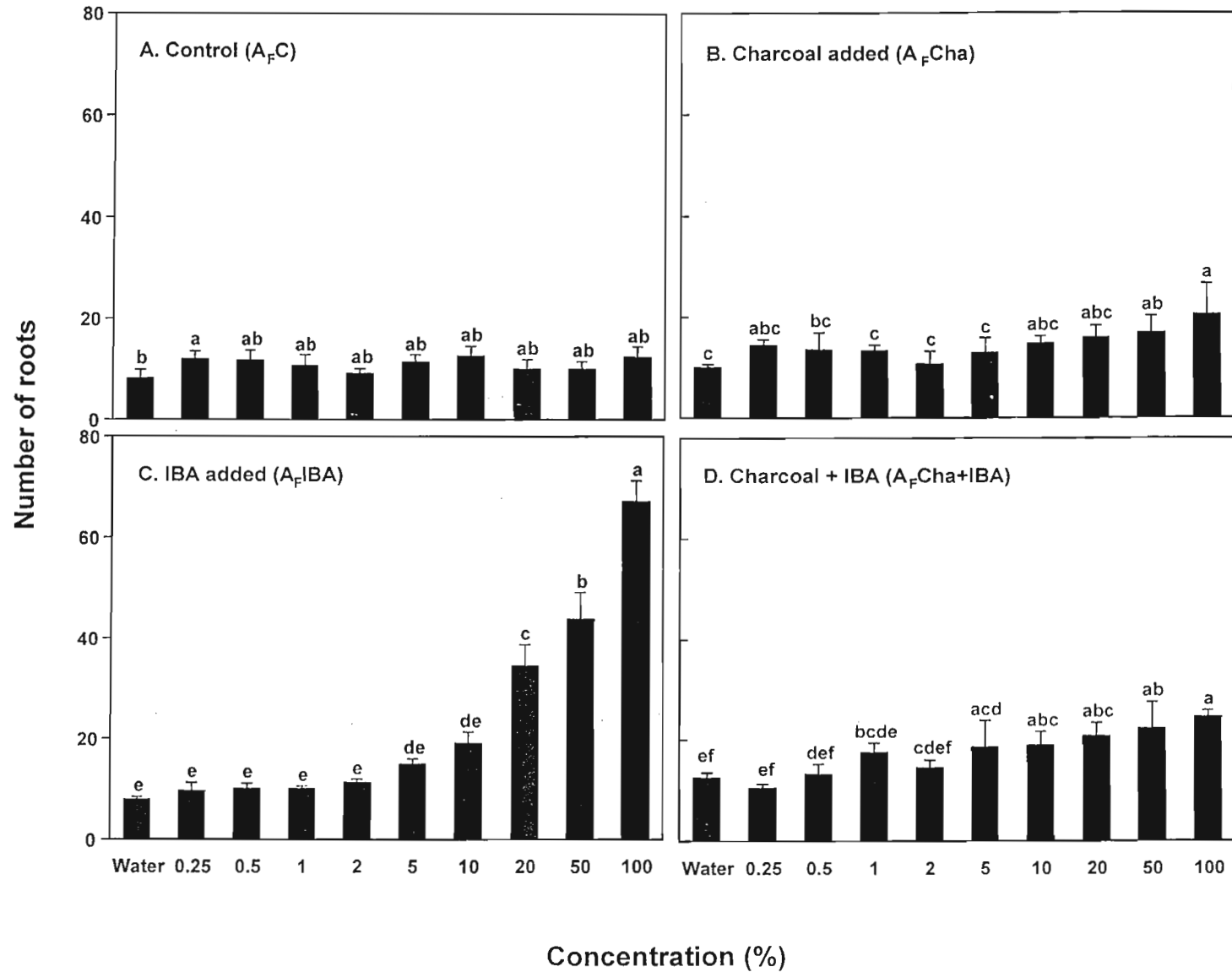


Figure 4.5. Average number of roots produced by mungbean cuttings with various concentrations of autoclaved frozen Agar Bacteriological water extracts in different treatment combinations. Different letters indicate significant differences ($p=0.05$) within each treatment.

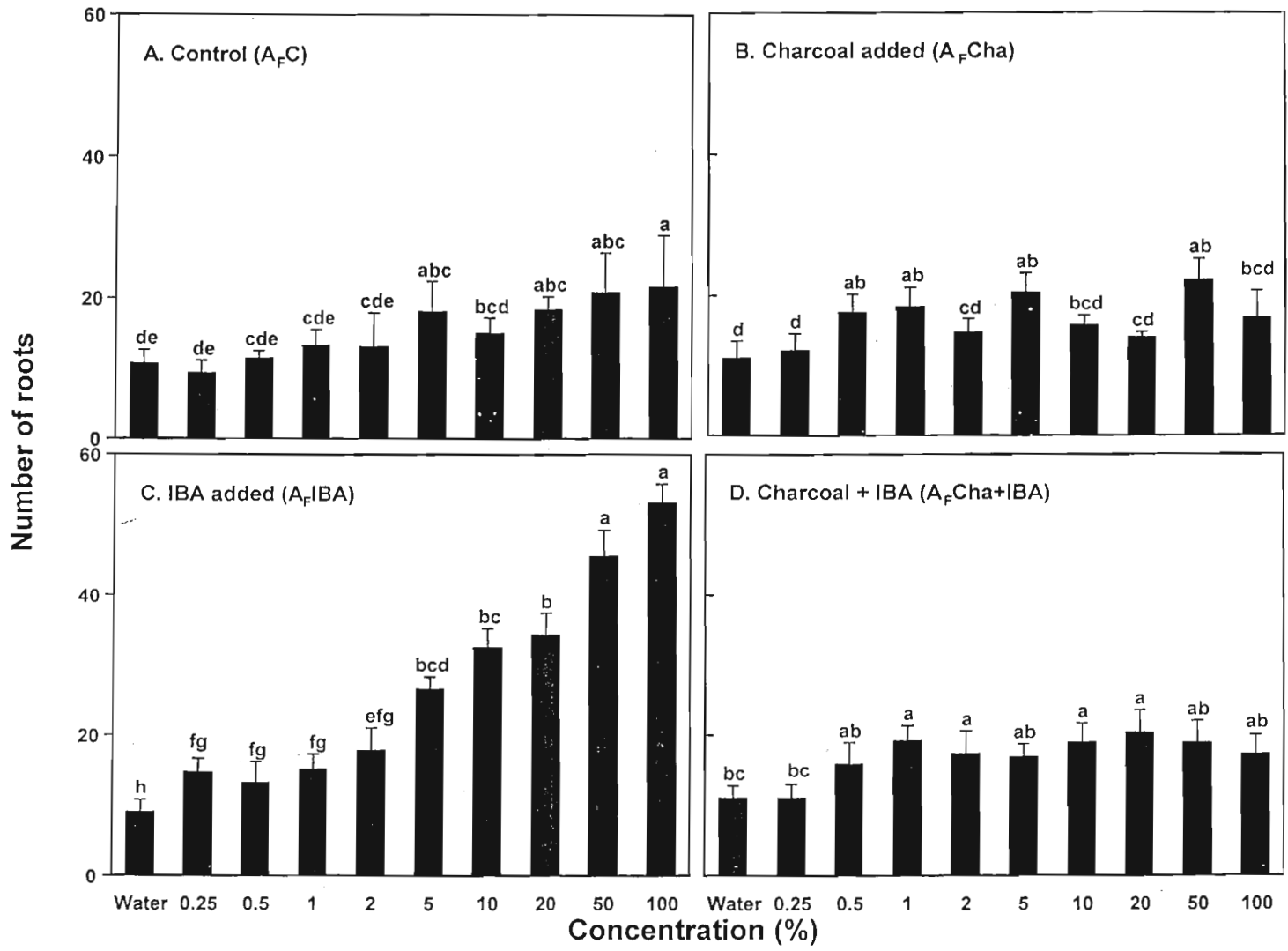


Figure 4.6. Average number of roots produced by mungbean cuttings with various concentrations of autoclaved frozen Agar Commercial Gel water extracts in different treatment combinations. Different letters indicate significant differences (p=0.05) within each treatment.

