THE EFFECT OF CHARCOAL ON TISSUE MORPHOGENESIS *IN VITRO*

by

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PREFACE

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg, from January 1998 to July 2000 under the supervision of Professor J van Staden.

The results have not been submitted in any other from to another University and except where the work of others is acknowledged in the text, are the result of my own investigation.

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November 2000

I declare that the above statement is correct.

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ABSTRACT

Pan, Manjing (2000) Effect of Charcoal on Tissue Morphogenesis *In Vitro*. Ph.D thesis, University of Natal Pietermaritzburg, Republic of South Africa.

The effect of activated charcoal, autoclaving and culture media on sucrose hydrolysis in tissue culture media was investigated. Activated charcoal acidified an aqueous sucrose (5%) solution and culture media by about 1 to 2 units after autoclaving. Sucrose hydrolysis in tissue culture media and/or aqueous sucrose (5%) solutions containing activated charcoal (buffered to pH 5.8) was dependent on both the hydrogen ion concentration (pH) and autoclaving. After autoclaving, 70%, 56% and 53% sucrose hydrolysis were respectively recorded in a 5.0% sucrose solution, Murashige and Skoog (MS) and Gamborg B5 (B5) liquid media in the presence of 1.0% activated charcoal, added before autoclaving. In the absence of activated charcoal, autoclaving resulted in about 20% of the sucrose being hydrolysed

The adsorption of 2, 4-dichlorophenoxyacetic acid (2,4-D) by activated charcoal from methanol and aqueous solutions was determinated using HPLC. The amount of the added 2,4-D decreased in both methanol and aqueous solutions in the presence of activated charcoal, compared with those in the absence of activated charcoal. In methanol and aqueous solutions, activated charcoal used at the level of 0.1% significantly reduced 2,4-D. About 68.4% and 60.9% respectively of the added 2,4-D was adsorbed by activated charcoal (1.0%) from

these solutions. The changes of inorganic elements in MS-salt solutions, in the presence of activated charcoal, were analysed by SEM-EDX. The concentrations of magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn) deceased in the presence of activated charcoal, while the concentrations of potassium (K), copper (Cu), manganese (Mn), phosphorus (P), and sulphur (S) increased in the MS salt solution in the presence of activated charcoal compared with no activated charcoal in the medium. This suggests that activated charcoal adsorbed calcium, magnesium, iron and zinc and released copper, manganese, phosphorus and sulphur.

Rooting occurred when 7-day-old seedling hypocotyls of *Daucus carota* L. Cape Market were placed on MS medium supplemented with 2,4-D, and IAA/NAA in the presence of activated charcoal. Hypocotyls did not produce roots on the 2,4-D- containing media in the absence of activated charcoal. The roots were produced polarly on the NAA/IAA-containing media in the presence of activated charcoal. No-polarity of root formation was observed on media supplemented with NAA/IAA without activated charcoal. Different responses of hypocotyls to a series of 2,4-D concentrations (0.5, 1.0, 3.0 5.0, 8.0, and 10.0 mg l^{-1}) were observed on media supplemented with 0.02, 0.1 and 0.5% activated charcoal. In the NAA/IAA-containing media in the presence of activated charcoal, root number per hypocotyl decreased. Root number per hypocotyl, on the media supplemented with NAA and IAA, increased when hypocotyls were pre-cultured on MS medium supplemented with 2,4-D (1.0 mg l^{-1}) for 2-3 days. When hypocotyls were pre-cultured on a 2,4-D-containing MS medium for 5 days, embryos emerged from the hypocotyls directly on the medium supplemented with 2,4-D in the presence of activated charcoal.

Addition of activated charcoal to MS medium supplemented with 2,4-D resulted in somatic embryogenesis of *Daucus carota.* Somatic embryos were not formed on the medium in the absence of activated charcoal. In suspension culture, the incorporation of 0.01 to 1.0% concentrations of activated charcoal to the MS medium, irrespective of 2,4-D, increased the number of somatic embryos produced. The maximum number of somatic embryos were produced with 1.0% activated charcoal. Further development of embryos of *Daucus carota* occurred on the media in the presence of activated charcoal, and the embryos subsequently regenerated normal plantlets. Abnormal somatic embryos followed the addition of 3.0% activated charcoal to the medium.

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ABBREVIATIONS

AC	Activated charcoal
B5	Gamborg B_5 medium (1968)
BA	Benzyladenine
EDX	Energy Dispersive X-ray
GC	Gas-chromatography
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige & Skoog medium (1962)
NAA	α-Naphthaleneacetic acid
P/B	Peak-to-background ratios
PVP	Polyvinylpyrrolidone
SEM	Scanning electron microscopy
2,4-D	2,4 - Dichlorophenoxy acetic acid

CHAPTER ONE

Literature Review

The use of charcoal in *in vitro* culture

1.1 Introduction

All normal living cells within the plant body posses the potential to regenerate the entire organism. This totipotency has been exploited through the culture of protoplasts, cells, tissues and organs *in vitro*. The *in vitro* growth and development of a plant is determined by a number of complex factors: (a) the genetic make-up of the plant; (b) nutrients: water, macro- and micro-elements and sugars; (c) physical growth factors: light, temperature, pH, O_2 and CO_2 concentrations; and (d) some organic substances: hormones and vitamins. The genetic make-up is a decisive factor at every stage of plant development. The expression of the genome also depends on the physical and chemical conditions to which the material is subjected while maintained *in vitro*.

Success in plant cell culture is determined by many factors. Subcultured callus and explants can be induced to form organs or embryos through manipulation of the above factors. Both embryogenesis and organogenesis may occur in culture. The tendency for organogenesis and/or embryogenesis to occur in culture appears to be species specific. Plant growth regulating substances play a most important role in these processes.

1.1.1 Organogenesis

Organogenesis involves *de novo* production of adventitious shoots on explants from many different sources. Plants are multiplied by organogenesis for various reasons, including improved multiplication rate, genetic engineering, most importantly however, to maintain clonal characteristics. Many factors can affect shoot organogenesis. Most of these factors can be: a) environmental conditions such as light and temperature; b) the culture medium including components from salts to plant growth regulators; and c) explant material including explant type and physiological development of the tissue. Each factor can affect species and cultivars differently.

Many plant growth regulating substances are included in the culture medium to manipulate organogenesis *in vitro*. A large number of plant species respond to a suitable auxin/cytokinin balance by forming shoots and roots. A variety of substituted purines, pyrimidines and ureas have also been used successfully in place of cytokinins to bring about organogenesis (THORPE 1980; CHANDLER and THORPE 1986). Similarly, various auxin-like compounds can satisfy the auxin requirement *in vitro*. Other phytohormones, including gibberellins and abscisic acid added to the medium play a role in organogenesis. No generalisations can be made with respect to the effects of these substances in shoot and root formation, as they have been shown to repress, enhance or be without effect in different plant species (THORPE 1980).

In addition to phytohormones, other metabolites including adenine, guanine, uracil, uridine, amino acids, various phenolic acids, nicotine, and anti-auxins, stimulate organogenesis in different species (THORPE 1980; CHANDLER and THORPE 1986). The interaction between these different substances is in agreement with the basic ideas of Skoog and Miller (1957) on plant morphogenesis. It is possible that many of the mentioned metabolites alter the level of endogenous phytohormones. Lastly, polyamines (BAGNI and BIONDI 1987; TORRIGIANI *et al.* 1987; 1989), oligosaccharides (TRAN THANH VAN *et al.* 1985) and the primary cell wall itself (FRY 1990) have been implicated in organogenesis.

1.1.2 Embryogenesis

Somatic embryogenesis is the formation of an embryo from a cell other than a gamete, or the product of gametic fusion, and is a process analogous to zygotic embryogenesis, but one in which a single cell or a small group of vegetative cells are the precursors of the embryo (AMMIRATO 1983). In manipulating embryogenesis, it seems clear that two media components, auxin and nitrogen, play crucial roles (KOHLENBACH 1978). The importance of auxin was first recognized by Halperin and Wetherell (1964). Unlike organogenesis, somatic embryogenesis can recapitulate in zygotic embryogenesis with the production of embryos having shoots and roots (bipolarity). Since the development of somatic embryogenesis in carrot tissue cultures (REINERT 1958; STEWARD *et al.*1958), much has been written on the potential application of embryogenic cultures for a number of purposes, including mass clonal propagation, genetic engineering, protoplast culture and production of potential useful somaclonal variants.

Further studies showed that the process of somatic embryogenesis normally takes place in two stages: (a) the induction of cells with embryogenic competence in the presence of high concentrations of auxins, and (b) the development of the embryogenic masses into embryos in the absence of, or in the presence of, a lowered concentration of auxin (NOMURA and KOMAMINE 1985). 2,4-Dichlorophenoxyacetic acid (2,4-D) is the auxin most commonly used to bring about somatic embryogenesis. The process of somatic embryogenesis is often initiated in media containing high levels of auxins, but embryos do not develop further until the auxin concentration is reduced. Somatic embryogenesis has been reported for a number of plants such as monocotyledonous and dicotyledonous floricultural genera, woody perennials and conifers. The formation of somatic embryos is the predominant mode of *in vitro* regeneration for a large number of woody and monocotyledonous species which are considered to be highly recalcitrant. This is generally achieved by placing tissue explants from young plant organs, that comprise largely of meristematic and undifferentiated cells, on media containing high concentrations of strong auxins (such as 2,4-D), and sometimes, certain cytokinins.

1.2 Charcoal

Charcoal has been used as a purifying and decolourizing compound for liquids since the 18th Century. Charcoals used prior to the present century were produced by pyrolysis only. They were not subsequently oxidized and then purified to produce the activated charcoal (AC) which is widely used at present. Activation

of charcoal by treatment of the pyrolysis product with a stream of carbon dioxide to produce a charcoal of superior adsorping properties is covered by patents dated 1900/1 by the Russian inventor Ostrejko (YAM *et al.* 1990).

Charcoal is any form of carbon characterized by a high adsorptive capacity for gases, vapors and colloidal solids. Charcoal is produced by destructive distillation of woods, peat, lignite, nut shells, bones, vegetables or other carbonaceous matter. Different kinds of activated charcoal are prepared for different purposes. Charcoals activated for the adsorption of gases are harder and more dense than those used for purification of liquids. They possess a large number of small pores. Activated vegetable charcoals can be produced from wood, wood waste, paper-mill waste liquors and peat. They are frequently used in culture media. Activated carbon can also be produced from petroleum coke and coals.

Activated carbons are characterized by very large specific areas ranging from 600 to 2000 m^2 g l^{-1} and pore distributions ranging from 10 µm to 500 µm (YAM *et al.* 1990). All activated charcoal derived from wood has a particularly large surface area (most of it internal). Activated charcoal used in nutrient media has an adsorption preference for moderately polar rather than apolar or highly polar organics. They show greater adsorption for aromatic than olefinic unsaturated products (YAM *et al.* 1990). Therefore, aromatic compounds such as the phenolics and their oxidates, auxins [indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA)], cytokinins [benzyladenine (BA)], and hormones (having heterocyclic and unsaturated ring structures (e.g. kinetin), could have great adsorption affinity with activated charcoals. In contrast, the highly polar and readily

water-soluble sugars and sugar alcohols (sorbitol, mannitol and inositol) might not be removed from the medium and/or solution. Sparingly soluble organics are dispersed in the aqueous solvent, and tend to separate on prolonged standing, or change of pH. They could also be adsorbed.

Solutes in a solution in contact with activated charcoal will be adsorbed. This will continue until an equilibrium between adsorbed and desorbed molecules is established (adsorption isotherm). The adsorptive capacity of charcoal generally is dependent on a variety of factors such as density, purity of charcoal, and pH. Inorganic salts may affect the adsorption capacity of charcoal. Halhouli *et al.* (1995) found that pH and three inorganic salts (KCl, KI, and NaCl) affected the adsorption isotherms of phenol (from a dilute aqueous solution) by activated charcoal. The authors noted that different concentrations of each salt (0.1, 0.02, and 0.005 M) produced different effects. The effect of pH (in the pH range 3 -11) in the presence of KI, KCl and NaCl was also different.

The structure of activated charcoal is an important factor which intimately affects its adsorptive capacity. The structure investigation of commercial activated charcoal by X-ray diffraction showed that it is amorphous in nature and has a micro-crystalline structure (QADEER *et al.* 1994). The pore size distribution curve and the analysis of the nitrogen adsorption isotherm indicated the microporous nature of the activated charcoal with a surface area of 1000 $m^2 g^{-1}$, porosity 75.7% and pore volume of 1.4 $cm^2 g^{-1}$ (QADEER *et al.*1994). Wurster *et al.* (1994) used a molecular modeling program to stimulate the adsorption of various barbiturates by an activated charcoal surface. The activated charcoal surface was modeled

using graphitic crystallites which had either no oxygen-containing functional group, or a C=O functional group. Estimates for heat of adsorption ranged from -62.3 $kJmol^{-1}$ for barbituric acid to -91.1 $kJmol^{-1}$ for mephobarbital on the hydroxylated surface. It demonstrated the greater importance of the C-O functional state for barbiturate adsorption compared to the C=O functional state.

Many grades of activated charcoal are available from chemical suppliers. Some are acid-washed to meet certain chromatographic requirements. Powder grades, usually average about 10 µm in particle size. They are easily dispersed in media. An important factor is the surface area per gram of charcoal. Grades with large surface areas are more promising for culture media.

1.3 Use of Charcoal in in vitro culture

Just as in the case of agar, there are various brands of activated charcoal used in plant tissue culture of fruit and forest trees, grasses, vegetables and field crops (Table 1.1). Effects of charcoal on tissue response in *in vitro* culture appear to be dependent not only on the kind of charcoal and its degree of activation, but also on the plant species cultured. The addition of activated charcoal to tissue culture media may have either a beneficial or an adverse effect on growth and development, depending upon the medium, tissue used, and/or objective(s) of the researcher.

Use of charcoal can make a major difference in the success or failure of a

given culture attempt. In general, effects of charcoal on *in vitro* cultures can be dealt with under the following headings:

1.3.1 Providing a dark environment

Charcoal provides a degree of darkness during *in vitro* culture. Light is a major factor of the culture environment and has been shown to have an effect on organised development *in vitro*. Light requirements for differentiation involve a combination of several components, including intensity, daily light period and quality. The properties of light affect tissue cultures, and influence their growth and development.

Table 1.1: Literature survey on the use of charcoal in plant tissue culture.

Species	Application	Effect	References
Allium cepa	rooting	promotion	FRIDBORG & Eriksson 1975
Arachis hypogaea	rooting	NC	KANYAND *et al.* 1994
Arnica montana	shoot development	promotion	CONCHOU *et al.* 1992
	shoot initiation	inhibition	
Anemone spp.	embryogenesis	promotion	JOHANSSON *et al.* 1990
Beta vulgaris	callus formation	inhibition	GEYT *et al.* 1987
	rooting	promotion	TOLDI *et al.* 1996
Cenchrus cillaris	embryogenesis	promotion	DETHIER-ROGERS *et al.* 1993
Cicer arietinum	regeneration	NC	ADKINS *et al.* 1995
Citrus.	rooting	promotion	OMURA & HIDAKA 1992
	shoot growth	inhibition	
Cupressus sempervirens	shoot elongation	NC	CAPUANA & GIANNINI 1997
Cucumis sativus	embryogenesis	promotion	CHEE & TRICOLI 1988
Cymbidium forrestii	rhizome growth	promotion	PAEK & YEUNG 1991
	shoot formation	inhibition	
Daucus carota	embryogenesis	promotion	FRIDBORG & ERIKSSON 1975
Diuris longifolia	rooting	promotion	COLLINS & DIXON 1992
Elaeis guineensis	embryogenesis	NC	TOUCHET *et al.* 1991
	callus producing	NC	NWANKO & KRIKORIAN 1983
Equisetum arvense	protoplast growth	promotion	KURIYAMA *et al.* 1990
Eucalyptus regnana	rooting	promotion	BLOMSTEDT *et al.* 1991
Eustoma grandiflorum	protoplast growth	promotion	KUNITAKE *et al.* 1995
Glycine max	rooting	promotion	MOHAMED-YASSEEN & SPLITTSTOESSEER 1990
Iris ensata	rooting	NC	YABUYA *et al.* 1991
Lupinus mutabilis	growth of callus	inhibition	PHOPLONKER & GALIGARIPD 1993
Morus alba	shoot growth	promotion	SHARMA & THORPE 1990
Picea engelmannii	shoot elongation	promotion	PATEL & THORPE 1986
Pinus pinaster	rooting	promotion	DUMAS & MONTEUUIS 1995
Pinus caraiensis	bud development	promotion	MARTINEZ-PULIDO *et al.* 1990
Pinus strobus	shoot growth	inhibition	KAUL 1990
Pinus pinea	explant elongation	no effect	GARCIA-FERRIZ *et al.* 1994
Poncirus trifoliata	growth of cryopreserved axes	promotion	RADHAMANI & CHANDEL 1992
Psidium guajava	rooting	promotion	JAISWAL & AMIN 1987
Quercus robus , Q. rubur	rooting	promotion	SANCHEZ *et al.* 1996
Solanum melongena	shoot regeneration	inhibition	PERRONE *et al.* 1992
Triticum aestivum	embryogenesis	promotion	JONES & PETILINO 1988
Thuja occidentalis	shoot growth	promotion	NOUR & THORPE 1993
Vanilla planifolia	rooting	promotion	GWORGE & RAVISHANKAR 1997

NC: used but unclear effect

Reduction of light at the base of a shoot can provide an environment conducive to the accumulation of photosensitive auxin or co-factors. Dumas and Monteuuis (1995) found that during the *in vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinaster*, addition of activated charcoal to the rooting medium improved the potential for adventitious rooting. Not only in terms of rooting rates, but also an enhancement of the number and the length of the roots, as well as root score. This stimulating effect of activated charcoal on rootability was mostly associated with mature explants.

Charcoal may affect the activity and/or stability of plant growth regulators by reducing or excluding light in *in vitro* culture. Light promoted degradation of IAA and IBA in both liquid and solid agar media (NISSEN and SUTTER 1990).

1.3.2 Adsorption of undesirable or inhibitory substances in in vitro cultures

A problem frequently encountered during initial stages of culture is browning and eventual death of the tissue due to the excessive production of polyphenols, possibly by triggering of defense reactions. Phenolics frequently have the connotation of being inhibitory substances that should be avoided or eliminated from *in vitro* environments. Various methods of preventing the build up of phenolics have been proposed. The most widely used method is to transfer explants to fresh medium. However, increasing the number of subcultures sometime results in accumulation of mutations of cells and may cause loss in the ability of the affected cells to undergo embryogenesis. Incorporation of charcoal or polyvinylpyrrolidone

(PVP) to cultures can alleviate this problem. Activated charcoal prevented discoloration by adsorbing phenolics and rendered polyphenol oxidase and peroxidase inactive. Activated charcoal reduced browning of palm explants and culture media (TISSERAT 1979), thus increasing explant survival and organogenesis. Other antioxidants, such as PVP, ascorbic acid or dihydroxynaphathalene were ineffective (TISSERAT 1979). Activated charcoal also controlled browning and stimulated shoot growth of *Strelitzia reginae* and *Anemone oronaria* (MENSUALI-SODI *et al.* 1993). Activated charcoal improved potato protoplast culture by reducing browning (CARLBERG *et al.* 1983). Addition of activated charcoal to European orchid media stimulated growth of those species which exhibited browning, but had no effect on those which had no visible browning in the medium (VAN WAES 1987). Activated charcoal was more effective than ascorbic acid or PVP in reducing browning in *Dipterocarpus alatus* and *D. intricatus* (LININGTON 1991). Johansson *et al.* (1990) reported that PVP stimulated embryogenesis, but was not as effective as activated charcoal. Light exclusion by activated charcoal may have a secondary effect in reducing discoloration, since light was suggested to increase the activity of enzymes associated with phenol oxidation (LININGTON 1991).

It is widely accepted that some of the beneficial effects of activated charcoal can be attributed to the removal of inhibitory substances from the media, produced either on autoclaving the media (WEATHERHEAD *et al.* 1978) or released by the tissue itself (FRIDBORG *et al.* 1978). Activated charcoal may be able to adsorb toxic brown/black pigments (phenol-like compounds and melanin) as well as other

unknown colourless toxic compounds. The production of phenolic compounds by *in vitro* cultures was demonstrated by several research groups (SUGANO *et al.* 1975; BUTCHER 1977; HABAGUCHI 1977). Fridborg *et al.* (1978) found that differentiation occurred in *Daucus* and *Allium* cultures in activated charcoal-containing media. The media without activated charcoal contained phenolic compounds and other metabolites which inhibited embryogenesis and morphogenesis.

A major breakdown product of autoclaved sugars, which has been shown to reduce growth of plant tissue *in vitro*, is HMF (5-hydroxymethyl-2-furaldehyde) (RÉDEI 1974; RAO and PATTABIRAMAN 1990). The inhibition of growth of tobacco anther cultures by 100 mg l^{-1} HMF was eliminated by addition of activated charcoal, while the addition of 0.3% activated charcoal increased plantlet yield by 200-300% in all the anther cultures (WEATHERHEAD *et al.* 1978).

1.3.3 Adsorption of plant growth regulators in in vitro culture

In *in vitro* culture, plant regulators, especially auxins and cytokinins, are frequently used. Whether an auxin and/or cytokinin has to be added to a nutrient medium to obtain cell extension and/or cell division is dependent on the type of explant and the plant species cultured.

Table 1.2: A semiquantitative spectroscopic analysis of activated charcoal (Nuchar C) and its water extract (ERNST 1975).

Element	Not extracted, %[a]	Water extracted, %[a]
Al	0.11	0.063
B	0.0004	Trace
Ca	0.034	Trace
Cu	0.001	0.0007
Fe	0.041	Trace
K	0.17	0.139
Mg	0.048	0.024
Mn	0.020	0.003
Mo	Trace	—
Na	0.35	0.344
Si	0.36	0.24
Sn	0.006	0.003
Zn	0.002	Trace

[a]Based on weight of charcoal

The concentration and combination of auxins and cytokinins in the nutrient medium is usually a key factor which determines successful plant regeneration. ; The use of activated charcoal as a culture component for adsorption of toxic plant metabolites is known. Activated charcoal is able to adsorb high concentrations of the growth regulators BA, IAA, IBA, NAA and Kinetin (CONSTANTIN *et al.* 1977 WEATHERHEAD *et al.* 1978) in both liquid and solid media (NISSEN and SUTTER 1990).

Pretreatment of embryogenic callus with activated charcoal increased the level of precocious germination of somatic embryos in red fescue (ZAGHMOUT and TORELLO 1988). This may be due to the adsorption of 2,4-D along with other inhibitors. Johansson *et al.* (1982) and Johansson (1983) proposed that the stimulatory effect of activated charcoal in embryogenesis may be due not only to the adsorption of phenolic compounds and/or inhibitory substances from the agar but also adsorption of ABA and ethylene which were released by anthers during culture. These compounds normally inhibit embryogenesis.

Ethylene is a gaseous hormone produced by plant tissue and/or the medium. Activated charcoal was able to adsorb ethylene released by the medium and by *Nicotiana tabacum* nodes (HORNER *et al.* 1977). More ethylene was produced by *Anemone caronaria* seedlings than agar alone (MENSUALI-SODI *et al.*1993). The authors also noted that the effect of activated charcoal on ethylene is dependent on culture conditions, such as container volume and shape, medium volume, and surface exposed to the inner atmosphere.

Table 1.3: Analysis of inorganic ions and metals present in water treated by activated charcoal (Merck, p.a.) (JOHANSSON *et al.* 1990).

Substances	mg /l	Substances	mg /l
NH_4	<0.1	Be	<0.01
NO_2	34	Pb	<0.03
NO_3	940	Cd	<0.002
PO_4	9.9	Co	<0.03
F	<0.1	Cu	0.015
Cl	8	Cr	<0.02
SO_4	28	Mo	<0.10
Ca	10.3	Ni	<0.02
Mg	2.2	Se	0.002
Na	20.6	Ag	<0.005
K	690	Tl	<0.03
Fe	0.033	Sn	<0.5
Mn	0.090	Vn	<0.3
Al	0.12	Zn	1.34
As	0.013	Sb	<0.10
Ba	0.57	Ti	<1.0

Ebert *et al.* (1993) reported that 6-benzylaminopurine (BA) and 2,4-D in the medium decreased with the addition of activated charcoal to the medium. In addition, the different BA concentrations added to the medium interfered with the adsorption pattern of 2,4-D by activated charcoal. The authors noted that adsorption to activated charcoal involved various media components. Other components also compete with 2,4-D in the adsorption process.

Takayama and Misawa (1980) reported that root formation and growth were inhibited by higher concentrations of BA, but this inhibition was completely reversed by the addition of activated charcoal. Root formation and growth were better in BA-free medium containing activated charcoal than in the medium without activated charcoal. Similar results were observed in bulb formation. This could be due to activated charcoal regulating internal physiological processes. It is also possible that a substance(s), which antagonizes cytokinin activity, was adsorbed by activated charcoal.

1.3.4 Substances released from charcoal

It is well known that minerals are an important group of nutrient materials for *in vitro* growth. There is a large choice of combinations of macro- and micro-salt mixtures which is dependent on the experimental plant. The possibility that the effects of charcoal are due to the contribution of minerals was among the first to be investigated (ERNST 1975). A semiquantitative spectroscopic analysis of activated charcoal (Nuchar C) (Table 1.2) did not provide evidence in support of this possibility. A subsequent, more detailed analysis (Table 1.3) confirmed the earlier

report (JOHANSSON *et al.* 1990).

Activated charcoal can promote somatic embryogenesis (LOU and KAKO 1994) and embryogenesis in anther cultures of *Anemone* and *Nicotiana* (JOHANSSON 1983). Many research workers have shown that the addition of activated charcoal often has a promotive effect on the growth and organogenesis of woody species. It is possible that activated charcoal releases substances which promote growth. This requires further study.

Many materials are adsorbed or desorbed to colloidal soil particles and activated charcoal acts similarly (PROSKAUER and BERMAN 1970). Desorption from activated charcoal is generally a very slow process depending on solvent and solution conditions. Many factors could affect this process. They include (a) the grade of activated charcoal (based on raw material and process of preparation), (b) temperature, (c) pH of the solution, and (d) type of solvent. However, adsorbed compounds may be less mobile than in soil, due to the larger internal surface of activated charcoal. Johansson and Eriksson (1977), Litz and Conover (1980) and Johansson *et al.* (1990) postulated that activated charcoal gradually released adsorbed products and Jaiswal and Amin (1987) and M'kada *et al.* (1991) suggested that these products were available to explants by active uptake.

The source of activated charcoal may be important for morphogenesis (PATEL and THORPE 1984) and its effectiveness may be altered by impurities (BONGA 1982). However, others suggested that the stimulative effect of activated charcoal

was due to those same impurities (MISSION *et al.* 1982). Some recommended that activated charcoal be acid washed and neutralised prior to use. Many elements (ERNST 1975) are present in activated charcoal, and Johansson *et al.* (1990) demonstrated that many of these were released from activated charcoal in significant amounts. However, the addition of a water extract of activated charcoal to orchid culture medium had no effect on seedling growth (ERNST 1975).

1.3.5 Other effects

Sugar is a very important component in any nutrient medium and its addition is essential for *in vitro* culture growth and development.

Tissue culture media or aqueous sucrose solutions containing activated charcoal buffered to pH 5.5 and autoclaved did not undergo appreciable sucrose hydrolysis. The extent of sucrose hydrolysis in media containing activated charcoal is proportional to the hydrogen ion concentration (DRUART and De WULF 1993; WANN *et al.* 1997). This finding is consistent with the known mechanism of acid-catalyzed hydrolysis of acetals such as sucrose. Several types of charcoal were identified that acidified culture media to the extent that considerable acid-catalyzed sucrose hydrolysis occurred under autoclaving conditions. Autoclaved sugar promoted or inhibited growth *in vitro*, depending on species and tissue (LEVI and SINK 1990). Growth promotion has been attributed to hydrolysis into glucose and fructose (BALL E 1953; De LANGE 1989). Fructose has previously been identified as inhibitory to the *in vitro* growth of some species (DREW 1992). However, it was

reported (LEVI and SINK 1990) that in asparagus, glucose promoted embryogenesis and root growth; and fructose promoted shoot growth. Alteration of medium pH to an optimum level for morphogenesis has also been reported as a beneficial effect of activated charcoal (OWEN *et al.* 1991).

1.4 Conclusions

Activated charcoal is commonly used in tissue culture media due to its structure, and its addition may have either beneficial or harmful effects, especially on organogenesis and embryogenesis. The effects of activated charcoal can be attributed to; (a) providing a dark environment in the medium; (b) adsorption of certain inhibitory substances in culture, produced by either media or explants; (c) adsorption of plant growth regulators and other organic compounds; and (d) the release of substances naturally present in, or adsorbed by, activated charcoal, which are beneficial to growth in *in vitro* culture.

Use of charcoal in the culture medium may either promote or inhibit growth *in vitro*, depending on a number of factors. Most publications have concentrated on the promotive effects on tissue response during *in vitro* culture. Addition of activated charcoal to culture media may affect growth, especially rooting, shoot elongation and embryogenesis. Activated charcoal can provide a dark environment and adsorb substances presumed to be deleterious and/or inhibitory to *in vitro* culture. However, adsorption by activated charcoal of growth regulators being supplied to the tissue could also occur at the same time. The non-selective

adsorption effect of activated charcoal may result in negative effects on cultured explants. Some researchers noted that activated charcoal adsorbs thiamine, nicotinic acid (WEATHERHEAD *et al.* 1978), pyridoxine, folic acid (JOHANSSON *et al.* 1990), growth regulators, iron chelates (HERBERLE-BORS 1980; JOHANSSON *et al.*1990), and Zn. Both Fe and Zn were only slightly adsorbed in the presence of sucrose (MISSION *et al.* 1983). The adsorption of the plant regulators may result in inhibitory effects on growth *in vitro.* It was reported that callus growth of *Glycine max* and *Haplopappus gracilis* was totally inhibited by activated charcoal (FRIDBORG and ERIKSSON 1975; FRIDBORG *et al.* 1978). Addition of activated charcoal to the medium used for European orchids resulted in lower germination rates and slower development (VAN WAES 1987). When activated charcoal was used, shoot elongation and leaf size of *Eucalyptus citriodora* increased but the number of shoots decreased (AHUJA 1985). Webb *et al.* (1988) noted that shoot elongation was promoted by charcoal but charcoal inhibited shoot induction when it was included with BA. Activated charcoal also inhibited root formation when included in the final rooting medium. Activated charcoal had no beneficial effects on maturation of chicory somatic embryos or the growth of isolated adventitious shoots of *Picea abies*; moreover, long incubation times in activated charcoal-containing medium caused shoots to become abnormal with long whirled needles (VON ARNOLD 1982). Activated charcoal used in rooting medium for *Prunus silicina* had a marked negative effect on the percentage of rooted shoots. In some cases chlorosis and severe leaf drop was induced (ROSATI *et al.*1980).

Activated charcoal did result in hyperhydricity in *Picea abies* and *Sequoia sempervirens* cultures (DENCSO 1987; VON ARNOLD 1982). However, it significantly reduced hyperhydricity in shoot-tip cultures of onion (SCHLOUPT 1994). Debergh *et al.* (1981) showed that hyperhydricity in artichoke was not reduced by adding activated charcoal, which was used to adsorb the metabolites of cytokinin catabolism which were thought to induce hyperhydricity.