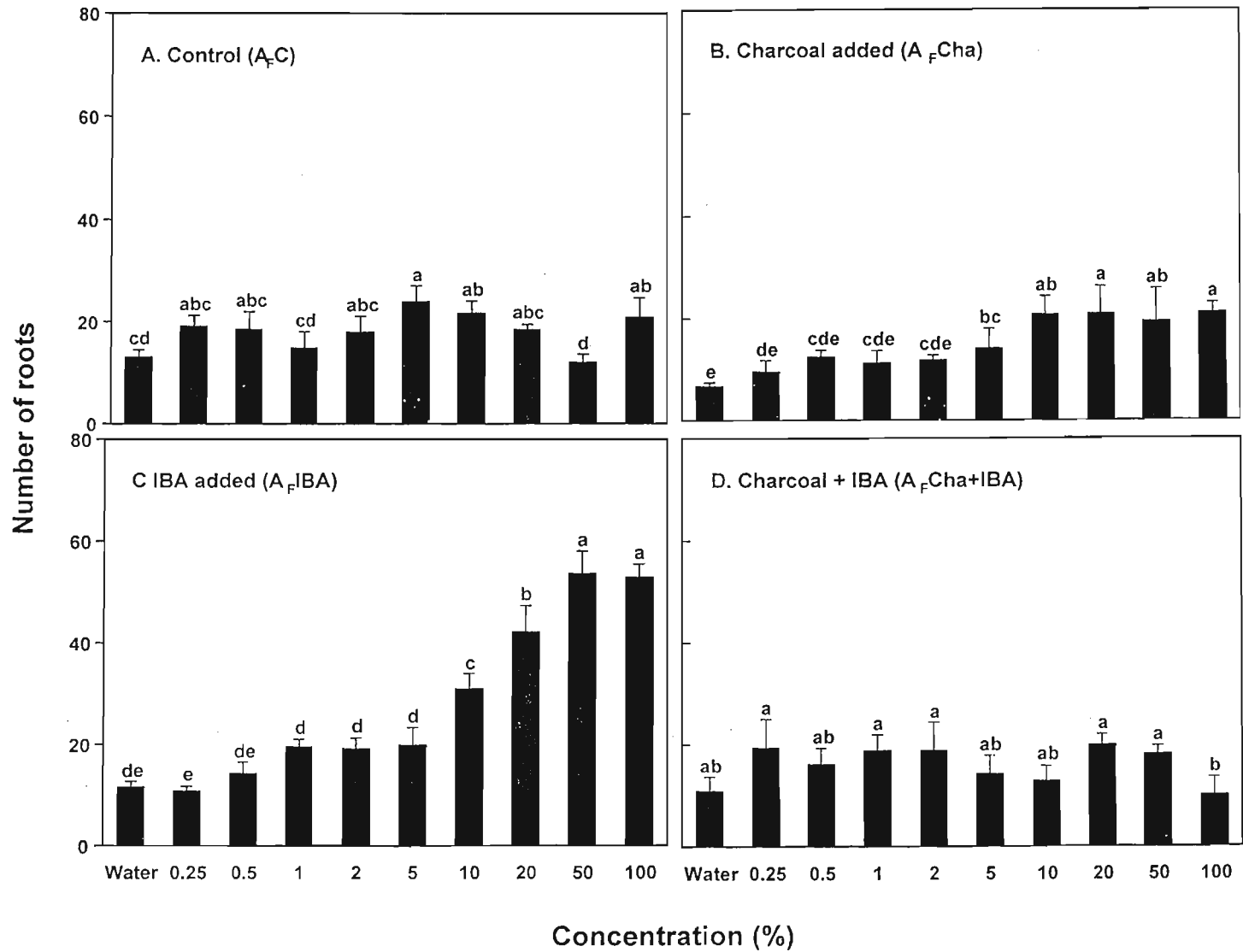


Figure 4.7. Average number of roots produced by mungbean cuttings with various concentrations of autoclaved frozen Difco Bacto Agar water extracts in different treatment combinations. Different letters indicate significant differences (p=0.05) within each treatment.

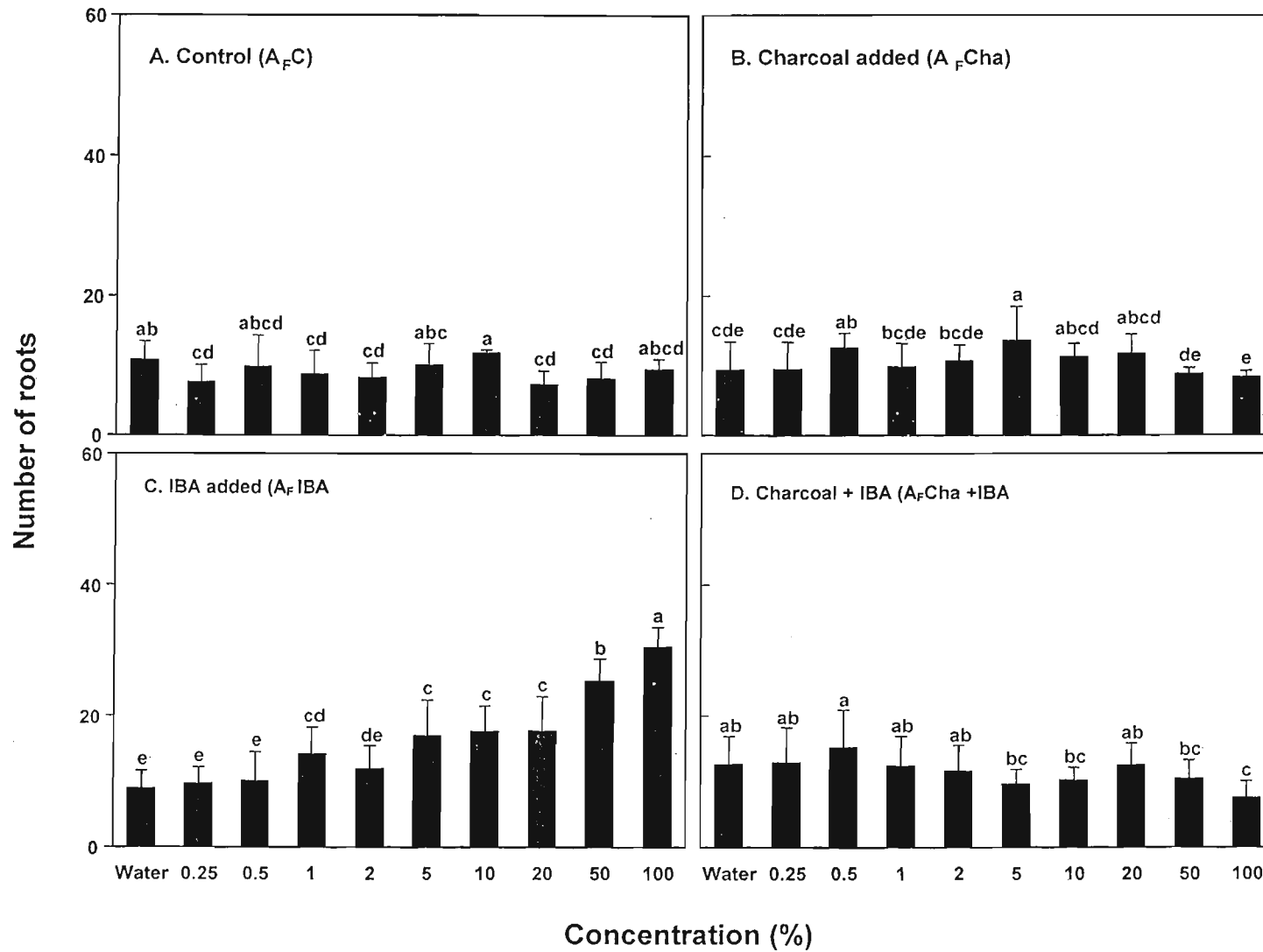


Figure 4.8. Average number of roots produced by mungbean cuttings with various concentrations of autoclaved frozen Gelrite water extracts in different treatment combinations. Different letters indicate significant differences ($p=0.05$) within each treatment.

4.4.4. RELEASE OF ADSORBED METABOLITES FROM THE CHARCOAL

The IBA recovered from the charcoal using acidic methanol was measured in terms of rooting. Rooting was promoted at the higher concentrations for all the gelling agents except Gelrite where rooting was low across the board (Figure 4.9D). From Table 4.3, it can be seen that rooting in 20, 50 and 100% concentrations of A_FCha + IBA_r was either half or a little more than half of the respective rooting compared to the A_FIBA treatments, which gave the highest rooting. With Agar Bacteriological and Agar Commercial Gel, there was no significant difference between 50 and 100% concentrations whereas in Difco Bacto Agar there was no difference at the three highest concentrations. Rooting in the 2% concentration of Gelrite was significantly higher than the 50 and 100% concentrations. The extract from Agar Bacteriological had a pH of 8.4, Agar Commercial Gel pH 7.1, Difco Bacto Agar pH 7.4 and Gelrite pH 9.1. With the exception of Gelrite, rooting increased as the extract concentration increased (Figure 4.9).

4.5. DISCUSSION

4.5.1. IBA AS A ROOTING FACTOR

IBA solutions (IBA-S) significantly promoted rooting of mungbeans at higher concentrations (10^{-5} M- 10^{-3} M; Figure 4.1A). Similar results were obtained when the extracts were autoclaved (IBA-A) (Figure 4.1B), although at 10^{-3} M IBA there was a significant difference between treatments (Table 4.1). Thus, autoclaving the media slightly reduce the biological effect of IBA but the results indicate that IBA is reasonably thermostable. The addition of charcoal to the medium (IBA-Cha) significantly reduced the rooting of mungbeans (Figure 4.1C; Table 4.1) compared to rooting of mungbeans at similar IBA concentrations of the IBA-S treatment (Figure 4.1A). A similar trend was observed in the charcoal added and autoclaved treatment (IBA-Cha). This reduction in root number can be attributed to the effect of charcoal and not autoclaving.

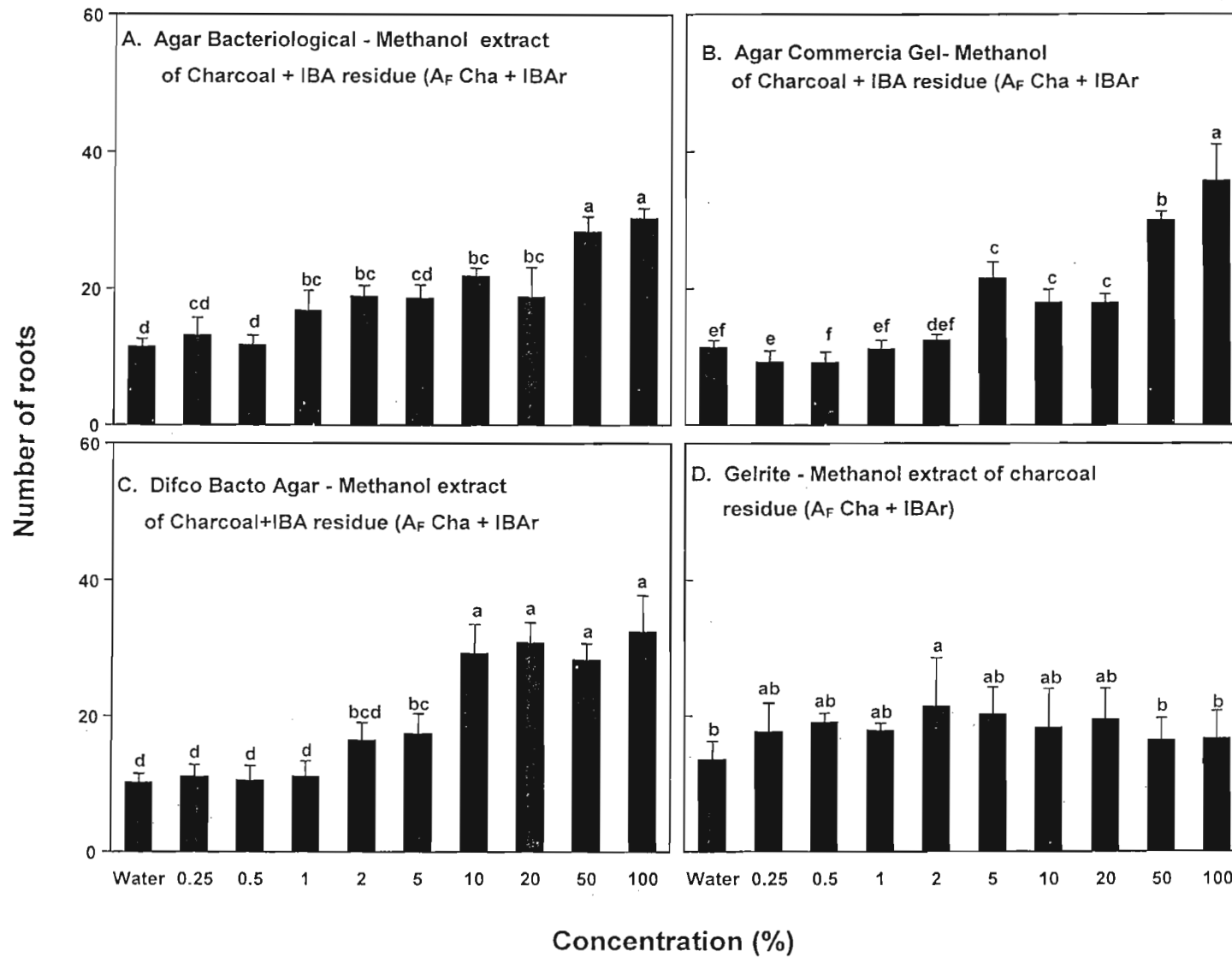


Figure 4.9. Average number of roots produced by mungbean cuttings with various concentrations of methanol extracts from residue autoclaved frozen gelling agents with charcoal and IBA. Different letters indicate significant differences ($p=0.05$) within each treatment.

Table 4.3 Statistical analysis of the average number of roots in mungbean cuttings with various autoclaved frozen extracts of gelling agent (20-100% concentrations) across all treatments. Different letters indicate significant differences (P=0.05)

Agar type	Conc. (%)	Treatments				
		A _F C	A _F Cha	A _F IBA	A _F Cha+IBA	A _F Cha+IBA _R
Agar	20	10.1±1.8 ⁱ	15.9±2.4 ^{ghi}	34.6±4.1 ^c	20.8±2.7 ^{fg}	18.8±4.3 ^{gh}
Bacteriological	50	10.0±1.5 ⁱ	16.8±3.4 ^{ghi}	43.7±5.3 ^b	22.3±5.3 ^{efg}	28.4±2.2 ^{cd}
	100	12.3±1.9 ^{hi}	20.7±6.1 ^{fg}	67.2±4.8 ^a	24.3±1.4 ^{def}	30.4±1.4 ^{cd}
Agar	20	16.4±1.8 ^{de}	14.0±7.0 ^e	34.3±2.5 ^c	20.4±3.2 ^{de}	18.0±1.2 ^{de}
Commercial Gel	50	20.8±5.8 ^{de}	22.2±3.0 ^d	45.5±5.6 ^b	16.8±3.8 ^{de}	30.1±1.2 ^c
	100	21.6±7.3 ^{de}	16.8±3.8 ^{de}	53.1±4.8 ^a	17.3±2.7 ^{de}	35.8±5.3 ^c
Difco Bacto Agar	20	16.4±1.0 ^{ef}	20.8±5.3 ^{de}	42.5±5.0 ^b	19.9±2.0 ^{de}	30.8±3.0 ^c
	50	12.0±1.6 ^f	19.4±6.4 ^{de}	53.7±4.3 ^a	18.1±1.0 ^{ef}	28.4±2.4 ^{cd}
	100	20.7±3.8 ^{de}	21.2±1.8 ^{de}	52.0±2.5 ^a	9.9±3.5 ^f	32.4±5.3 ^c
Gelrite	20	7.3±1.8 ^h	11.7±2.8 ^{ef}	17.8±5.3 ^{cd}	12.5±3.3 ^e	19.0±4.6 ^c
	50	8.1±2.3 ^{gh}	9.8±0.8 ^{fgh}	25.4±3.3 ^b	10.5±2.8 ^{efg}	16.5±3.2 ^d
	100	9.5±1.4 ^{fgh}	8.4±0.8 ^g	30.6±3.0 ^a	7.6±2.5 ^{gh}	16.7±4.1 ^d

A_FC - Autoclaved frozen gelling agent Control; A_FCha - Autoclaved frozen gelling agent Charcoal added; A_FIBA - Autoclaved frozen gelling agent IBA added; A_FCha+IBA - Autoclaved frozen gelling agent Charcoal added + IBA; A_FCha + IBA_R - Methanol extract of charcoal added + IBA residue (Autoclaved frozen gelling agent)

4.5.2. EFFECT OF AUTOCLAVING AND CHARCOAL ON THE ABILITY OF THE GELLING AGENT EXTRACT TO PROMOTE ROOTING

With Agar Bacteriological and Agar Commercial Gel, there was an increase in root number as concentration increased in the control treatments (C) (Figures 4.2A and 4.3A) and when charcoal was added (Cha) (Figures 4.2C and 4.3C). The charcoal did not have a marked effect on reducing rooting indicating the root stimulating substances desisted adsorption. The increase in the root number suggests that there are root-

stimulating substances present in agar preparations. In the control treatment (C) (Figure 4.4A) of Difco Bacto Agar, rooting was lower than in Agar Bacteriological and Agar commercial Gel (Figure 4.2A and 4.3A). In contrast to the rooting in IBA Charcoal added (IBA-Cha) treatment (Figure 4.1C), the addition of charcoal to Agar Bacteriological and Agar Commercial Gel (Figures 4.2C and 4.3C) did not have an effect on rooting. Control autoclaving (CA) reduced rooting at the 20-100% concentrations in Agar Bacteriological and Agar Commercial Gel (Figures 4.2B and 4.3B) showing the negative effect that autoclaving had on the root-stimulating substance(s) present. That the charcoal had no effect on rooting suggests that other substances in the media, that do not stimulate rooting or are less effective, were adsorbed by the charcoal instead of the root stimulating substances or the charcoal did not absorb anything. The control autoclaved (CA) (Figure 4.4B), Charcoal added (Cha) (Figure 4.4B) and Charcoal added plus autoclaved (ChaA) (Figure 4.4D) treatments reduced rooting in the presence of Difco Bacto Agar but not the other two agars. This indicated that the root-stimulating substances in Difco Bacto Agar might be different from those in the first two agars where the addition of charcoal had a negligible effect on rooting.

Even though many researchers have shown that the addition of activated charcoal to tissue culture media may have promotive effects, in this rooting experiment this was not observed. Rooting with charcoal added and autoclaved media (ChaA) (Figures 4.2D, 4.3D and 4.4D) was lower and the effect could mainly be attributed to autoclaving, which might have inactivated the rooting stimulating substances.

Many elements are present in activated charcoal in significant amounts. However, the addition of a water extract of activated charcoal to an orchid culture medium had no effect on seedling growth (ERNST, 1975). This report confirms the low rooting obtained with the activated charcoal extract (ACE) (Figure 4.1C) which was just like the rooting in DW (control) (Figure 4.1A).

4.5.3. EFFECT OF AUTOCLAVED FREEZE-THAWED GELLING AGENT EXTRACTS ON ROOTING

In the autoclaved frozen treatments, rooting was low in the control (A_FC) and charcoal-

added (A_F Cha) treatments. The best root growth was obtained in the IBA-added treatments (A_F IBA) (Figures 4.5C; 4.6C; 4.7C and 4.8C). The average number of roots, at the 100% concentration level for the gelling agents was five fold and three fold more than rooting in the control and charcoal-added treatments respectively. High rooting in the IBA + gelling agents is more likely to be an additive effect. The charcoal + IBA treatment (A_F Cha + IBA) (Figures 4.5D; 4.6D; 4.7D and 4.8D) reduced rooting greatly and this confirmed the reports that activated charcoal is able to adsorb high concentrations of the growth regulators BA, IAA, IBA, and kinetin (CONSTANTIN, HENKE & MANSUR, 1977; WEATHERHEAD, BURDON & HENSHAW, 1978; FRIDBORG & ERIKSSON, 1975). In a tissue culture medium, 0.1% activated charcoal can effectively adsorb 10 μ M IAA (1.75 mg l⁻¹) and 10 μ M IBA (2.03 mg l⁻¹) from the liquid medium (NISSEN & SUTTER, 1990).

In Table 4.3 there was a significant difference between rooting in 100% and the lower concentrations (50 and 20%) of A_F IBA of the gelling agents, apart from Difco Bacto Agar where rooting in the 100 and 50% concentrations were not significantly different. There were no marked significant differences in rooting within and between the other treatments. Mungbean cuttings in the Gelrite treatments consistently produced fewer roots than the others. In the IBA-added treatment (Figures 4.8C), rooting at 20, 50 and 100% concentrations was better but still lower than rooting in the other gelling agents of the same treatments.

The rooting ability of the shoots of Norway spruce varied depending on the agar concentration used (VON ARNOLD & ERIKSSON, 1984). ROMBERGER & TABOR (1971) showed that increasing the agar concentration restricted diffusion of macromolecules. STOLTZ (1971) noted that the decreased growth at higher agar concentration was due to reduced water availability. DEBERGH, HARBAOUI & LEMEURE (1981) found that agar is responsible for the matrix component of the medium water potential and that changes in the water potential are responsible for the agar effects. This could be related to the IBA incorporated Gelrite treatment which was in the liquid state as were the other gelling agents. The relative rigidity of Gelrite could have made the release of IBA difficult and therefore was not available to the cuttings to

ensure adequate rooting. For all the agar treatments that gave good rooting, rooting increased progressively as extract concentration increased.