The capacity of cells in culture to undergo organogenesis and somatic embryogenesis has allowed for the widest application of tissue culture technology such as micropropagation via organogenesis. In plant tissue culture, it is well known that no two genotypes give similar responses under a given set of culture conditions (NEHRA *et al.* 1989; NEHRA *et al.* 1990). Use of activated charcoal in *in vitro* culture may either promote or inhibit growth *in vitro*, depending on a number of factors. Investigation of the mechanism(s) of adsorption of activated charcoal , and identification of the substances adsorbed and/or released by activated charcoal will help our understanding as to how charcoal acts in plant growth and development.

This study was undertaken to elucidate some of the effects of activated charcoal on plant growth in culture conditions.

CHAPTER TWO

Effect of activated charcoal, autoclaving and culture media on sucrose hydrolysis

Abstract

The effect of activated charcoal, autoclaving and culture media on sucrose hydrolysis in tissue culture media was investigated. Activated charcoal acidified an aqueous sucrose (5%) solution and culture media by about 1 to 2 pH units after autoclaving. Sucrose hydrolysis in tissue culture media and/or an aqueous sucrose (5%) solution containing activated charcoal (buffered to pH 5.8) was dependent on both the hydrogen ion concentration (pH) and autoclaving. After autoclaving, 70%, 56% and 53% sucrose hydrolysis were respectively recorded in a 5% sucrose solution, Murashige and Skoog (MS) and Gamborg B5 (B5) liquid media in the presence of 1% activated charcoal, added before autoclaving. In the absence of activated charcoal, autoclaving resulted in about 20% of the sucrose being hydrolysed.

2.1 Introduction

Sugar is an important component of nutrient media. Its addition is essential for *in vitro* growth and development, as photosynthesis is insufficient to supply the energy needs of growing explants. Glucose and fructose can also be used as carbon sources for *in vitro* culture, depending on the type and age of plant material cultured.

Sucrose can undergo changes as a result of autoclaving; it may be hydrolysed to glucose and fructose. Autoclaved sucrose either promotes or inhibits growth *in vitro*, depending on species and tissue used (NASH and BOLL 1975; De LANGE 1989; DREW 1992). Growth promotion by autoclaved sucrose is widely attributed to its hydrolysis into glucose and fructose (BALL 1953; De LANGE 1989). Fructose can be inhibitory to the *in vitro* growth of some species (DREW 1992). In papaya, substitution of fructose for sucrose reduced growth rates of rooted papaya shoots in culture (DREW 1992). In asparagus, glucose promoted embryogenesis and root growth; and fructose promoted shoot growth (LEVI and SINK 1990). The effect of sugars on shoot multiplication of sour cherry (*Prunus cerasus* L.) *in vitro* (BORKOWSKA and SZCZERBA 1991) showed that sucrose and glucose brought about a similar rate of proliferation. However, in the presence of fructose, proliferation was lowest. However, it was coupled with the highest degree of long shoot formation. In *Taxus* cell suspension cultures, fructose used as the sole sugar, promoted cell growth better than equimolar concentrations of glucose or sucrose (WICKREMESINHE and ARTECA 1994). In shoot multiplication of mature hazelnut

(*Corylus avellana* L.) *in vitro*, plants grown on 3% glucose or fructose-containing media produced more and longer shoots than those cultured on sucrose (YU and REED 1993).

Activated charcoal (AC) is commonly used in tissue culture media. It may have either beneficial or harmful effects on the culture, depending upon the medium, and tissue used. The beneficiary effects of activated charcoal on tissue responses *in vitro* can be attributed to: (a) establishing polarity by darkening the medium (DUMAS and MONTEUUIS 1995); (b) adsorption of inhibitory substances, produced by either the media or explant (FRIDBORG and ERIKSSON 1975; FRIDBORG *et al.* 1978); (c) adsorption of plant growth regulators and other organic compounds (CONSTANTIN *et al.* 1977; WEATHERHEAD *et al.* 1978; NISSEN and SUTTER 1990); or (d) the release of substances naturally present in or adsorbed by the charcoal (ERNST 1975; JOHANSSON *et al.* 1990).

Several types of charcoal are able to acidify culture media to the extent that considerable acid-catalysed sucrose hydrolysis to fructose and glucose occur upon autoclaving (DRUART and De WULF 1993; WANN *et al. 1997)*. Wann *et al.* (1997) report that the extent of sucrose hydrolysis in media containing activated charcoal was directly proportional to the hydrogen ion concentration.

Although alteration of medium pH to an optimum level for morphogenesis has been reported as a beneficial effect of activated charcoal (OWEN *et al.* 1991), activated charcoal is a complex substance. The entire range of its effects on tissue

culture media and the subsequent growth and morphogenesis of tissue cultures need to be studied. The objective of this part of the study was to determine the influence of activated charcoal in combination with autoclaving and culture media, on sucrose hydrolysis.

2.2 Materials and methods

2.2.1 Materials

A five percent (w/v) sucrose solution in water, Murashige and Skoog (1962) (MS-salts, MS-vitamins, 30 g l^{-1} sucrose) , Gamborg B5 (GAMBORG *et al.* 1968) (B5-salts, B5-vitamins, 30 g l^{-1} sucrose) media, or buffers (0.1 M phosphate buffer; 0.1M acetate buffer) were autoclaved at 121^{o}C (1.05 kg per cm^2) for 20 min in the presence of various concentrations (w/v) of AC (BHD, England). The pH of the solutions and the culture media was adjusted after the addition of AC. After autoclaving, solutions were cooled to room temperature and the AC removed by filtration. The pH and degree of sucrose hydrolysis were determined. The sucrose solution and the culture media were sampled daily up to 28 days and then processed further for determination of pH and the degree of sucrose hydrolysis. Solid media were frozen at -70^{o}C overnight and then thawed at room temperature before sampling. The pH of the sucrose solutions and media were adjusted after addition of AC. Liquid media (MS and B5) were used unless otherwise indicated.

For the AC added after autoclaving, an aqueous AC (1%) suspension was autoclaved separately and added to the autoclaved sucrose solution and/or media. The concentration(s) of the other components in the sucrose solution and /or media were calculated to ensure that the final concentrations were not diluted after mixing with the AC suspension.

2.2.2 Sugar analysis by gas-chromatography (GC)

Sugar oximes (TANOWITZ and SMITH 1984) were silylized (SWEELEY *et al.* 1963). The standard sugars (glucose , fructose and sucrose; Sigma, Germany) at 1 mg ml^{-1} each were dissolved in 80% ethanol. Five hundred microliters (0.5 mg) of each standard were placed into a pill vial, and dried under nitrogen. Two hundred microliters pyridine with hydroxylamine monohydrochloride were added to each dry standard and then incubated at 40°C for 20 min. One hundred microliters of the pyridine solution were transferred into an Eppendorf tube, and dried under nitrogen flow. Fifty microliters of Sil-A (Sigma, Germany) were added and the mixture allowed to react for 15 min at room temperature, and then microcentrifuged and kept at 4°C until GC analysis. Sugar samples were prepared by the same method as the sugar standards. Sucrose standards and samples were separated by gas chromatography on a 1.8 m x 3 mm (internal diameter) glass column packed with OV - 17 on Chromosorb HP 80/100 and detected by flame ionization detection. The column was held at 125°C for 3 min, followed by ramping at 4°C per min to 270°C. The final temperature was maintained for 5 min. Individual sugars were tentatively identified by co-chromatography with authentic standards.

All experiments were performed at least twice and the values reported are the average from at least two determinations.

2.3 Results

Activated charcoal added to 5% sucrose solution , MS and B5 culture media, affected both the pH and degree of sucrose hydrolysis that occurred. The effect of AC on the pH of the aqueous sucrose solutions and culture media is shown in Table 2.1. The pH of all solutions and media (MS and B5) decreased by about 1 to 2 units after autoclaving in the presence of different concentrations of AC. The decrease was highest when 5% AC was added to the test solution and media. The culture media clearly had some buffering capacity as the initial decrease in pH was less pronounced with MS and B5 media than with a 5% sucrose solution (Table 2.1; Figure 2.1). Only small changes in the pH of the sucrose solutions and culture media (MS and B5) were recorded when 0.01-0.1% AC were added (Table 2.1).

After autoclaving, the sucrose solution and culture media were cooled, the pH recorded, and then stored at room temperature for a period of 28 days. The pH of the respective media and solution was measured at weekly intervals. After the initial relatively large decrease in pH immediately after autoclaving the pH of all treatments increased slightly with time, the trends being similar for all treatments (Figure 2.1)

Table 2.1: The effect of different concentrations of activated charcoal, added prior to autoclaving, on the pH of a sucrose solution and different liquid culture media (MS and B5)*

Activated charcoal concentration (%)	Media pH immediately after autoclaving			
	Sucrose solution B5 (5%)	MS (Full strength)	MS (Half strength)	
0.00	5.5 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.4 ± 0.1
0.01	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
0.05	5.0 ± 0.1	5.4 ± 0.1	5.4 ± 0.1	5.2 ± 0.1
0.10	4.7 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.1
0.50	3.8 ± 0.1	5.0 ± 0.1	4.9 ± 0.1	5.1 ± 0.1
1.00	3.6 ± 0.1	4.9 ± 0.1	4.8 ± 0.1	5.1 ± 0.1
2.00	3.4 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	5.0 ± 0.1
5.00	3.4 ± 0.1	4.6 ± 0.1	4.2 ± 0.1	4.8 ± 0.1
1.00[a]	3.6 ± 0.1	4.8± 0.1	4.7 ± 0.1	5.0 ± 0.1

[a] activated charcoal autoclaved by itself

* after the addition of activated charcoal, but before autoclaving, the pH of all sucrose solutions and media were adjusted to 5.8

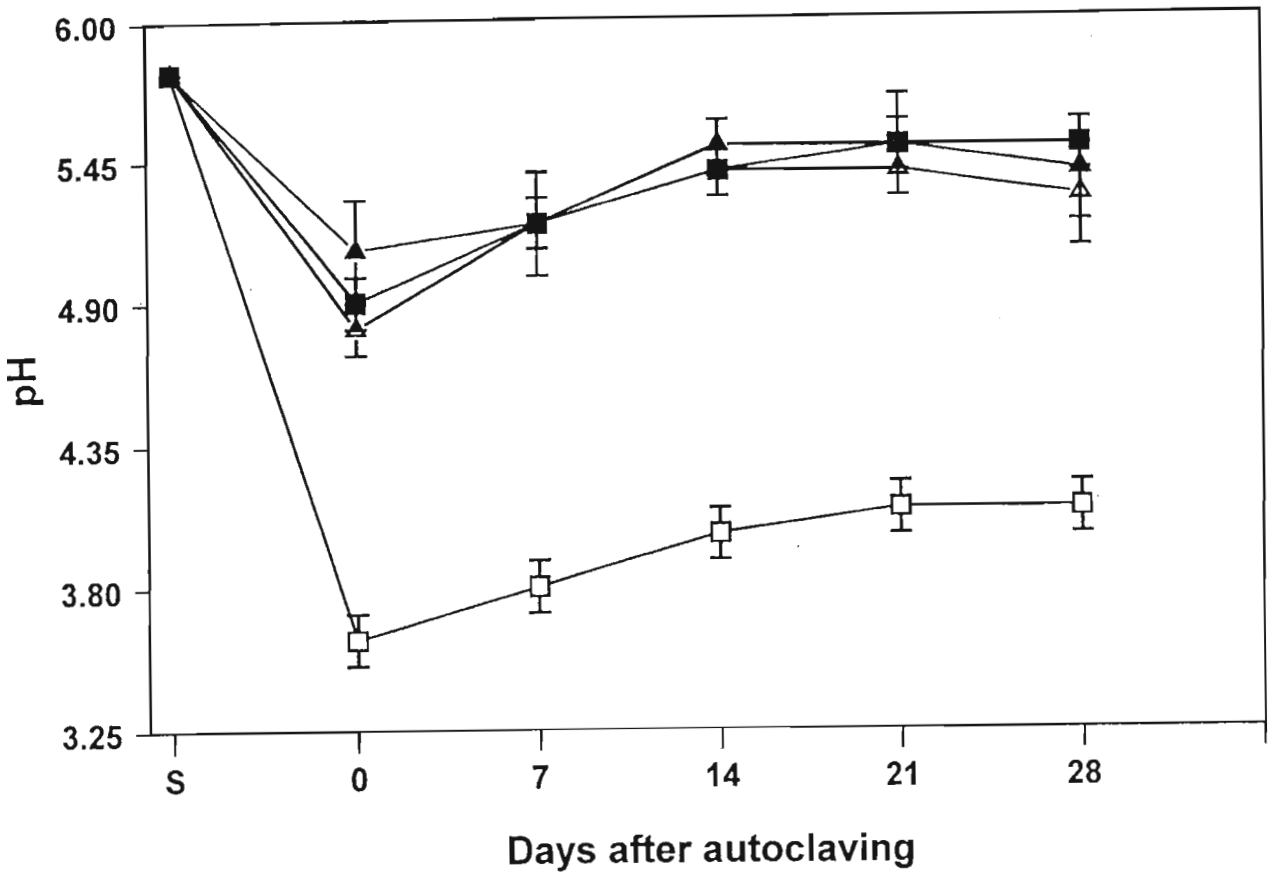

Figure 2.1: Changes in the pH of a 5% sucrose solution and liquid culture media in the presence of 1% activated charcoal for a period of four weeks after autoclaving. 5% sucrose solution (□); Full strength MS liquid medium (■); Half strength MS liquid medium (△); Gamborg B5 liquid medium (▲); The starting pH for all solutions and media was 5.8 (S).

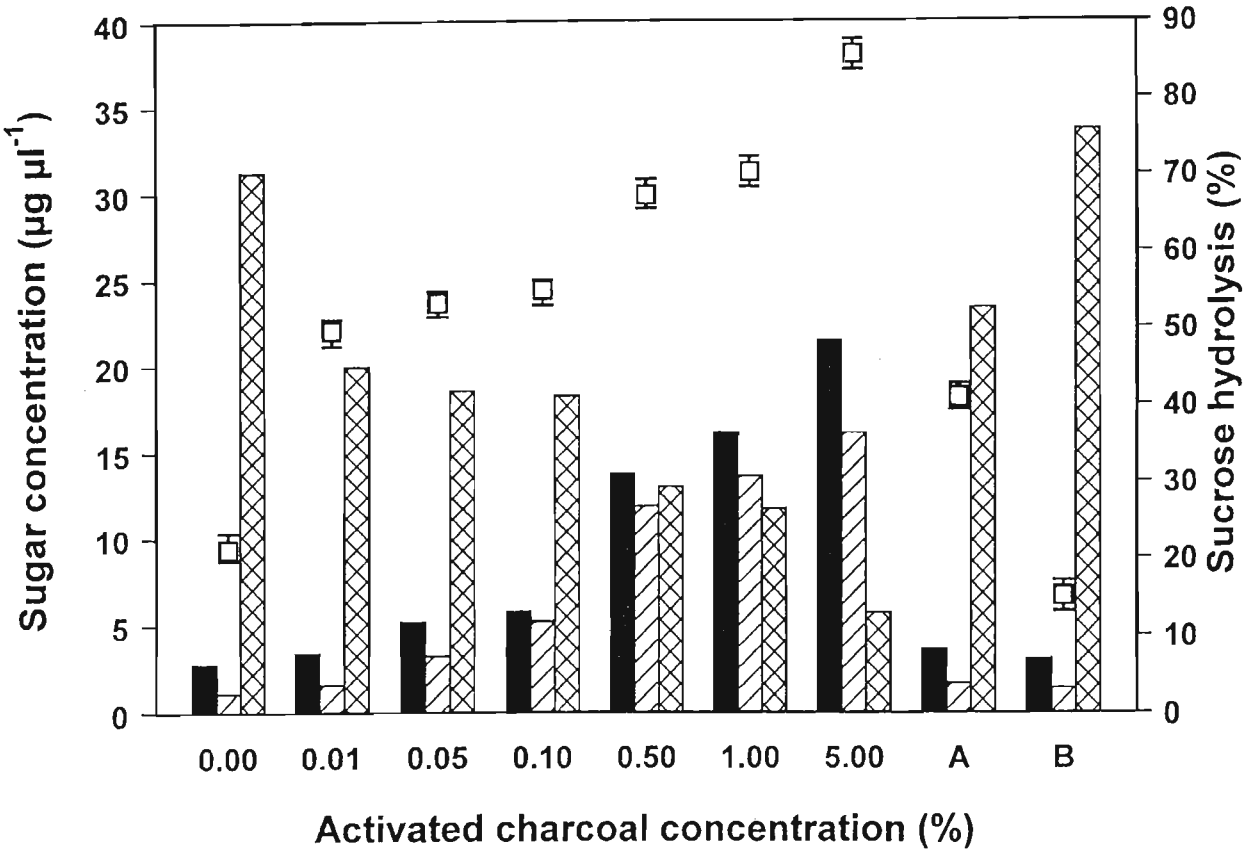

Figure 2.2: Sugar content in sucrose solutions (5%) to which various concentrations of activated charcoal had been added prior to autoclaving. (A). Activated charcoal (1%) was autoclaved in water and subsequently added to the sugar solution. (B). Activated charcoal (1%) was added to a sugar solution, which was buffered to pH with phosphate, prior to autoclaving. In all cases the starting pH was 5.8. Bar graphs represent the amount of sugar measured by GC analysis. ■ = fructose; ▨ = glucose; ▩ = sucrose: The open squares (□) represent the percentage of sucrose hydrolysed.

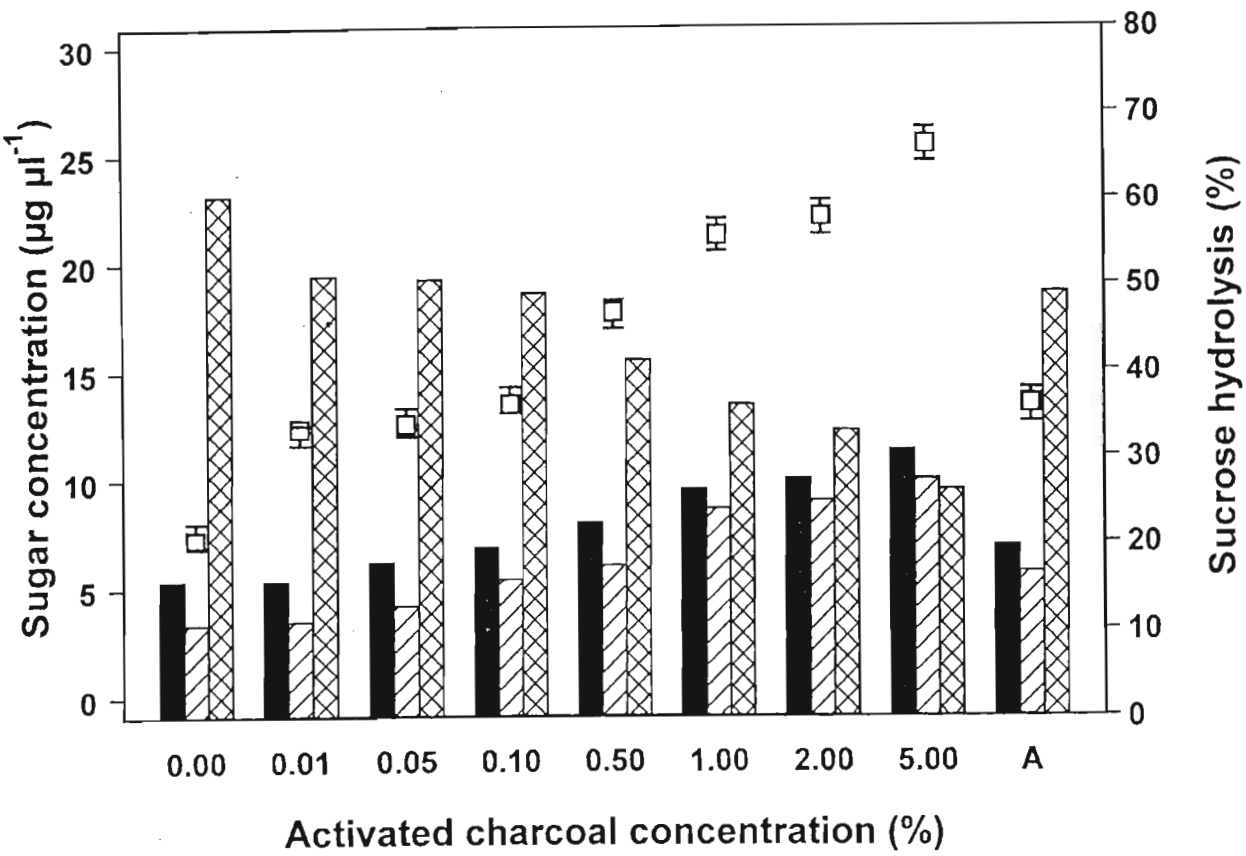

Figure 2.3: Sugar content in full strength MS liquid media to which various concentrations of activated charcoal had been added prior to autoclaving. (A). Activated charcoal (1%) was autoclaved in water and subsequently added to the medium. In all cases the starting pH was 5.8. Bar graphs represent the amount of sugar measured by GC analysis. ■= fructose; ▨ = glucose; ▩ = sucrose: The open squares (□) represent the percentage of sucrose hydrolysed.

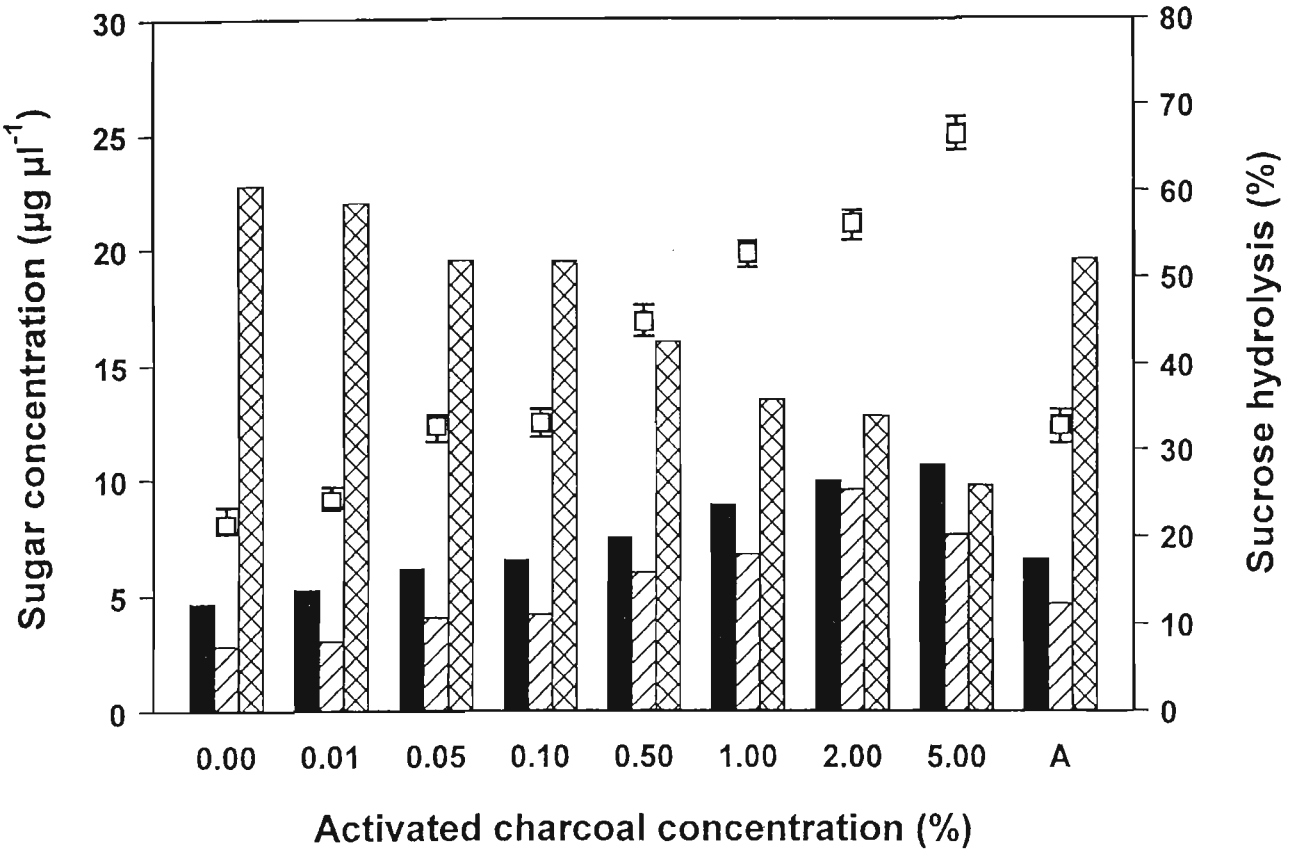

Figure 2.4: Sugar content in Gamborg B5 liquid media to which various concentrations of activated charcoal had been added prior to autoclaving. (A). Activated charcoal (1%) was autoclaved in water and subsequently added to the medium. In all cases the starting pH was 5.8. Bar graphs represent the amount of sugar measured by GC analysis. ■= fructose; ▨ = glucose; ▩ = sucrose: The open squares (□) represent the percentage of sucrose hydrolysed.

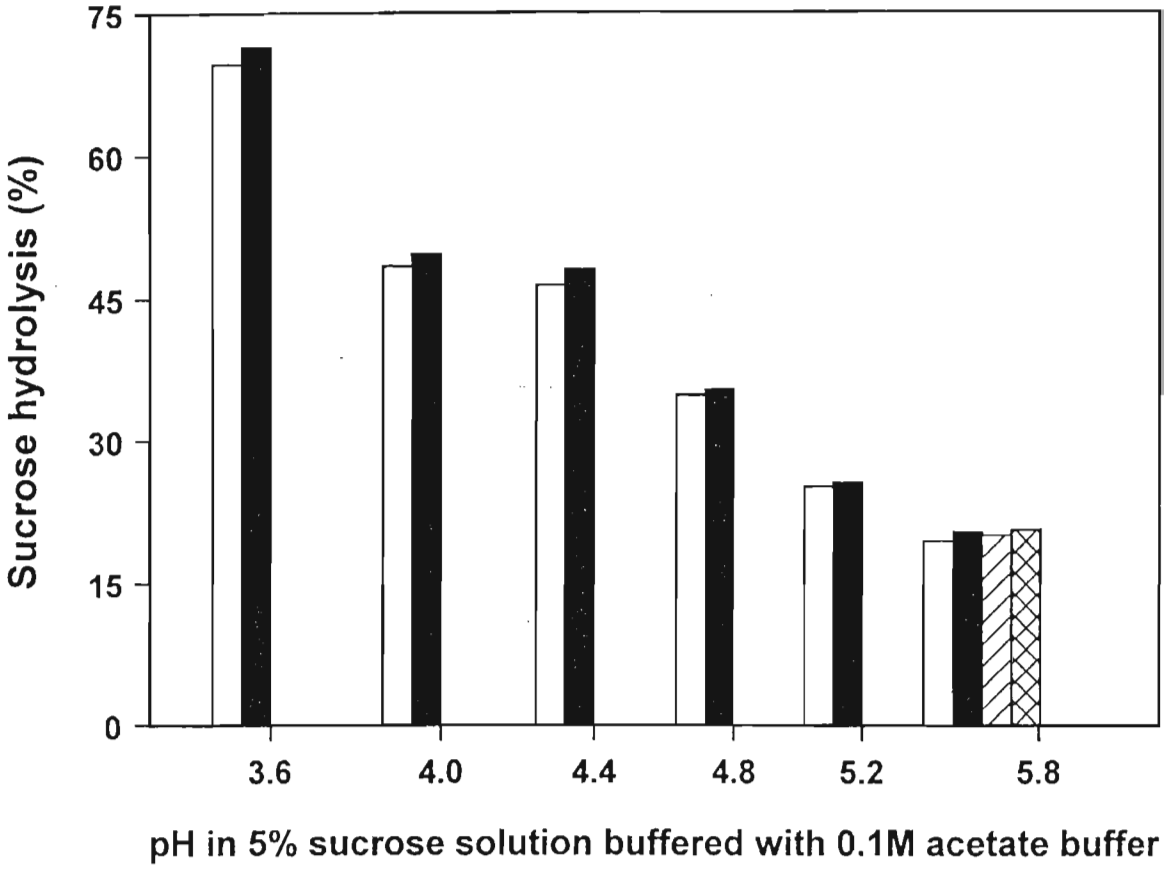

Figure 2.5: The degree of sucrose hydrolysis in the 5% sucrose solution. The pH was buffered with 0.1 M acetate buffer to different levels, both in the absence (□) and presence (■) of 1% activated charcoal. The effects of 1% pre-autoclaved activated charcoal added to the buffered (pH 5.8) sucrose solution (▨) and re-autoclaving the buffered sucrose solution with 1% pre-autoclaving activated charcoal (▩) are shown.

The results presented in Figure 2.2 indicate that irrespective of treatment autoclaving did result in a degree of sucrose hydrolysis. The non-autoclaved sucrose solution yielded a single peak which co-chromatographed with sucrose following GC analysis (not shown). In the absence of AC, autoclaving resulted in as much as 20% of the sucrose being hydrolysed (Figures 2.2 to 2.4).

The effect of AC on sugar hydrolysis in a sucrose solution (Figure 2.2), MS (Figure 2.3) and B5 (Figure 2.4) media showed similar trends in that increased AC concentrations increased the degree of hydrolysis, reaching values as high as 86% in the 5% sucrose solution (Figure 2.2). The "buffering capacity" of the media can again be seen as the final hydrolysis was much lower with 5% AC when it was added to the sucrose solution (66% compared to 86%).

When 1% AC was autoclaved in water by itself and subsequently added to the sucrose solution and culture media, the degree of sucrose hydrolysis was much less (A in Figures 2.2 to 2.4). It varied between 41% (sugar solution), 36% (MS medium) and 33% (B5 medium) compared to 70%, 56% and 53% where the AC was added prior to autoclaving. When the 5% sucrose solution was buffered with a phosphate buffer sucrose hydrolysis amounted to only 15% (B in Figure 2.2). Lower degrees of sucrose hydrolysis were observed by autoclaving acetate buffered (pH 3.6 - 5.8) 5% sucrose solutions in the absence of AC (Figure 2.5). There was no effect when pre-autoclaved AC was added to a sucrose solution buffered to pH 5.8 and/or then re-autoclaved (Figure 2.5).

During storage for 28 days after autoclaving only small changes in sucrose

hydrolysis were observed with 1% AC, in that, the media and sucrose solution used, and the degree of sucrose hydrolysis in the solid media, was slightly higher than in the liquid media.