4.5.4. RELEASE OF ADSORBED METABOLITES FROM THE CHARCOAL

The IBA and/or rooting substances eluted from the charcoal residue using acidic methanol promoted rooting in mungbeans. The methanol extract from the charcoal + IBA residue of Difco Bacto Agar (Figure 4.9C) produced the best rooting. The difference in terms of low rooting with the Gelrite (Figure 4.9D) might stem from the high degree of rigidity, which reflects the inherent nature of the Gelrite. In contrast to this, MACRAE & VAN STADEN (1990) noted that in *in vitro* propagation of *Eucalyptus grandis* from axillary buds, that shoot multiplication and elongation on Gelrite-containing media was superior to obtained on agarose and agar-containing media. Rooting was also enhanced with Gelrite as a gelling agent. With the recovery of IBA using acidic methanol, a further reduction in the pH could have eluted more rooting substances from the IBA-added treatment but its effect on rooting might have been undesirable. Further investigation is needed to clarify how recovery of root stimulating substances and/or incorporated phytohormones could be achieved with a gelling agent such as Gelrite. It has been reported that an acidic pH increases the carrier mediated uptake of IAA (LIU, MUKHERTJEE & REID, 1993). This might allow for auxin accumulation at the target sites responsible for rooting. Low pH increased the number of adventitious roots formed by the hypocotyls of sunflower seedlings and the acidic conditions may in part, promote root formation by increasing the movement of IAA to the root zone (LIU, MUKHERTJEE & REID, 1993). Even though desorption from charcoal is generally a slow process, there might be considerable reduction of the bonding and/or adsorptive effect between the charcoal and IBA and/or stimulating substances at the low pH of 4.5.

4.5.5. ROOTING OF MUNGBEAN WITH REGARD TO MEDIUM pH

4.5.5.1. IBA as a rooting factor

Rooting occurred over a wide range of pH values (pH 6.7-9.5). The high pHs of 9.4 and 9.5 might have contributed to the minor reduction of root number at 10^{-3} M in the IBA-

Cha treatment (Figure 4.1C) and by more than half in the IBA-ChaA treatment (Figure 4.1D) respectively.

4.5.5.2. Gelling agents

A) Ordinary water extraction

High root stimulation occurred in media with a high pH; for example, Charcoal Added treatments (Cha) of pH 9.7 (Agar Bacteriological) and pH 8.7 (Agar Commercial Gel) as well as in media with acidic to neutral pH; for example Control (C) treatment of pH 7.3 (Agar Bacteriological) and 6.6 (Agar Commercial Gel). This showed that rooting was independent of pH. TANIMOTO & WATANABE (1986) stated that root growth was affected by pH in the medium but that pH was changed only slightly by agar. ICHIMURA & ODA (1998) reported that root growth-stimulating activity seems not to be involved in changes in pH. With the low rooting in the control autoclaved (CA) and charcoal autoclaved (ChaA) treatments of Agar Bacteriological, Agar Commercial Gel and Difco Bacto Agar (Figures 4.2; 4.3 and 4.4), autoclaving could be the possible cause of the reduction in root number.

B) Freeze- thaw technique

Similar results were observed using this technique with; Agar Bacteriological Control treatment ($A_F C$), of pH 6.6, charcoal added ($A_F Cha$) of pH 6.1 and charcoal added + IBA ($A_F Cha+IBA$); Agar Commercial Gel Control treatment ($A_F C$) of pH 6.4, charcoal added ($A_F Cha$) of pH 7.2 and charcoal added + IBA ($A_F Cha+IBA$) 7.7, which all gave low rooting. The pHs of $A_F IBA$ treatments for the four gelling agents (under Section 4.4.3) were slightly acidic, (pH range between 5.5-6.9) and just as for some of the above treatments, high rooting was achieved. The contrast in rooting with media of similar pHs confirms that pH plays no significant role in rooting. Rooting in the $A_F IBA$ treatment could be attributed solely to the potency of the IBA and not the pHs.

CHAPTER 5

SEPARATION, PURIFICATION AND QUANTIFICATION OF ROOT-STIMULATING SUBSTANCES IN GELLING AGENTS

5.1. INTRODUCTION

5.1.1. SOLVENT SYSTEMS, EXTRACTION, PURIFICATION AND ISOLATION OF IAA

There are various procedures that can be used to isolate, identify and measure IAA and other compounds with similar biological activities. IAA is freely soluble in methanol and ethanol, soluble in acetone, ethyl acetate and diethyl ether and sparingly soluble in water and chloroform. However, methanol appears to be the most efficient solvent tested even though it decolorizes the tissue indicating plastid breakdown. These denaturing properties allow rapid entry and it appears to give a higher yield of IAA from tissue than the other solvents mentioned (McDOUGALL & HILLMAN, 1978). However, one potential drawback when using any of these solvents is the risk of esterification of the carboxylic acid. However this is regarded as an insignificant problem (McDOUGALL & HILLMAN, 1978). ATSUMI, KURAISHI & HAYASHI (1976) claimed on the basis of simple and non-definitive purification methods that transamination of tryptophan to indole-3-pyruvic acid followed by decarboxylation to IAA could occur during acidic methanolic extraction of plant tissue.

All solvents including methanol, must be redistilled to remove impurities before extracting IAA. Absolute or 80% methanol can be used. Usually a ratio of 1 g fresh weight of tissue to a minimum of 5-10 ml of solvent is used (McDOUGALL & HILLMAN, 1978). The following criteria must be strictly considered during extraction of IAA: 1) extraction from tissue must be done with minimal contamination; 2) no synthesis,

conversion and degradation of the extracted IAA should occur during the extraction process; 3) only free IAA should be extracted such that a separate assessment can be made of the bound fraction in the residue; and 4) IAA in the extract must not arise as an artifact through microbial activity (McDOUGALL & HILLMAN, 1978). Enhancement of extraction rate is very important and McDOUGALL & HILLMAN (1978) state that this can be achieved by occasionally agitating the solution and using fresh solvents. The maximum extraction time for large and small quantities of material should not exceed 24 h and 1-2 h respectively.

The analysis of plant growth regulatory substances in plant extracts involve several steps. Initially the extracts are tested for biological activity using a bioassay system. Unequivocal identification can only be made by using physicochemical methods. The method used depends very much upon the individual growth regulators, its concentration and the spectrum of contaminants that are present (CROZIER & MORITZ, 1999). Further research must therefore involve accurate and quantitative techniques of sample purification and identification. The initial purification step after extraction of plant material normally involves partitioning between an aqueous phase and an immiscible organic solvent (CROZIER & MORITZ, 1999). The neutral compounds are distributed between the two phases according to their partition co-efficient (pK). The distribution of ionizable molecules depends upon their pK s and the pH of the aqueous phase. They will migrate into the organic phase when uncharged. Amphoteric compounds, however, tend to remain in the aqueous phase because they exist as dissociated structures regardless of pH (YOKOTA, MUROFUSHI & TAKAHASHI, 1980). Examples of amphoteric compounds are given in an partitioning flow chart (Figure 5.1).

5.1.1.1. Paper chromatography

This technique is used in combination with other bioassays. It is a good chromatographic method which is used initially to purify large quantities of extract and for separating compounds from each other. With this technique other compounds, originating from the extract with similar R_f values, can interfere with the hormones being isolated. In the isolation of ABA using paper and iso-propanol: ammonium hydroxide: water (10:1:1 v/v) as a solvent system, it was shown that the inhibitors in the zone R_f 0.6

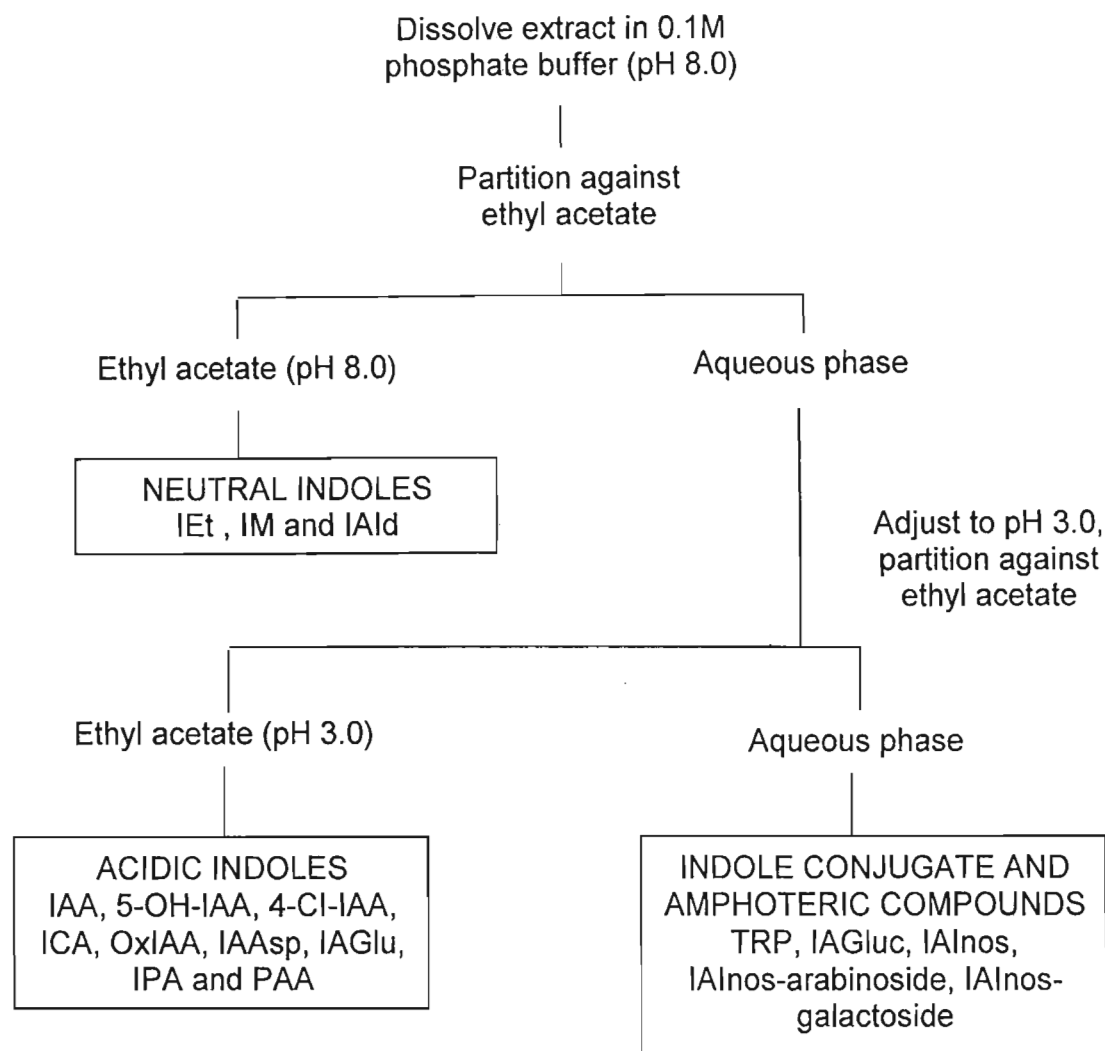


Figure 5.1. Partitioning procedures for the separation of indoles into neutral, acidic and conjugated fractions. Adapted from SANDBERG, CROZIER & ERNSTSEN (1987).

Abbreviations; IEt, Indole-3-ethanol; IM, Indole-3-methanol; IAld, Indole-3-aldehyde; IAA, Indole-3-acetic acid; 5-OH-IAA, 5-Hydroxy-3-indoleacetic acid; 4-Cl-IAA, 4-Chloroindole-3-acetic acid; ICA, Indole-3-carboxylic acid; OxIAA, Oxindole-3-acetic acid; IAAs, Indole-3-acetylaspartic acid; IAGlu, Indole-3-acetylglutamic acid; IPA, Indole-3-pyruvic acid; PAA, *p*-Phenylacetic acid; TRP, Tryptophan; IAGluc, Indole-3-acetylglucose; IAlnos-arabinoside, 5-O-L-arabinopyranosyl-2-O-(indole-3-acetyl) *myo*-inositol; IAlnos-galactoside, 5-O--L-galactopyranosyl-2-O-(indole-3-acetyl) *myo*-inositol.

to 0.7 at which ABA is eluted, contained several compounds including inhibitors such as salicylic acid, as well as promotive substances such as gibberellins and indoles. Interference of these compounds in the bioassays can lead to misinterpretation of the results (DÖRFFLING & TIETZ, 1983).

5.1.1.2. Mixed-mode solid phase extraction

Solid-phase extraction (SPE) using C₁₈ bound silica is frequently used as an efficient method for preparation of plant hormones on the basis of reversed-phase interactions (MORRIS, ZAERR & CHAPMAN, 1976; GUINN & BRUMMETT, 1990). However, cytokinins cannot be separated from auxins and abscisic acid, and cytokinin ribotides are poorly retained on this sorbent (GUINN & BRUMMETT, 1990). Since this single step does not produce samples pure enough for HPLC, it is mostly used in conjunction with ion-exchange or immunoaffinity chromatography prior to hormone determination by HPLC-MS or HPLC-ELISA (RIA) (REDIG, SCHMÜLLING & VAN ONCKELEN, 1996). The availability of SPE sorbents bearing both reversed-phase and cation exchange characteristics enables one step purification, increased retention and ensures high recoveries of a wide spectrum of hormones. DOBREV & KAMINEK (2002) noted that this efficient method which allows simple and fast separation of cytokinins from auxins and ABA, after their simultaneous extraction, has the following advantages over the commonly used procedures:

- i) The separation of cytokinins from auxins and ABA and their purification is achieved on a single column using step elution with different solvents which can be removed by evaporation;
- ii) The column may be allowed to run dry with no loss of capacity. This allows high sample through-put and potential automation; and
- iii) The extract is separated into three fractions containing 1) IAA and ABA; 2) cytokinin nucleotides; and 3) cytokinin bases, ribosides and glucosides.

5.1.1.3. HPLC analysis of endogenous IAA in gelling agents

Analytical and preparative HPLC is used to detect IAA and ABA in extracts with detection by either UV absorbance or fluorescence together with the appropriate retention time, giving up to 90% recoveries (BAUSHER & COOPER, 1976; BRENNER, ANDERSEN, CIHA, MONDAL & BRUN, 1976; DURLEY & KANNANGARA, 1976). MANN and JAWORSKI (1970) demonstrated that losses incurred during the purification of IAA can be established by the addition of radioactive IAA to the initial extract. The accuracy of this approach is dependent upon the fact that the radioactivity in the purified sample behaves in the same manner as the endogenous compounds and is exclusively associated with it. Therefore the labelled/endogenous compound ratio is maintained irrespective of sample losses during purification. Thus quantitative analysis of endogenous compounds extracted from the plant tissue can be carried out based on the recovered labelled material.

5.2. AIMS

To purify and quantify root stimulating substances in gelling agents.

5.3. MATERIALS AND METHODS

5.3.1. PAPER CHROMATOGRAPHY

Duplicate extractions of the Agar Bacteriological, Agar Commercial Gel and Difco Bacto Agar were carried out as in Chapter 2, using water and 80% ethanol respectively. Gelrite was extracted using 80% ethanol only. The air-dried extracts of the gelling agents were resuspended in 5 ml of the solvent used for extraction. These gelling agent extracts were strip-loaded onto a half sheet of Whatman No.1 chromatography paper and separated using a solvent system of iso-propanol:ammonium hydroxide:water (10:1:1 v/v). The chromatograms were left to run overnight in a descending manner. They were dried in an oven at 50°C overnight and divided into ten R_f zones. Each R_f zone was cut into small pieces and eluted with 10 ml distilled water. These were left overnight on a shaker and the paper removed and the extracts air dried. Dried samples were

resuspended in 20 ml distilled water and bioassayed using the mungbean assay as outlined in Chapter 2. A standard of 0.0203 g IBA and IAA (10^{-3} M) were dissolved in 5 ml distilled water and the above methodology used to obtain chromatograms. These were bioassayed.

5.3.2. ETHYL ACETATE PARTITIONING

Thirteen grams of gelling agent extract, prepared as outlined in Chapter 2 but using methanol instead of water, were resuspended in 100 ml 0.1 M phosphate buffer (pH 8.0) and partitioned against ethyl acetate according to the method of SANDBERG, CROZIER & ERNSTSEN (1987), as in Figure 5.1. To obtain the neutral, acidic and aqueous fractions, as indicated in the flow chart, each step in the partitioning procedure was against 3 X 100 ml ethyl acetate. These extracts were then dried *in vacuo* at 40°C and resuspended in 60 ml distilled water. Dilution series for the mungbean bioassay were as follows: 2 X 20 ml extract (100% of extract) and 2 X 10 ml extract + 10 ml distilled water (50% extract). The mungbean bioassay was carried out as described in Chapter 2. IBA was used as the standard.

5.3.3. MIXED MODE SOLID-PHASE EXTRACTION

Waters Sep-Pak Plus \dagger C₁₈ and Waters Oasis MCX cartridges were obtained from Waters Co. (Milford, MASS, USA). Each sample of gelling agent (preparation as in Chapter 2 using 80% methanol) was resuspended in 5 ml 80% methanol containing [1-¹⁴C] IAA as an internal standard (50000 dpm/sample; Amersham International, Buckinghamshire, UK) and passed through a \dagger C₁₈ cartridge which had been pre-conditioned with 5 ml distilled water, 5 ml methanol and 20 ml 80% methanol. The eluate was dried down to the aqueous phase at 35°C *in vacuo* and made up to 5 ml with 1M formic acid (pH 1.4). This was subsequently applied to an Oasis MCX column which had been pre-conditioned with 5 ml methanol and 5 ml formic acid. After sample application, the cartridge was washed with 5 ml formic acid and 5 ml methanol at a flow rate of 5 ml min⁻¹. The methanol wash was collected and dried under nitrogen, as it potentially contained the IAA. All procedures were carried out in dim light as IAA is degraded in light.

5.3.3.1 Methylation of samples

The residue from the methanol wash was resuspended in 100 µl methanol, and 2 ml ethereal diazomethane (preparation shown below) was added and left for 30 min to allow for methylation of plant hormones. The ether phase was subsequently removed under nitrogen and resuspended in 2 ml ethyl acetate. This was partitioned against 2 ml distilled water and the ethyl acetate fraction retained. This process was repeated until the ethyl acetate phase was colourless. The combined ethyl acetate fractions were passed through a Nucleosil strata (NH₂) column (Phenomenex®, Torrance, CA, USA) which had been pre-conditioned with 5 ml methanol and 8 ml ethyl acetate and subsequently dried under nitrogen. Methylation is a useful derivation step as methyl esters are stable and can be purified easily prior to analysis. It also enhances HPLC separations and improves detection limits (CROZIER & MORITZ, 1999).

A) *Ethereal diazomethane preparation*

Ethereal diazomethane was prepared according to the small scale technique described by FALES, JAOUNI & BABASHAK, (1973) in a Wheaton Diazomethane Generator (Pierce Co. Rockford, ILL, USA). *N*-nitroso-*N*-methylurea (0.35 g) was placed in the inner tube and 0.5 ml distilled water added to cool the reaction. Dry diethyl ether (3.5 ml) was placed in the outer tube. Both inner and outer tubes were clamped together and cooled on ice for 25 min. Subsequently 1 ml 5 N NaOH was injected through the teflon septum into the inner tube. The reaction was allowed to proceed for approximately 1 h until the ether developed a deep yellow colour. Dry diethyl ether was prepared by passing diethyl ether through a charcoal /diatomaceous earth (50:50, v/v) column (1 cm x 5 cm), upon which the dry diethyl ether was stored in a bottle containing iron filings, to remove peroxides. This is important as water and peroxides in the diethyl ether can cause an explosion during ethereal diazomethane generation.

5.3.4. HPLC QUANTIFICATION OF ENDOGENOUS IAA

Quantification of IAA was carried out by HPLC using a semi-preparative 5 µm C₁₈ column (250mm x 10 mm i.d., Hypersil 5ODS) eluted over 55 min with a linear gradient of 20-80% methanol in water at a flow rate of 2 ml min⁻¹. Compounds of interest were detected at 260 nm, using a Spectra System® UV6000LP detector (Thermo Separations

Products Inc., San Jose, CA, USA) and quantified after calibration with an authentic standard of IAA methyl ester. For estimation of losses incurred during extraction, fractions were collected at 1 min intervals, and mixed with 4 ml Beckman Ready Value Liquid Scintillation Cocktail and radioactivity determined by using a Beckman LS 6000LL Scintillation Counter.