2.4 Discussion

Previous reports indicated that activated charcoal catalysed the hydrolysis of up to 90% of the sucrose in culture media to fructose and glucose (DRUART and De WULF 1993). A recent report (WANN *et al.* 1997) suggested that AC do not catalyse sucrose hydrolysis in tissue culture media during autoclaving but that it deceased the pH which may affect hydrolysis. The present results indicated that both lowering of pH and autoclaving results in sucrose hydrolysis both in solution and when incorporated in tissue culture media. The degree of hydrolysis was much less in the culture media. A lower, non-significant degree of sucrose hydrolysis, was observed by autoclaving sucrose in acetate buffers in the absence of activated charcoal. Hydrolysis in the sucrose solutions was reduced when buffered with either a phosphate or acetate buffer. Very little effect was noticed when adding pre-autoclaved AC to buffered sucrose and/or then re-autoclaving. This is in agreement with the fact that when acetals are subjected to hydronium ion catalysis, the rate of hydrolysis is directly proportional to the hydrogen ion concentration (HINE 1962). This process was accelerated by autoclaving but considerably reduced when there was some buffering capacity in the medium. Sucrose hydrolysis in a medium will result in changing the ratio of medium components which may subsequently influence plant growth and development *in vitro*. Activated charcoal is often used in tissue culture to improve cell growth and development. In most cases the

reason(s) for this is unclear. It has been suggested that the primary benefit is its adsorption capacity of inhibitory substances in culture media (FRIDBORG *et al.* 1978; WEATHERHEAD *et al.* 1978). However, the degree of sucrose hydrolysis may well be the causative effect in many instances, particularly where different levels of glucose and/or fructose may have either harmful or beneficial effects on tissue growth (ANAGNOSTAKIS 1974; ZAGHMOUT and TORELLO 1988; LEVI and SINK 1990; DREW 1992; WICKREMESINHE and ARTECA 1994). These responses need to be investigated further in line with the effect of AC on the physical environment of cultured tissue and the adsorptive capacity of the charcoal used.

CHAPTER THREE

Changes of 2, 4-Dichlorophenoxyacetic acid and inorganic salt levels in media in the presence of activated charcoal

Abstract

The adsorption of 2, 4-dichlorophenoxyacetic acid (2,4-D) by AC from methanol and aqueous solutions was determinated using HPLC. The level of the added 2,4-D decreased in both methanol and aqueous solutions in the presence of AC. In methanol and aqueous solutions, AC used at the level of 1.0% resulted in significant reduction in 2,4-D. About 68% and 61% of the added 2,4-D was adsorbed respectively by AC (1.0%) from these two sources. The changes of inorganic elements of MS-salt solutions, in the presence of AC, were analysed by SEM-EDX. The concentrations of magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn) in MS salt solution deceased in the presence of AC, while the concentrations of potassium (K), copper (Cu), manganese (Mn), phosphorus (P), and sulphur (S) increased in the MS salt solution in the presence of AC when compared with the controls. This suggests that AC adsorbed calcium, magnesium, iron and zinc from and released copper, manganese, phosphorus and sulphur into the culture media.

3.1 Introduction

Charcoal is any form of carbon characterised by a high adsorptive capacity for gases, vapors and colloidal solids and is produced by destructive distillation of woods, peat, lignite, nut shells, bones, vegetables or other carbonaceous matter. Different kinds of AC are prepared for different purposes. The properties of AC are attributed mainly to its highly porous structure and relatively large surface area. Activated charcoals are characterized by very large specific areas ranging from 600 to 2000 m^2 g l^{-1} and pore distributions ranging from 10 µm to 500 µm (YAM *et al.* 1990). The adsorptive capacity of AC generally is dependent on a variety of factors such as density, purity of charcoal, and pH (HALHOULI *et al.*1995). All AC derived from wood has a particularly large surface area (most of it internal). They are generally used in culture media.

Auxins are very widely used in micropropagation work and are incorporated into nutrient media to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis. The effects of auxins are generally not absolute and specific. The responses of cells, tissues and organs *in vitro* can vary according to culture conditions, the type of explant and the plant genotype. Activated charcoal used in nutrient media has an adsorption preference for moderately polar rather than apolar or highly polar organics. They show greater adsorption for aromatic than olefinic unsaturation products (YAM *et al.* 1990). Therefore, aromatic compounds such as the phenolics and their oxidates, auxins [2, 4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), α-naphthaleneacetic

acid (NAA), indole-3-butyric acid (IBA)], cytokinins [benzyladenine (BA)], and hormones (having heterocyclic and unsaturated ring structures (e.g. kinetin), could have great adsorption affinity for AC. Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the auxins supplied in the medium, and the growth substances produced endogenously. In contrast, the highly polar and readily water-soluble carbohydrates (glucose, sorbitol, mannitol and inositol) might not be removed from the medium and/or solution (PAN and VAN STADEN 1998).

It is known that minerals are an important group of nutrient materials for *in vitro* growth. Plant tissue absorb the nutrients they require from media, almost entirely as ions. Macro-nutrients are provided for plant culture media from salts. Calcium, magnesium, and potassium are absorbed by plant cells as their respective cations (Ca^{2+}, Mg^{2+}, and K^{+}); phosphorus as the phosphate ions $(HPO_4)^{2-}$ and $(H_2PO_4)^{-}$; and sulphur as the sulphate ion $(SO_4)^{2-}$. The micro-nutrients provides the elements that are required only in trace amounts for plant growth and development. Micronutrients commonly added to plant cell culture media include manganese (Mn), copper (Cu), boron (B), iron (Fe), molybdenum (Mo), zinc (Zn) and iodine (I) (GEORGE 1993). Several authors have stressed the importance of Ca^{2+} for rooting (DE FOSSARD and BOURNE 1976; McCOMB 1985; GROSSER *et al.* 1988). In tissue culture medium, there is a large choice of combinations of macro- and micro-salt mixtures which is dependent on the experimental plant. There is the possibility that the effects of AC on *in vitro* growth may be due to a contribution of minerals (ERNST 1975). Ernst (1975) reported that many elements are present in AC, and

Johansson *et al.* (1990) demonstrated that many of these were released from AC in significant amounts, which could affect *in vitro* growth. However, the addition of a water extract of AC to orchid culture medium had no effect on seedling growth (ERNST 1975).

In view of the results described in Chapter 2 it was necessary to investigate whether the addition of different concentrations of AC to the medium interferes with the availability of 2,4-D, and the extent to which 2,4-D can be adsorbed by AC. It was also necessary to establish to what extent inorganic elements which were adsorbed are released by AC.

3.2 Materials and methods

3.2.1 HPLC determination of 2,4-D

Determination of 2,4-D was achieved by reversed phase high performance liquid chromatography (HPLC). The column used was a Hypersil 25 x 0.4 cm ODS C18, 5 mm particle size with a flow rate of 1.0 ml per minute maintained by a 3500 p.s. single piston reciprocating pump. Absorbance was recorded with a Varian variable wavelength monitor (Cspectra system UV 3000HR) at 280 nm which was fitted with a 8 µl flow through cell. Separation was achieved using a Varian 5000 liquid Chromatograph and the data recorded using a Vista 4000 data system. Authentic 2,4-D (BDH) was dissolved in HPLC methanol (BDH). Samples were prepared by adding 0.5 mg ml^{-1} of 2, 4-D (BDH) to both methanol and HPLC water

(BDH) in the presence of 0.01%, 0.1% and 1.0% AC (BDH or Sigma). The samples were dried under nitrogen and then re-dissolved in HPLC methanol. The standard and samples were filtered with 0.2 µM Millipore filters (Millipore Corporation) whereafter 10 to 80 µl aliquots were injected into the chromatograph. The aqueous buffer consisted of 0.2 M acetic acid adjusted to pH 3.5 with triethylamine. A linear gradient of methanol:aqueous buffer (5:95 to 95:5 over 30 minutes, flow rate: 1 ml min^{-1}) was used for separation. 2,4-D in the samples were tentatively identified and analysed on the basis of co-chromatography with an authentic sample. Samples were taken after 24 hours.

3.2.2. Substances released /adsorbed from/by AC

Ten grams of AC (BDH or Sigma) was added to MS-salts (MURASHIGE and SKOOG 1962) solution with a pH of 5.8. After autoclaving for 20 min at 121 ^{o}C (118 kPa) the solutions were cooled at room temperature, and AC was removed by sterile filtration. The remaining solution was collected and gelled with 1.0% agarose (Techcomp LTD) and air dried for Scanning Electron Microscopy- Energy Dispersive X-ray (SEM-EDX) analysis (LIU and VAN STADEN 2000). Specimens were prepared for SEM by air drying overnight and carbon coating to prevent charging as a clear picture was needed to enable selection of specific points. Samples were analysed by an energy-dispersive SiLi detector (Nuclear Semiconductors) combined with a Link exl II EDX system , attach to a Hitachi S-570 SEM operating at an accelerating voltage of 15 Kv. Specimens in the SEM were tilted to an angle of 15^{o} towards the X-ray detector, and the working distance in the microscope was set at 15 mm to obtain optimum results. X-ray counts based

on the peak-to-background ratios (P/B) of a specimen were compared with the standard. X-ray spectra were recorded with an electron beam of 15 Kv and spectra were collected at 1000 X mag within 100 sec live time in a scanning mode over the area of callus for at least 8 locations each. The X-ray signal intensity was quantified by counts (Y-axis label) and was indicative of the relative concentration of the elements in the samples.

3.3 Results

3.3.1 Effect of different levels of activated charcoal on the adsorption of 2,4-D from methanol and aqueous solutions.

The levels of AC used in experiments reported in the literature vary from 0.2% to 3.0% (PIERIK 1987; EBERT and TAYLOR 1990). It is not clear, how critical these levels are and how little may be used without exceeding its capacity to adsorb 2,4-D from the medium. To examine the capacity of AC to adsorb the standard concentrations of 2,4-D (0.5 mg ml^{-1}), two types of AC (Sigma and BDH) and four different concentrations of AC (0.01%, 0.1%, 1.0% and 3.0%), were added to the methanol or aqueous solutions which were supplemented with 0.5 mg ml^{-1} or 1.0 mg ml^{-1} 2,4-D. Samples of methanol and aqueous solutions were taken on the first, third, fifth and seventh day after preparation. The changes of the added 2,4-D in both methanol and aqueous solutions in the presence of AC are shown in Figure 3.1. The results shown in Figure 3.1 indicates that AC adsorbs 2,4-D rapidly at all the levels used. The level of added 2,4-D decreased in both methanol and aqueous solutions in the presence of AC (Sigma and BDH). Figure 3.1A shows

that the 2,4-D (0.5 mg ml^{-1}) adsorbed from the methanol solution by adding 0.01% AC (Sigma) was approximately 35%, while it was 31.7% in the aqueous solution when adding 0.01% AC (Sigma). About 29.5% of the added 2,4-D was adsorbed by adding 0.01% AC (BDH) in methanol solution and about 26.7% of the added 2,4-D was adsorbed by 0.01% AC (BDH) in aqueous solution (Figure 3.1B). Activated charcoal (Sigma and BDH) used at the level of 1.0% resulted in significantly reducing the added 2,4-D (0.5 mg ml^{-1} and 1.0 mg ml^{-1}) in both methanol and aqueous solutions (Figure 3.1A, B, C, D).

The changes of the added 2,4-D in methanol and aqueous solutions in the presence of AC are shown in Figure 3.2. The results show that AC adsorbs 2,4-D rapidly at all the levels used. The adsorption process took place progressively during the first day with about 28% to 68% of the added 2,4-D adsorbed depending on the type of AC and solution. On the fifth day the level of AC normally had established an equilibrium with 34% to 70% of the 2,4-D adsorbed by AC (Figure 3.2). The capacity of adsorption of 2,4-D did differ between Sigma and BDH AC. This was, however, not statically significant (Figure 3.2). There was no significant difference in the level of 2,4-D adsorbed from methanol and aqueous solutions (Figure 3.2).

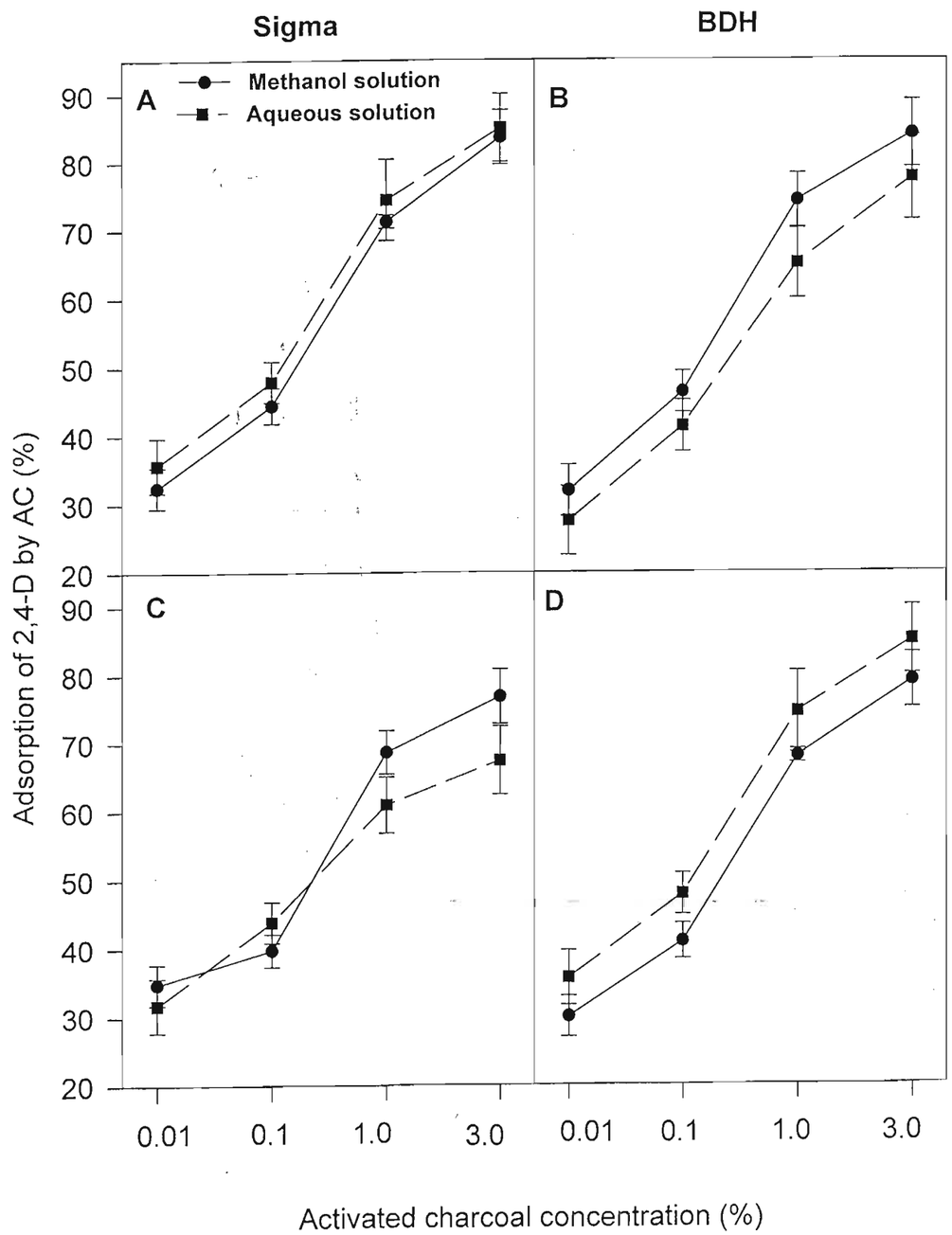

Figure 3.1: The changes of 2,4- D in the methanol or aqueous solutions in the presence or absence of activated charcoal determinated by HPLC on the first day after preparation. A: 0.5 mg ml^{-1} 2,4-D added to AC-containing (Sigma) solutions; B: 0.5 mg ml^{-1} 2,4-D added to AC-containing (BDH) solutions; C: 1.0 mg ml^{-1} 2,4-D added to AC-containing (Sigma) solutions; D: 1.0 mg ml^{-1} 2,4-D added to AC-containing (BDH) solutions. Values are the means of three replicates with bars indicating ±SE.