5.4. RESULTS

5.4.1. PAPER CHROMATOGRAPHY

Both auxin standards gave well-defined activity in the mungbean bioassay after being run on paper. IBA ran at R_f 0.5-0.6 (Figure 5.2H) and IAA at R_f 0.4-0.5 (Figure 5.2I). There were two rooting peaks for Agar Bacteriological when extracted using 80% ethanol (Figure 5.2A). These were eluted at R_f 0.2, 0.3 and 1.0. For the water gelling agent extract, there was a peak of high auxin-like activity at R_f 0.1 (Figure 5.2B). Low rooting activity was detected for Agar Commercial Gel when extracted using 80% ethanol (Figure 5.2C). However, two peaks with good rooting were detected in the water gelling agent extract. These peaks co-eluted at R_f 0.2, 0.3 and 0.9 (Figure 5.2D). The highest peak in the 80% ethanol extract of Difco Bacto Agar occurred at R_f 0.1-0.2 (Figure 5.2E). Rooting in the water gelling agent extract consisted of three peaks at R_f s 0.2 and 0.4 with the largest peak of activity at R_f 0.8 (Figure 5.2F). Auxin-like activity in the 80% ethanol extract of Gelrite was low. However, two active zones were detected at R_f 0.2 and 0.6.

5.4.2. ETHYL ACETATE PARTITIONING

5.4.2.1. Agar Bacteriological

The highest rooting was observed in the EA-pH 8.0 fraction at 100% concentration (Figure 5.3A) which was comparable to 10^{-4} M IBA (Figure 5.3E). There was no significant difference in rooting between EA-pH 3.0 fraction at the 50 and 100% concentrations (Figure 5.3A). The lowest rooting was noticed in the EA-pH 3.0 of the 50% concentration and this was comparable to 10^{-5} M IBA. All rooting in the agar

Figure 5.2.

Auxin-like activity detected using the mungbean bioassay after paper chromatographic separation of water and 80% ethanol extracts of various gelling agents. The extracts were separated by descending chromatography using iso-propanol:ammonium hydroxide:water (10:1:1v/v). IBA and IAA standards are represented in graphs H and I. Different letters indicate significant differences ($P=0.05$)

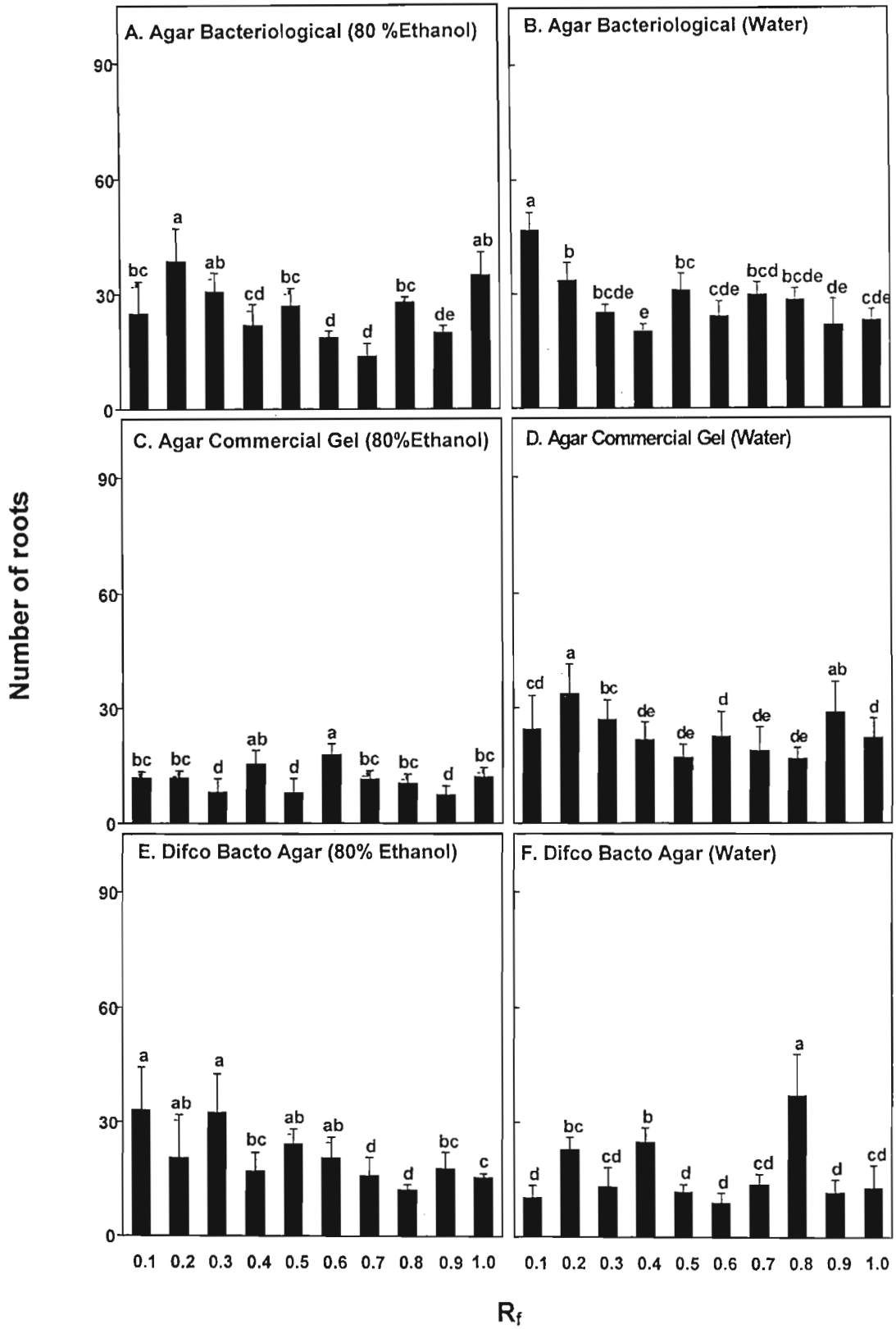
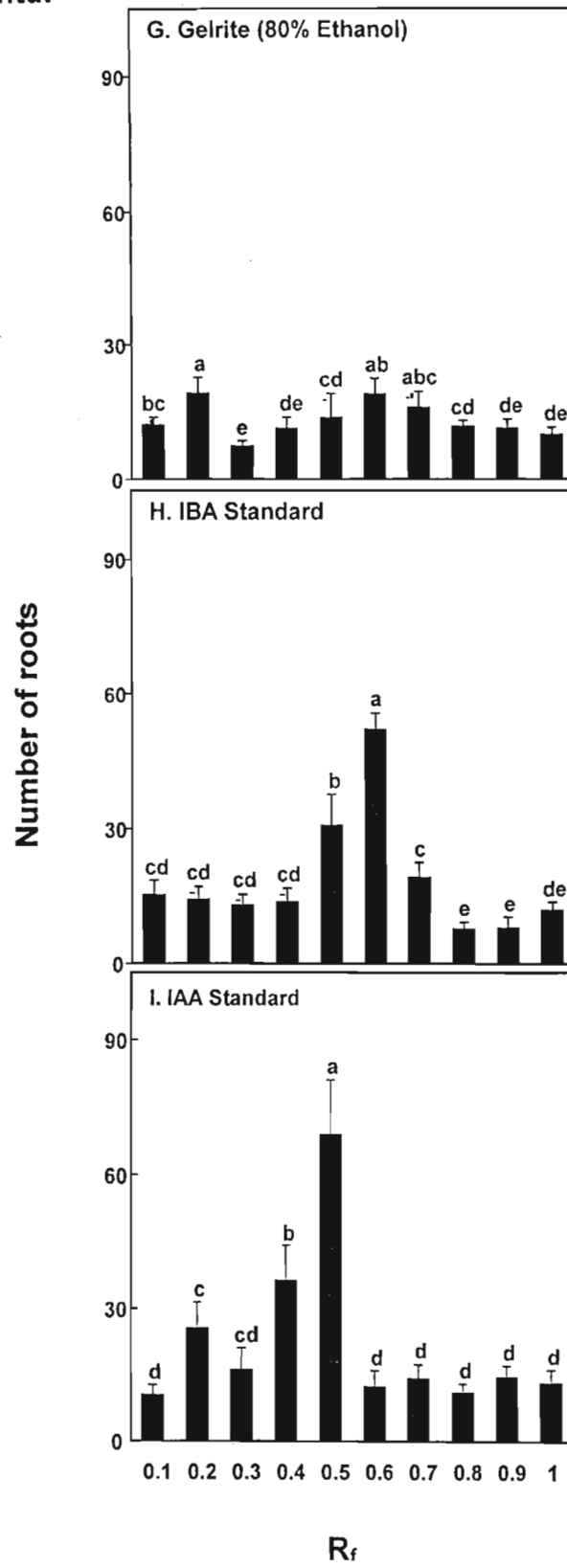


Figure 5.2.contd. Auxin-like activity detected using the mungbean bioassay after paper chromatographic separation of water and 80% ethanol extracts of various gelling agents. The extracts were separated by descending chromatography using iso-propanol:ammonium hydroxide:water (10:1:1v/v). IBA and IAA standards are represented in graphs H and I. Different letters indicate significant differences ($P=0.05$)

Figure 5.2. contd.