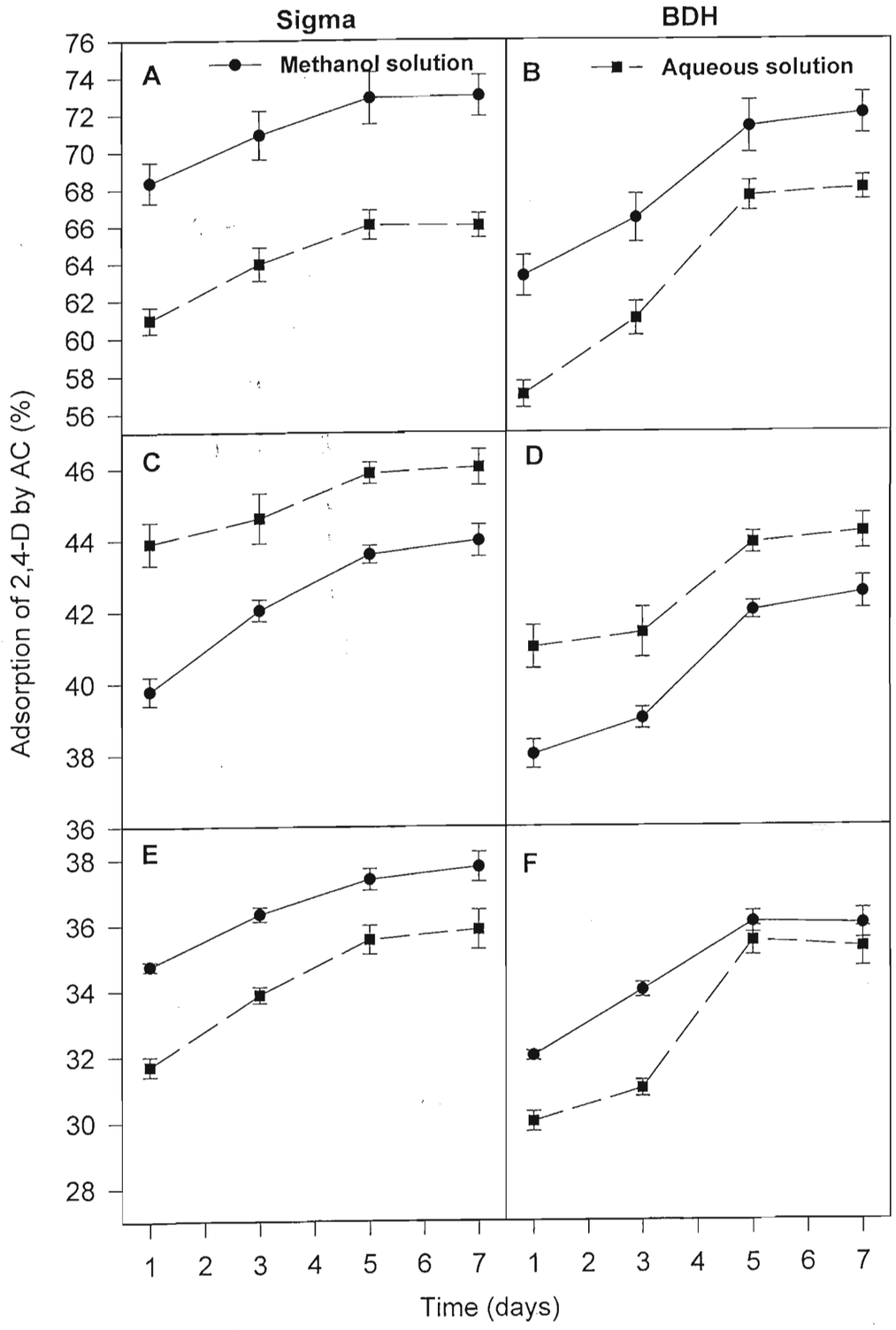

Figure 3.2: The percentage of 2,4-D (0.5 mg ml^{-1}) adsorbed by Sigma and BDH activated charcoal from methanol and aqueous solutions over seven days. A: adsorption of 2,4-D by Sigma AC (0.01%); B: adsorption of 2,4-D by BDH AC (0.01%); C: adsorption of 2,4-D by Sigma AC (0.1%); D: adsorption of 2,4-D by Sigma AC (0.1%); E: adsorption of 2,4-D by Sigma AC (1.0%); F: adsorption of 2,4-D by BDH AC (1.0%). Values are the means of three replicates with bars indicating ±SE.

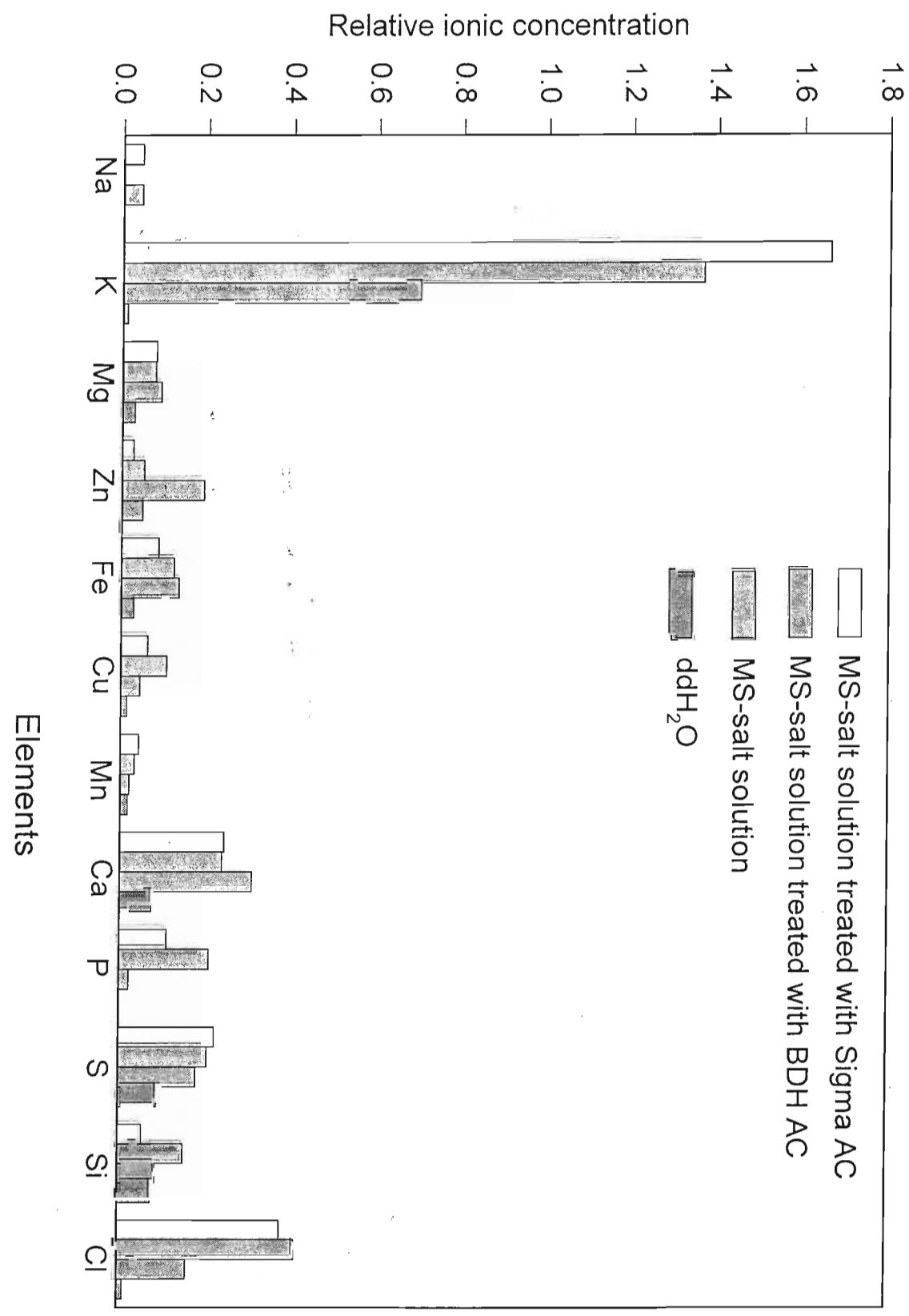

Figure 3.3 Changes in inorganic elements of MS-salt solutions in the presence or absence of activated charcoal as shown by SEM-EDX analysis.

3.3.2. Comparison of the adsorption/release of inorganic elements by different AC from /to MS salt media.

The changes in inorganic elements of MS-salt solutions, which were treated with or without AC, were determined by SEM-EDX (Figure 3.3). The concentrations of inorganic salts such as magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn) deceased, while the concentrations of other inorganic salts such as potassium (K), copper (Cu), manganese (Mn), phosphorus (P), and sulphur (S) increased in the presence of AC. The results suggests that the inorganic salts (Mg, Ca, Fe, Zn) might be adsorbed by AC while K, Cu, Mn, P, S are released into the medium from AC.

3.4 Discussion

Addition of AC to a culture medium is a recognized practice in plant tissue culture. The beneficial effects of AC are frequently attributed to its adsorptive properties. Activated charcoal is able to adsorb substances presumed deleterious and/or inhibitory to *in vitro* culture. However, adsorption of growth regulators being applied to the tissue by AC could also occur at the same time. Auxin is generally required to be incorporated into the nutrient medium for the induction of callus from explants. The auxin most frequently employed to initiate somatic embryogenesis cultures is 2,4-D. For somatic embryogenesis induction from broad-leafed plants, 2,4-D is generally used at levels between 1.0-3.0 mg l^{-1}. 2,4-D promote cell dispersion in suspension cultures, the relatively high levels of 2,4-D added to liquid

media for this purpose will prevent morphogenesis, but might induce an embryogenesis if the cells are still competent. The process of somatic embryogenesis is often initiated in media containing high levels of 2,4-D, but embryos do not develop further until the auxin concentration is reduced. It was shown that 2,4-D was effectively adsorbed by AC from methanol and aqueous solutions. Two types (Sigma and BDH) and four different levels (0.01%, 0.1%, 1.0% and 3.0%) of AC used resulted in reducing the added 2,4-D levels in methanol and aqueous solutions. The adsorptive capacity of AC (Sigma) is stronger than the adsorptive capacity of AC (BDH), but the difference is not significant. There was no significant difference in the changes of the level of adsorption of 2,4-D by AC between methanol and aqueous solutions. The highest levels of adsorbed 2,4-D by AC (Sigma and BDH) occurred within the first day. The degree of adsorption of 2,4-D by four AC concentrations (0.01%, 0.1%,1.0% and 3.0% AC) in both methanol and aqueous solutions slightly increased with time after seven days.

Research workers have shown that the addition of AC often has a promotive effect on the growth and organogenesis of woody species (MARTINEZ-PULIDO *et al.* 1990; DUMAS and MONTEUUIS 1995; SANCHEZ *et al.* 1996). It is possible that AC releases substances, and/or AC adsorbs substances which promote and/or inhibit the *in vitro* growth of plants or explants. Although Weatherhead *et al.* (1979) analysed impurities in AC and studied effects of ions of a few metals in anther cultures of *Nicotiana tabacum*, little is known of a possible release from AC of substances that may affect embryogenesis in anther cultures. It can not be excluded that some of the elements that are not present in MS-medium, but are

released in minor amounts from AC, may be beneficial to embryogenesis, as suggested by Weatherhead *et al.* (1979). In that study, impurities in AC was determined by mass spectrometry, and in some case related to embryogenesis. However, the results may not be directly comparable to the present results as no statement was made as to the release of the impurities into culture media. The brand of AC was also not mentioned. SEM-EDX analysis of AC-containing MS medium indicates that the concentrations of calcium, magnesium, zinc, iron and potassium, manganese, phosphorus, sulphur in MS-medium were changed by the amounts released from AC or adsorbed by AC.

Calcium functions as a secondary messenger, and is an important enzyme co-factor and enzyme regulator. Calcium ions are also involved in establishing the structure and properties of both cell membranes and cell walls. Magnesium is a component of chlorophyll and is important biochemically as an enzyme cofactor. It is also a structural component of ribosomes. Iron is required for the formation of several chlorophyll precursors and is a component of ferredoxins which are important oxidation:reduction reagents. Iron is also an important component of proteins which carry out oxidation and reduction reactions. Zinc and copper are important components of some types of enzyme, such as oxidases and superoxide dismutase, which help to prevent damage to tissues due to superoxide radicals. Manganese is required to maintain chloroplast ultrastructure and for photosynthesis. Phosphorus plays a key role in the transfer of energy within the cell, regulates the activity of many enzymes and is a component of macro molecules such as DNA and RNA. Sulphur is an important part of some amino acids and as such it is involved in determining protein structure by the formation of

disulphide bridges. Sulphur is also an important part of some enzyme co-factors. It is clear that AC adsorbed calcium, magnesium, iron and zinc and released copper, manganese, phosphorus and sulphur. This is in agreement with the results of Johansson and Eriksson (1977), Litz and Conover (1980) and Johansson *et al.* (1990) who all postulated that AC gradually release adsorbed products.

CHAPTER FOUR

Effect of activated charcoal and auxins on root formation by hypocotyls of *Daucus carota* L.

Abstract

In vitro rooting of hypocotyls of *Daucus carota* L. Cape Market, using AC and auxins [2, 4 - Dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA)] was investigated. Rooting occurred when 7-day-old seedling hypocotyls were placed on Murashige and Skoog medium supplemented with 2,4-D in the presence of AC, and NAA or IAA in the presence and/or absence of AC. Hypocotyls did not produce roots on the 2,4-D-containing medium in the absence of AC. Hypocotyls produced roots polarly on the NAA or IAA-containing media in the presence of activated charcoal. No-polarity of root formation was observed on media supplemented with NAA or IAA without AC. Different responses of hypocotyls to various levels of 2,4-D (0, 0.5, 1.0, 3.0, 5.0, 8.0 and 10.0 mg l^{-1}) were observed on media supplemented with 0.02, 0.1 and 0.5% AC. Root number per hypocotyl decreased in the NAA or IAA-containing media in the presence of AC. However, root number per hypocotyl, on the media supplemented with NAA or IAA, increased when hypocotyls were pre-cultured on MS medium supplemented with 2,4-D (1.0 mg l^{-1}) for 2-3 days. When hypocotyls were pre-cultured on a 2,4-D-

containing MS medium for 5 days, embryos emerged from the hypocotyls directly on the medium supplemented with 2,4-D in the presence of AC.

4.1 Introduction

Roots formed *in vitro* can be of different kinds, depending on the treatment under which they were produced. Auxin is an essential factor in the initiation and promotion of root growth. It is widely used in micropropagation and is incorporated into nutrient media to promote the growth of callus, cell suspensions or organs and together with cytolinins to regulate morphogenesis. Auxins influence a number of plant functions such as the promotion of cell elongation and cell division, apical dominance, root initiation, differentiation of vascular tissue, ethylene biosynthesis and mediation of tropistic responses (KEY 1989; CHASAN 1993; SACHS 1993; WARREN-WILSON and WARREN-WILSON 1993). In general, the role of auxin in root development include the following essential features: (1) it is necessary for elongation of root cells, but may be inhibitory at concentrations which promote shoot growth; (2) IAA also regulates root growth by inhibiting the duration of cell elongation; (3) IAA may not be in the root as such, but may exist as a complex, or as a member of a group of other promoters and inhibitors; (4) auxin moves predominantly in a base to apex direction in the root tip in an active polar transport system; and (5) auxin metabolism in the root is complex, and some of its many features may be related directly to cell growth and differentiation (SCOTT 1972).