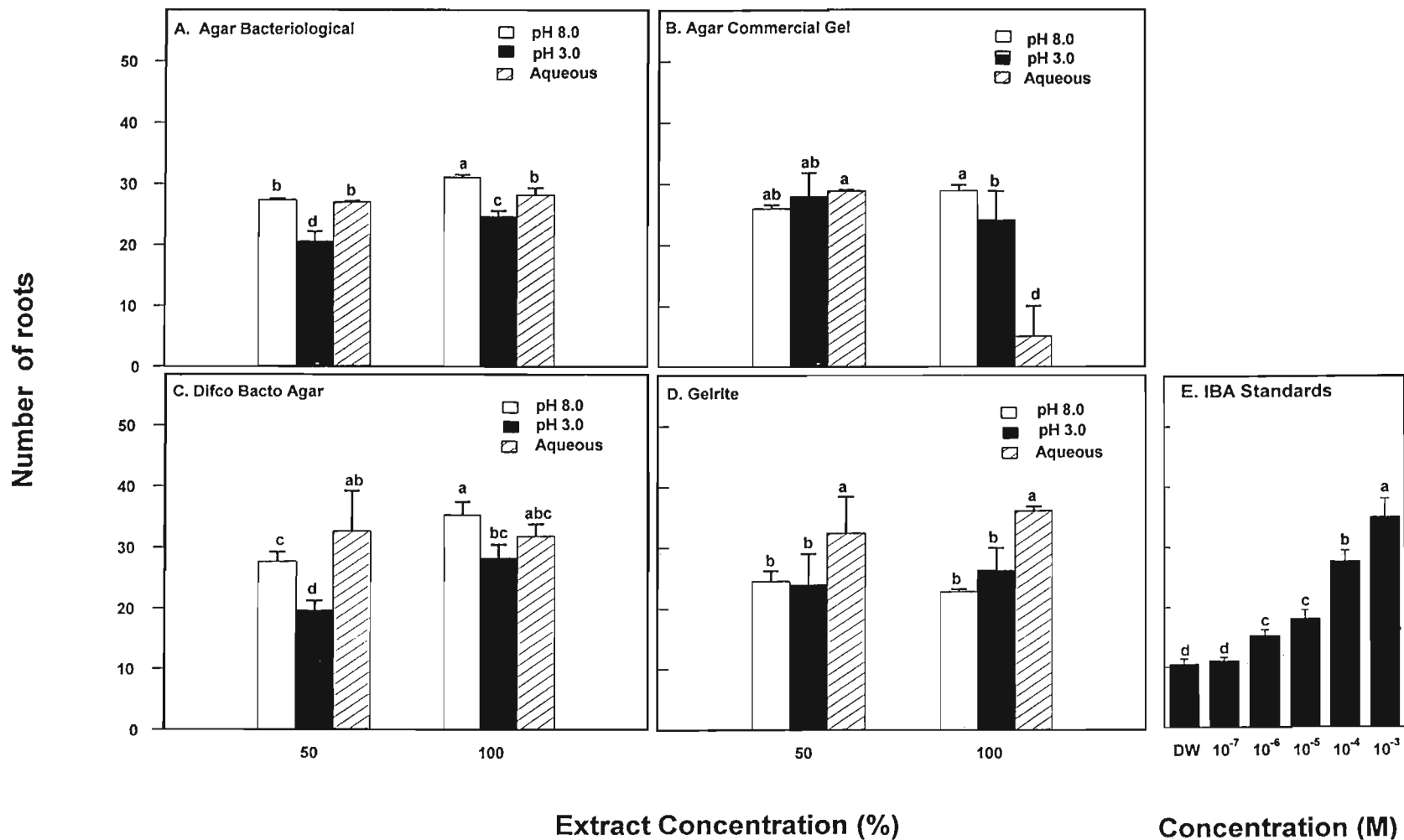


Figure 5.3. Auxin-like activity detected in the various gelling agent extracts separated by ethyl acetate partitioning and using the mungbean bioassay. EA-pH 8.0 is the initial ethyl acetate phase used to extract neutral indole. EA-pH 3.0 is the second ethyl acetate phase used to extract the acidic indoles and the aqueous phase contains the indole conjugates and amphoteric compounds (SANDBERG, CROZIER & ERNSTSTEN, 1987). E is the IBA standard, DW is distilled water. Different letters indicate significant differences (P=0.05) within each treatment.

extracts was higher than that in the distilled water (control).

5.4.2.2. Agar Commercial Gel

Rooting in the three fractions of the 50% concentration and EA-pH 8.0 fraction of the 100% concentration were not significantly different from each other (Figure 5.3B) and were comparable to 10^{-4} M IBA (Figure 5.3E). Rooting in EA-pH 3.0 of the 50% and 100% concentrations were not significantly different from each other. Rooting in the Aqueous fraction of the 100% concentration was greatly reduced and was lower than that in distilled water (control).

5.4.2.3. Difco Bacto Agar

The highest rooting was observed in the EA-pH 8.0 fraction of the 100% concentration (Figure 5.3C) which was comparable to 10^{-3} M IBA. However, there was no significant difference between this and rooting in aqueous fractions of 50 and 100% concentrations (Figure 5.3C). Rooting in the aqueous and EA-pH 3.0 fractions of the 100% concentration was not significantly different from rooting in the EA-pH 8.0 fraction of the 50% concentration. The lowest rooting was noticed in the EA-pH 3.0 fraction of the 50% concentration which was comparable to rooting in 10^{-5} M IBA.

5.4.2.4. Gelrite

The aqueous fractions at both the 50% and 100% concentrations gave the highest rooting and were not significantly different from each other (Figure 5.3D). It was comparable to rooting at 10^{-4} M IBA (Figure 5.3E). Rooting in the EA-pH 8.0 and EA-pH 3.0 fractions of the 50% and 100% concentrations were not significant from each other.

5.4.3. HPLC QUANTIFICATION OF ENDOGENOUS IAA

Figures 5.4 and 5.5 show chromatograms of separation of IAA methyl esters from Difco Bacto Agar and Gelrite. The results in Table 5.1 indicate endogenous IAA levels in the four gelling agents studied, using HPLC. Difco Bacto Agar possessed the highest concentration of IAA followed by Agar Commercial Gel. The levels in Agar Bacteriological and Gelrite were 3-fold and 5-fold lower than that in the Difco Bacto Agar respectively.

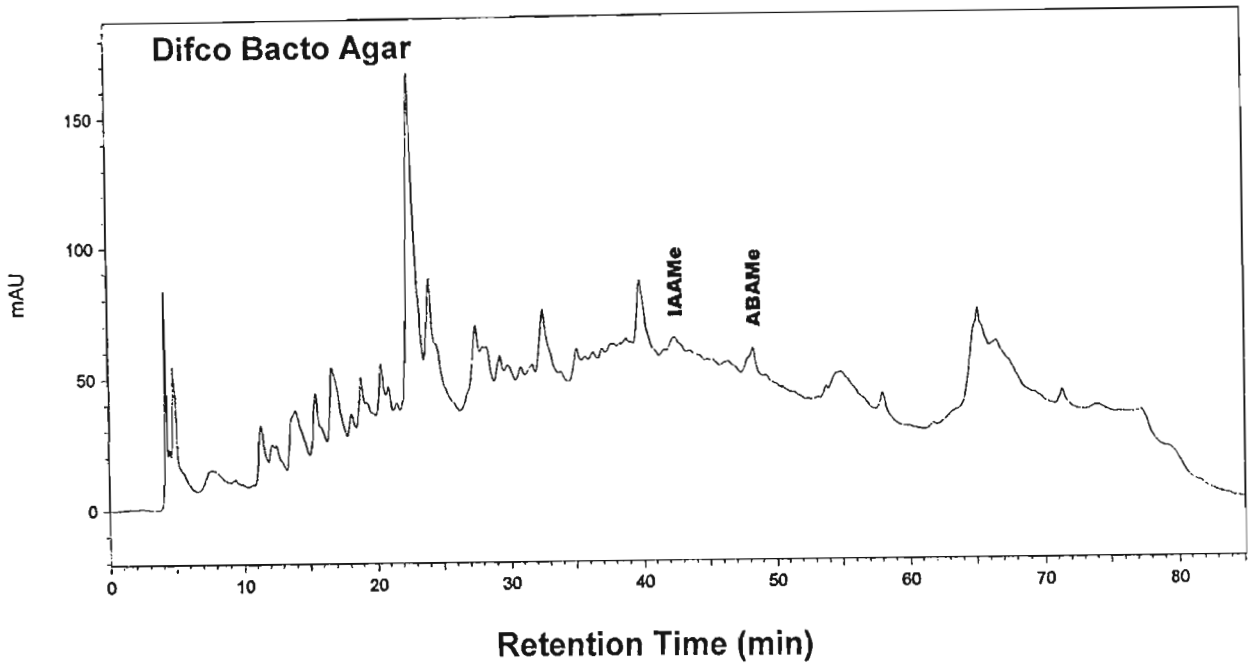


Figure 5.4. Chromatogram of separation of IAA methyl ester from Difco Bacto Agar

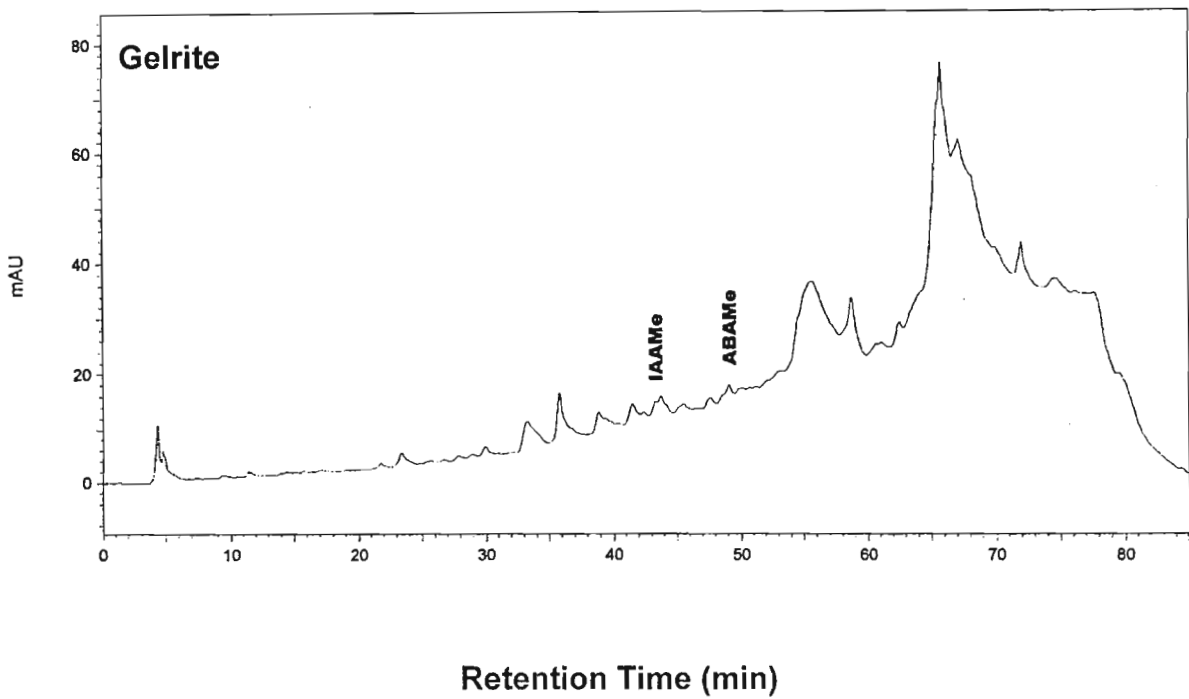


Figure 5.5. Chromatogram of separation of IAA methyl ester from Gelrite

Table 5.1. Endogenous level of IAA in gelling agents as quantified by reference to an authentic IAA standard obtained by HPLC.