Unlike IAA, NAA and IBA, 2,4-D is rarely used for root induction. There are however, reports of 2,4-D being used for this purpose. 2,4-D induced root formation on callus of birch (HUHTINEN 1976). Boyes and Sink (1981) found that

adventitious root formation on shoot in *Salpiglossis sinuata* was more effectively induced with 2,4-D than NAA. *In vitro* rooting on shoots of *Leptospermum flavescens* was obtained by placing shoots on a 2,4-D-containing medium (SHIPTON and JACKES 1986). However, 2,4-D did not induce any root formation on shoots of *Anigozanthos fulginosa* (SRISKANDARAJAH and MULLINS 1981). At concentrations less than 10 µM 2,4-D inhibited rooting of *Malus x domestica*, causing both shoots and medium to discolour within a few days (SRISKANDARAJAH and MULLINS 1981).

Activated charcoal (AC) is prepared by the controlled carbonisation of wood in a stream or air. It possesses strong adsorptive properties and is used in chemistry to adsorb both gases and dissolved solids. The use of AC in *in vitro* culture was reviewed in Chapter 1. Activated charcoal is used in plant tissue culture due to its structure and because its addition to culture medium may promote or inhibit *in vitro* growth, depending on a number of factors. The addition of AC to culture media promote *in vitro* rooting (FRIDBORG and ERIKSSON 1975; JAISWAL and AMIN 1987; BLOMSTEDT *et al.* 1991; DUMAS and MONTEUUIS 1995; GEORGE and RAVISHANKAR 1997). Stimulation of root growth by AC could be ascribed: (a) to the adsorbtion of undesirable/inhibitory substances and/or (b) preventing of tissue and medium browning.

Activated charcoal is able to adsorb substances presumed deleterious and/or inhibitory to *in vitro* culture. However, adsorption of growth regulators applied to the tissue by AC could also occur at the same time, and non-selective

adsorption may result in negative effects on cultured explants. Fridborg and Eriksson (1975) suggested that AC removed growth regulators, particularly auxins, from the medium. In tissue culture media 0.1% AC can effectively adsorb 10 µM IAA (1.75 mg l^{-1}) and 10 µM IBA (2.03 mg l^{-1}) from the liquid medium (NISSEN and SUTTER 1990).

Activated charcoal can provide a dark environment. Light can be kept away from the root zone by darkening the medium. The amount of light passing through a solidified medium is reduced by adding charcoal. If sufficient AC is added to the medium it can exclude light sufficiently to promote physiological reactions which occur in the dark. Photosynthesis provides carbohydrates needed for root initiation and root growth. However, keeping micropropagated shoots in darkness during the inductive phase is generally beneficial to rooting (GEORGE 1996). This is due to the fact that auxins are metabolised less rapidly in the dark than in the light (NORTON and BOE 1982). Darkness appears to result in increased peroxidase activity (FABIJAN *et al.* 1981). Auxin treatment did substitute for the promoting effect of low illumination on the rooting of *Kalanchoë* cuttings (GEORGE 1996). In shoot apices from the adult phase of *Hedera helix,* root formation was practically zero in light of 4300-5400 µmol $^{-2}$ s^{-1}, but could be promoted by 10 mg l^{-1} IAA when the illumination was only 540 µmol $^{-2}$ s^{-1}. Juvenile phase apices, on the other hand, could be rooted at the higher illuminance providing they were treated with 5-10 mg l^{-1} NAA (HACKETT 1970).

The experiments reported on this chapter were to investigate the effect of AC and auxins (2,4-D, NAA and IAA) on root formation on hypocotyls of *Daucus carota* L.

4.2 Materials and methods

4.2.1 Plant material and culture media

Seeds of *Daucus carota* L. Cape Market were decontaminated in 70% (v/v) ethanol for 2 min, followed by 30 min in 1.05% (v/v) sodium hypochlorite and rinsed three times with sterile distilled water. The seeds were then germinated in sterile petri dishes containing moist filter paper. Seven-day-old seedlings were subsequently surface-sterilized with 1.05% sodium hypochlorite solution for 20 min and then rinsed five times with sterile distilled water. Ten-mm-long segments were cut from the seedling hypocotyls and each of the segments was placed on a medium which included MS salts and vitamins (hereafter referred to as MS medium) (MURASHIGE and SKOOG 1962), supplemented with 30.0 g l^{-1} sucrose, 8.0 g agar l^{-1}, different levels of AC and 1.0 mg l^{-1} 2,4-D or 0.5 mg l^{-1} IAA or NAA (unless otherwise indicated).

4.2.2 Activated charcoal treatments

Various concentrations of AC (Sigma) (0.01, 0.05, 0.1, 0.5, 1.0 and 3.0%) were added to the MS media described previously (4.2.1) before adjusting the pH

to 5.8. Combinations of different concentrations of 2,4-D (0, 0.5, 1.0, 3.0, 5.0, 8.0, and 10.0 mg l^{-1}) and AC (0.02, 0.1, and 0.5%) were added to the MS medium.

For AC experiments for studying the removal of compounds, 0.5% AC was added to the MS media supplemented with 2,4-D (1.0 mg l^{-1}) or without 2,4-D. After the media were shaken at room temperature for 4 h, the AC was removed by filtration. The media were then autoclaved.

4.2.3 Culture conditions

All media were adjusted to pH 5.6 to 5.8 with KOH or HCl prior to the addition of agar and after addition of AC, and autoclaved for 20 min at 121^{o}C (118 kPa).

All explants were incubated at 25 ± 1^{o}C with 16:8 h/light :dark photoperiod under photosynthetically active radiation of 50 µmol m^2 s^{-1} provided by cool-white fluorescent tubes.

A minimum of 20 replicates were maintained for each treatment and the number of roots per explants were recorded after 4 weeks of culture. The experiment was repeated twice.

4.3 Results

The hypocotyls cultured on the medium supplemented with 2,4-D in the absence of AC produced callus (Figure 4.1A), while rooting resulted from the hypocotyls cultured on the 2,4-D containing medium in the presence of AC (Figure 4.1B), and IAA or NAA-containing media in the presence or absence of AC (Figure 4.2). Roots generally emerged after explants were cultured on media for 4 to 6 days. Roots were observed on explants with media which contained combinations of auxins (2,4-D and IAA or NAA) and different concentrations (0.01, 0.05, 0.1, 0.5, 1.0 and 3.0%) of AC. Roots appeared earlier on the 2,4-D-containing media to which were added 0.01% to 0.5% AC rather than those containing 1.0% and 3.0% AC. The number of roots per explant decreased with increased AC concentrations on 2,4-D and IAA or NAA-containing media (Table 4.1; Figure 4.3). Addition of 0.01% AC to the medium supplemented with 1 .0 mg l^{-1} 2,4-D resulted in a significantly increase in the number of roots produced per explant (Figure 4.1C and Figure 4.3). Rooting percentage of explants decreased on the IAA or NAA-containing media when increasing concentrations of AC were used (Table 4.1). However, there was no significant change in rooting percentage of explants on the 2,4-D-containing medium with increasing concentrations of AC used (Figure 4.3).

The results in Figure 4.2 show that the hypocotyls produced roots polarly on the NAA or IAA-containing media in the presence of AC (Figure 4.2A-a , 4.2B-b and 4.2D). Roots proliferated from the basal ends of the hypocotyls. No-polarity of root formation was observed on media supplemented with NAA or IAA without AC (Figure 4.2A-b, 4.2B-a, 4.2C, 4.2E, and 4.2F). Roots were observed on both ends

(basal and apical) and/or in the middle of the hypocotyl segments.

Results presented in Figure 4.1 D indicate that rooting also resulted with the hormone-free media in the presence and/or absence of AC. The rooting percentage of explants and the number of roots per explant on the 2,4-D-free medium in the absence of AC were lower than those on the 2,4-D-containing medium in the presence of AC (Figure 4.3).

Results presented in Figure 4.1E show that rooting occurred when AC was removed by filtration prior to media autoclaving [2,4-D (1.0 mg l^{-1}) was added to the medium before AC removing]. However, roots were not observed on the medium in which 2,4-D was added after removal of AC from the medium (2,4-D was not added to the medium before AC removal) before autoclaving.

Different responses of hypocotyls to various levels of 2,4-D (0, 0.5, 1.0, 3.0 5.0, 8.0, and 10.0 mg l^{-1}) were observed on MS media supplemented with 0.02, 0.1 and 0.5% AC (Table 4.2). Table 4.2 indicates that rooting occurred on the media containing 0.5 to 10.0 mg l^{-1} 2,4-D in the presence of 0.1% and 0.5% of AC.

Addition of 0.02% AC to the media containing 0.5 to 10.0 mg l^{-1} 2,4-D resulted in root, shoot and embryo formation (Table 4.2). Figure 4.4A shows that addition of 0.02% of AC to the medium containing 3.0 mg l^{-1} 2,4-D resulted in root and shoot formation. On the medium containing 5.0 mg l^{-1} 2,4-D in the presence

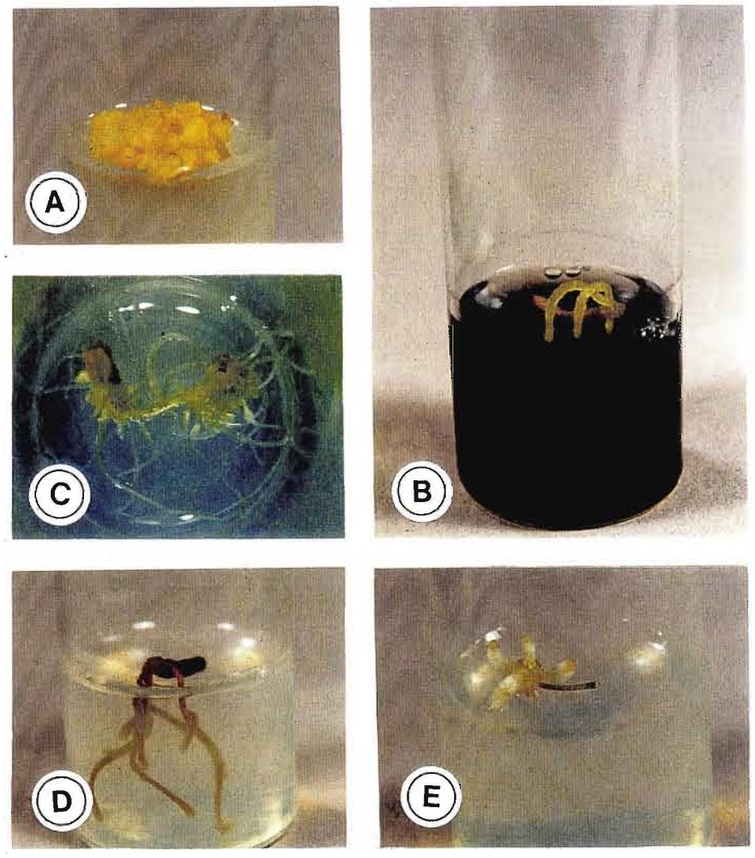

Figure 4.1: The response of hypocotyls of 7-day-old seedlings of *Daucus carota* L. cultured on MS media supplemented with 2,4-D in the presence or absence of activated charcoal. (A): Callus induced on the medium containing 2,4-D only; (B): Roots induced on the medium containing 2,4-D in the presence of 0.5% AC; (C): Roots induced on the medium containing 2,4-D in the presence of 0.01% AC; D: Roots induced on the 2,4-D-free medium in the absence of AC; E: Roots induced on the medium in which AC was removed before autoclaving.

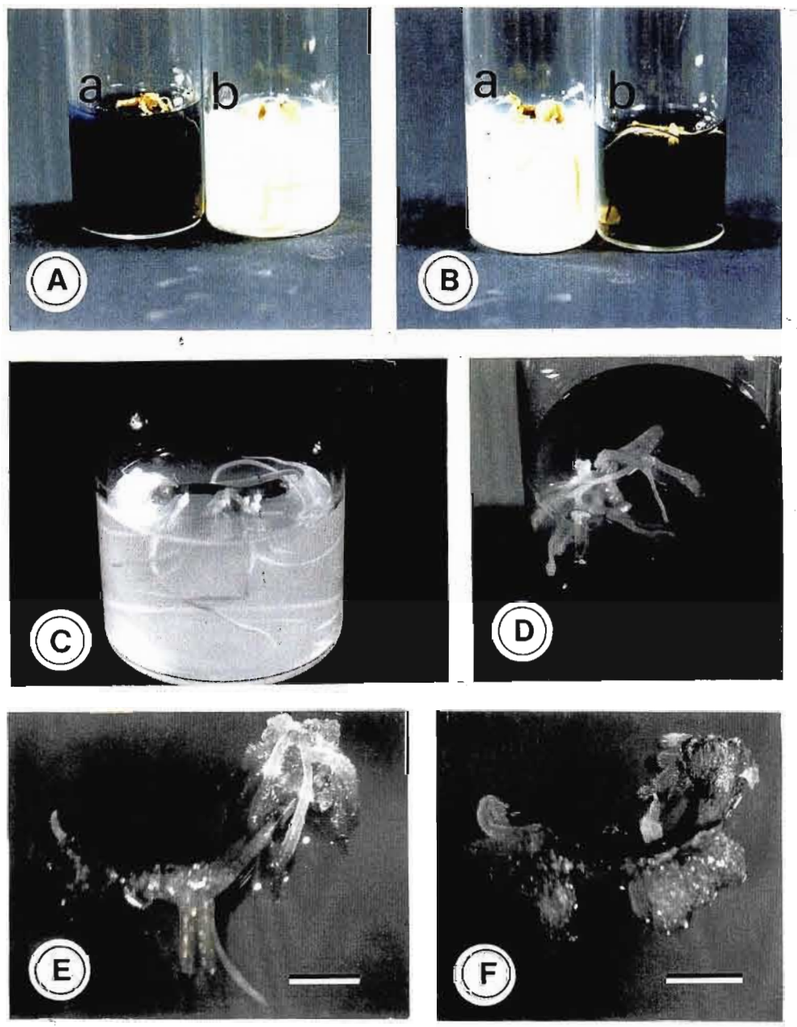

Figure 4.2: *In vitro* rooting of hypocotyls of *Daucus carota* L. A: on IAA-containing media, a: in the presence of AC; b: in the absence of AC. B: on NAA-containing media, a: in the absence of AC; b: in the presence of AC. C, E: on IAA-containing media in the absence of AC. Bar = 1 mm. D: on IAA-containing media in the presence of AC. F: on NAA-containing medium in the absence of AC. Bar = 1 mm.

Table 4.1: Effect of NAA, IAA and activated charcoal on root formation.

Treatment		No. of roots/ per explant	Rooting (%)
Auxin	AC (%)		
NAA (mg l^{-1})	0	7.3 ± 2.5	84.8
	0.1	4.2 ± 1.8	80.5
0.1	0.5	3.8 ± 1.5	78.0
	1.0	3.1 ± 1.5	62.0
	3.0	2.8 ± 1.0	60.3
NAA (mg l^{-1})	0	9.8 ± 1.9	86.7
	0.1	4.8 ± 1.7	78.5
0.5	0.5	3.7 ± 1.5	76.2
	1.0	3.0 ± 1.4	72.1
	3.0	2.9 ± 1.1	61.5
IAA (mg l^{-1})	0	6.7 ± 1.8	84.7
	0.1	4.0 ± 1.7	80.8
0.1	0.5	3.6 ± 1.4	79.5
	1.0	2.4 ± 1.2	66.7
	3.0	2.1 ± 1.0	60.9
IAA (mg l^{-1})	0	8.9 ± 1.9	85.4
	0.1	5.2 ± 1.8	80.2
0.5	0.5	4.3 ± 1.5	78.5
	1.0	3.1 ± 1.1	68.1
	3.0	2.0 ± 1.0	61.8

Table 4.2: The effect of 2,4-D and activated charcoal on the morphogenesis of the hypocotyls of *Daucus carota* L.