Gelling Agent	IAA-equivalents (nmol g ⁻¹)
Agar Bacteriological	0.40
Agar Commercial Gel	1.01
Difco Bacto Agar	1.29
Gelrite	0.25

5.5. DISCUSSION

5.5.1 PAPER CHROMATOGRAPHY

DÖRFFLING & TIETZ (1983) indicated that when using the solvent system of isopropanol : ammonium hydroxide : water (10:1:1 v/v), the peak for IAA was at R_f 0.37. In the current experiment the chromatograms of all the gelling agents had peaks of activity which co-eluted with IBA (R_f 0.5-0.6; Figure 5.2H) and IAA (R_f 0.4-0.5; Figure 5.2I). However, these peaks were not the most effective rooting substances present in the extracts. The results from the various gelling agents suggest that there are many other compounds which stimulate rooting in the gelling agents and these could be detected depending on the type of solvents and/or the method of extraction used.

With Agar Bacteriological rooting in both 80% ethanol (Figure 5.2A) and water (Figure 5.2B) extracts was high showing that there were both polar and non polar root stimulating substances present or possibly that the rooting substances could be eluted with both 80% ethanol and water. With Agar Commercial Gel, auxin-like activity in the water extract (Figure 5.2D) was greater than in the 80% ethanol extract (Figure 5.2C), suggesting that the most active rooting substances were highly polar and were therefore sparingly soluble in 80% ethanol. Alternatively, inhibitory substance(s) could have been eluted in the 80% ethanol which might have masked the effect of the root stimulating substances resulting in low rooting.

The highly polar compounds in Difco Bacto Agar were eluted at R_f 0.2-0.4 and 0.8 with the latter being the most active (Figure 5.2F). The less polar compounds were eluted with 80% ethanol which were active at R_f 0.1, 0.3 and 0.5 (Figure 5.2E). In the 80% ethanol extract of Gelrite (Figure 5.2G) compounds eluted were less polar and gave very low rooting. However, it is difficult to state whether there are highly polar compounds present since extraction with water was a problem due to the matrix of the Gelrite. The highly polar compounds detected in the Agar Bacteriological and Agar Commercial Gel partly confirmed the statement by ICHIMURA and ODA (1998) that root growth stimulating substances from agars are highly hydrophilic.

5.5.2. ETHYL ACETATE PARTITIONING

FIRN (1986) stated that the effects of PGRs in root growth are often a function of the concentrations and the types of PGR present. Poor rooting in the 100% aqueous fraction of Agar Commercial (Figure 5.3B) could either be the result of elution of an excessively high concentration of root-stimulating substances which suppressed rooting, or some other toxic substance which was eluted and thus had an adverse effect on rooting and/or increased the activity of other processes that inhibited rooting. The aqueous fraction of Gelrite at both 50 and 100% concentration (Figure 5.3D) contained strong activity and it is possible that the major rooting substances eluted were very soluble in this fraction which was supposed to be free from neutral and acidic auxins but contained indole conjugates and amphoteric compounds (Figure 5.1). The general trend of rooting in the fractions of the various gelling agents was good and thus showed how effective the ethyl acetate partitioning procedure is, particularly, for partitioning auxin-like substances from Gelrite.

5.5.3. HPLC QUANTIFICATION OF ENDOGENOUS IAA

Endogenous IAA appears to be present in all the gelling agents, but in various quantities. In previous experiments (Chapters 3, 4 & 5), rooting of mungbeans in Agar Bacteriological extracts had been consistently good, either comparable or higher than the 10^{-3} M IBA standard. Therefore, the relatively low IAA concentration of Agar Bacteriological 0.40 nmol g^{-1} (Table 5.1) detected by HPLC compared to that in Difco

Bacto Agar and Agar Commercial Gel which were higher (Table 5.1) suggest the involvement of more potent stimulating substances other than IAA in the rooting pattern observed with Agar Bacteriological.

The low IAA level in Gelrite (0.25 nmol g^{-1}) (Table 5.1) reflects the relative low rooting characteristic of mungbeans with extracts of Gelrite mentioned previously, except with the ethyl acetate partitioning where rooting was unexpectedly good (Figure 5.3D). This low rooting in Gelrite could be attributed to its complex network of polymers (jelly-like) which does not allow easy release of rooting substances with the solvents used.

CHAPTER 6

CONCLUSIONS

The use of gelling agents in tissue culture has been of paramount importance and it will be of great interest to know the hormonal content in them. Research in this area is very limited and increasing the scope of study with regards to the nutrient level, particularly the hormones, will be beneficial. In this regard the choice of gelling agents could essentially be based on the nutrient level relating to specific tissue culture techniques and not solely on the prices as stated by DEBERGH (1983).

It is well known that adventitious rooting is controlled by growth substances, and auxins are the main hormones that promote rooting and play a direct role in this process (FABIJAN, TAYLOR & REID, 1981; GASPAR & HOFINGER, 1988). It was evident that rooting substances in agars are water soluble and the higher the concentration of agar extract tested, the higher the rooting. Throughout the experiments gelling agent extracts for bioassay were in the liquid state to ensure free movement of molecules and to make absorption easy for enhanced rooting. Rooting was generally good with the agar extracts as in the control treatments (C) (Table 4.2). Gelrite, was generally observed to stimulate low mungbean rooting. This was in agreement with ICHIMURA & ODA (1998) who stated that Gelrite (gellan gum), cellulose and starch stimulated root growth of lettuce seedlings to a lesser extent.

The results obtained in the screening of the gelling agents (Figures 3.1; 3.2 and 3.3) show that different gelling agents have different root stimulating abilities depending on either the raw materials used and/or the preparation which includes the incorporation of additives.

Activated charcoal is able to adsorb high concentration of PGRs such as BA, IAA, IBA and kinetin (CONSTANTIN, HENKE & MANSUR, 1977, WEATHERHEAD, BURDON & HENSHAW, 1978; FRIDBORG & ERIKSSON, 1975) and is able to release them into

the medium to explants through active uptake. Desorption is based on temperature, pH of solution, and the amount and nature of compounds adsorbed (JOHANSSON & ERIKSSON, 1977; LITZ & CONOVER, 1980; YAM, ERNST, ARDITT, NAIR & WEATHERHEAD; 1990; JOHANSSON, CALLEBERG & GEDIN, 1990; M'KADA, DORION & BIGOT, 1991). In this study activated charcoal in extracts of Agar Bacteriological and Agar Commercial Gel did not significantly stimulate nor inhibit rooting (Figures 4.2C and 4.3C) as compared to the rooting in the controls (Figures 4.2A and 4.3A). However, it efficiently removed rooting substances when it was added to extracts of autoclaved frozen gelling agents + IBA (Figures 4.5D; 4.6D; 4.7D and 4.8D). The reason could be due to high levels of hormones present. Therefore, the use of activated charcoal in media containing a high amount of plant hormones must be carefully evaluated since it reduces rooting induced by IBA and other growth hormones. On the contrary, activated charcoal may exert a beneficial effect by reducing or eliminating the damaging effect of phytotoxic metabolites (HABAGUCHI, 1977; MENSUALI-SODI, PANIZZA, SERRA & TOGNONI, 1993).

Autoclaving is a standard procedure for sterilizing nutrient medium for tissue culture and most tissue cultures are grown at a pH of 5.6-5.8, with pH adjustment being made prior to autoclaving. Autoclaved gelling agent extracts reduced rooting of mungbean cuttings (Figures 4.2B; 4.3B and 4.4B) suggesting that the root promoting compounds in the gelling agents are not thermostable. Therefore, in tissue culture they may not have great impact on growth.

Partitioning with ethyl acetate indicated the presence of high auxin-like substances in EA-pH 8.0; EA-pH 3.0; and aqueous fractions of the four gelling agents, except the aqueous fraction of the 100% concentration of Agar Commercial Gel where rooting was very low (Figure 5.3). This implies that a wide range of compounds are responsible for root stimulation. Auxin-like activity of Gelrite was increased markedly as a result of ethyl acetate extraction (Figure 5.3D) and was evident in the high rooting activity in the three fractions of both 50 and 100% concentrations. The question one may ask is was the complex polymer nature of Gelrite reduced and therefore released substances which promoted good rooting in the gelling agent extract?.

Quantitative estimates of endogenous IAA in the four gelling agents using reversed phase HPLC showed Difco Bacto Agar and Agar Commercial Gel to contain the highest amount of IAA. Agar Bacteriological was relatively low with Gerite having the least IAA. Rooting in Agar Bacteriological had consistently been high in all its extracts as indicated in the control treatment (Figure 4.2A) and the paper chromatographic separation using both 80% ethanol and water extracts (Figure 5.2A & B). Thus the low IAA compared to Difco Bacto Agar and Agar Commercial Gel shows that IAA might not solely be related to its rooting response but there must be several stimulating substances (unidentified and estimated) contributing to rooting. The presence of different root stimulating substances is evident due to the auxin-like activity detected in the various R_f s on the paper chromatograms, the different fractions in ethyl acetate treatment (Figure 5.3) and the IAA detected using HPLC.

In the present work water extracts of the gelling agents generally increased rooting with increasing concentration. Rooting substances could influence each other to change their effectiveness resulting in root promotion as observed with the IBA + gelling agent complex (Figures 4.5C; 4.6C; 4.7C and 4.8C). It could also result in rooting suppression, but that was not confirmed in this experiment. This is in agreement with LEE, WETZSTEIN & SOMMER (1986) who stated that the concentrations and the types of gelling agents could influence tissue cultured *in vitro*. Even though treatments of the extracts (addition of charcoal and autoclaving) are very important, the agars greatly stimulated root growth in mungbean cuttings as found by ICHIMURA & ODA (1998) in lettuce seedlings.

This study has clearly revealed that root stimulating substances in agar are highly polar and IAA and other root stimulating substances (unidentified) are present in the four gelling agents tested. There is a need for further studies to ascertain not only the types and amounts of rooting substances and other hormones but also the inorganic nutrients available in the gelling agents. This knowledge is necessary so that informed decisions can be made on the gelling agent to be used in different tissue culture systems. This will also be beneficial in the medical field where agar is more often used in the preparations of capsules.

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