Activated Charcoal (%)	2,4-D Concentration (mg l^{-1})						
	0	0.5	1.0	3.0	5.0	8.0	10.0
0	R	C	C	PC	PC	EB	EB
0.02	R	R	R	R/S	C/E/R/S	R	R
0.1	R	R	R	R	R	R	R
0.5	R	R	R	R	R	R	R

C: Callus; E: Embryos; EB: Explant Browning; PC: Poor Callus; R: Root; S: Shoot

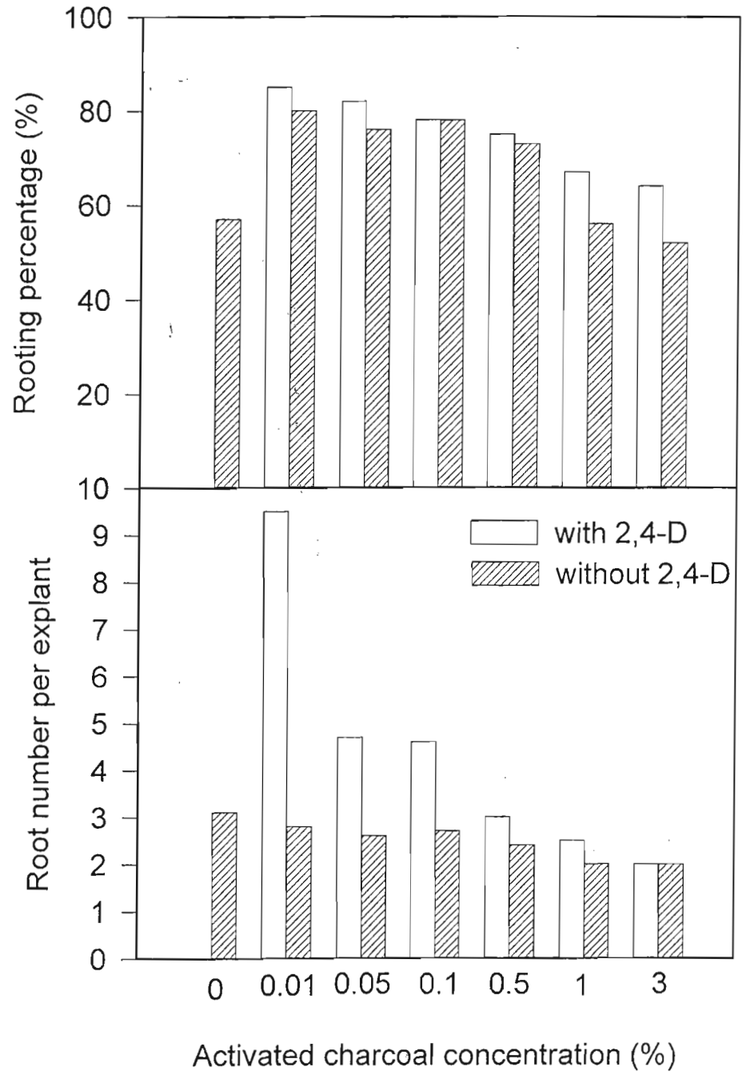

Figure 4.3: Root number per explant and percentage of hypocotyls rooting of 7-day-old seedlings of *Daucus carota* L. which were cultured on MS media with or without 2,4-D, in the presence or absence of activated charcoal.

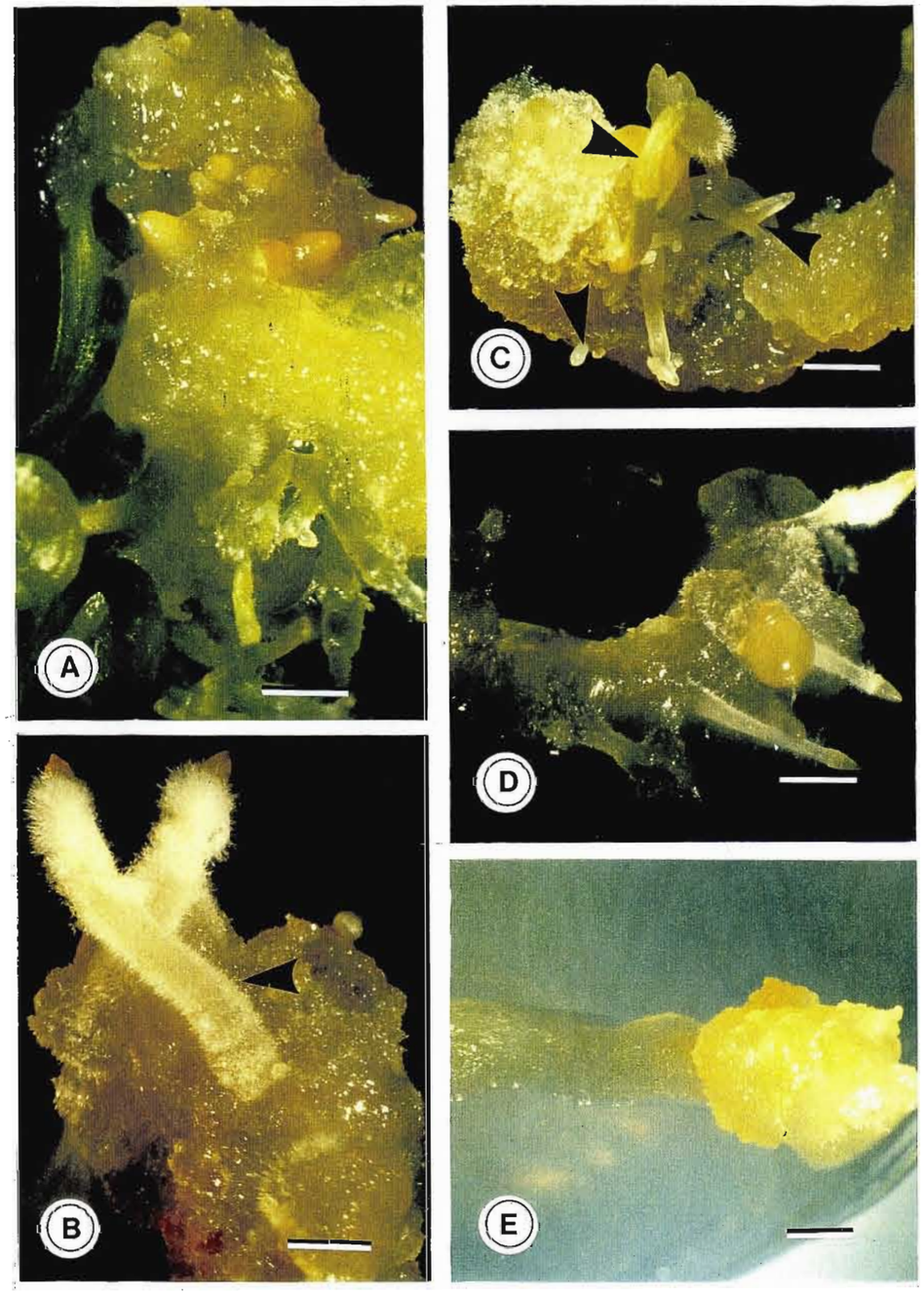

Figure 4.4: *In vitro* rooting on the hypocotyls of 7-day-old seedlings of *Daucus carota* L. which were cultured on MS media containing 2,4-D in the presence or absence of activated charcoal: (A): Root primordia and shoots on the medium containing 3.0 mg l^{-1} 2,4-D in the presence of 0.02% AC; (B):Callus and roots on the medium containing 5.0 mg l^{-1} 2,4-D in the presence of 0.02% AC; (C): Embryos and shoots induced on the medium containing 5.0 mg l^{-1} 2,4-D in the presence of 0.02% AC; (D): Roots induced on the medium containing 8.0 mg l^{-1} 2,4-D in the presence of 0.02% AC; (E): swollen explant on the medium containing 5.0 mg l^{-1} 2,4-D in the absence of AC. Bar = 500 µM.

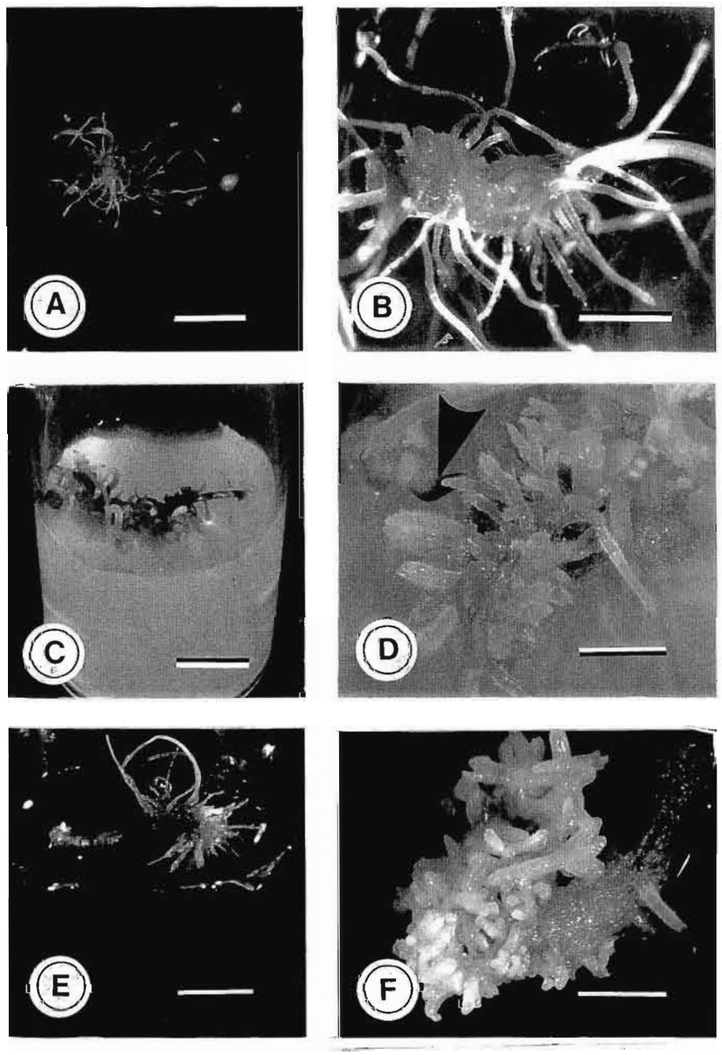

Figure 4.5: ***In vitro*** **rooting on hypocotyls of** ***Daucus carota*** **L. which were pre-cultured on a MS medium containing 2, 4-D for 2 to 5 days.** A: on the IAA-containing medium in the presence of AC. Bar = 5 mm B: amplification of A. Bar = 1mm. C: on the IAA-containing medium in the absence of AC. Bar = 5 mm. D: amplification of C showing shoot primordia as indicated by arrow. Bar = 1 mm. E: on the NAA-containing medium in the presence of AC. Bar = 5 mm. F: on the 2,4-D containing medium in the presence of AC. Bar = 1 mm.

of 0.02% of AC, callus and roots were observed (Figure 4.4B). Embryos and shoots were also induced from the explants (Figure 4.4C). Rooting also resulted from the hypocotyl on the medium containing 8.0 mg l^{-1} 2,4-D in the presence of 0.02% of AC (Figure 4.4D). However, the explants were swollen. Poor callus was observed on the hypocotyls in the medium supplemented with 5.0 mg l^{-1} 2,4-D in the absence of AC (Figure 4.4E).

2,4-D may influence root primordia indirectly by regulating cell division activity. It was found that root number per explant greatly increased on the IAA or NAA-containing media in the presence (Figures 4.5A, B and E) or absence (Figures 4.5C and D) of AC when the explants were pre-cultured on a MS medium containing 1.0 mg l^{-1} 2, 4-D for 2 to 3 days. Figure 4.5D also indicates that shoot primordia were induced on the IAA-containing medium in the absence of AC when the explant was pre-cultured on 2,4-D-containing medium for 3 days.

Results presented in Figure 4.5 show that when hypocotyls were pre-cultured on a 2,4-D-containing MS medium for 5 days, embryos emerged from the hypocotyls directly in the presence of AC (Figure 4.5F). This suggested that longer pre-culturing (5 days) onto 2,4-D lead directly to induction of embryos from explant hypocotyls.

4.4 Discussion

Activated charcoal has been used in plant tissue culture media to improve growth and/or promote morphogenesis in a wide variety of plant species (GEOGER

1993; PAN and VAN STADEN 1998). In this study, rooting occurred in the MS medium supplemented with 2,4-D in the presence of AC. Roots were not observed on the 2,4-D-containing medium in the absence of AC. Rooting also occurred with the medium containing high concentrations (0.5 to 8.0 mg l^{-1}) of 2,4 -D in the presence of 0.02% AC. The beneficial effect of AC on root production is mainly, but may not entirely, be due to the adsorption of 2,4-D by AC. This suggests that AC can adsorb comparatively high concentrations of growth regulators and make them unavailable to tissue explants. In agreement with this, Nissen and Sutter (1990) recommend that 0-100 times more auxins should be added to a medium if high concentrations of AC are used.

There are few reports that 2,4-D can be used for root induction (HUHTINEN 1976; BOYES and SINK 1981; SHIPTON and JACKES 1986). Pre-culturing explant on the 2,4-D-containing MS medium for 2 to 3 days resulted in an increased root number on the auxin-containing medium in the presence or absence of AC. Generally four phases can be distinguished in the rooting process (GEORGE 1996): (a) an induction phase, when the capacity for root formation is determined; (b) an initiation phase, when visible cytological changes occur; (c) an organisation phase, when root primodia can be seen to be produced histologically; and (d) a growth (root elongation) phase when primodia develop into roots. It seems that both 2,4-D pre-culturing and AC are directly or indirectly involved in root induction or initiation. It is unknown at which phase AC may exist its effect in the rooting process, and whether rooting might be stimulated by pre-culturing explants on the 2,4-D-containing MS medium for 2 to 3 days. This require further investigation.

This part of the study shows that *in vitro* rooting on hypocotyls of carrot occurred on MS medium supplemented with IAA or NAA in the presence or absence of AC. The present results shows that the hypocotyls of *Daucus carota* produced roots polarly on the NAA or IAA-containing media in the presence of AC. However, no-polarity of root formation was observed on media supplemented with NAA or IAA in the absence of AC. This suggests that AC might affect auxin transport.

Alteration of medium pH, to an optimum level for morphogenesis, has been reported as a beneficial effect of AC (OWEN *et al.* 1991). Liu *et al.* (1993) and Blakely *et al.* (1986) suggested that an increase in the acidity of the cell wall pH might facilitate auxin transport across membranes to the site of lateral root primordial formation. An acidic pH, increases carrier-mediated uptake of IAA and might allow 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 acidic conditions may in part promote root formation by increasing the movement of IAA to the rooting zone (LIU *et al.* 1993). The present study indicates that addition of AC to media resulted in a decrease in medium pH (Chapter 2). It is possible that rooting could be enhanced by the acidic pH provided by using AC. The pH of the medium dropped and may be accompanied by a decrease in sucrose with an corresponding increase in the glucose and fructose levels (PAN and VAN STADEN 1999).

Activated charcoal can provide a dark environment, and if sufficient AC is

added to the medium the amount of light passing through a solidified medium is reduced and/or light can be kept away from the rooting zone. This could promote some physiological reactions which occur in the dark. It is generally thought that auxins are metabolised less rapidly in the dark than in the light (NORTON and BOE 1982). Therefore, darkness is generally beneficial to rooting, especially during the inductive phase.

Addition of 0.01 to 0.5% AC to culture medium enhanced rooting ability. It is probably due to the fact that AC is also able to adsorb inhibitory substances which might be produced by cultures or be present in the medium. Activated charcoal adsorbed substances, such as products of sucrose breakdown, produced by autoclaving (WEATHERHEAD *et al.* 1978), and/or phenolic compounds which are released from culture cells (JOHANSSON 1983). Addition of 0.01% AC to the culture medium resulted in significantly increases in the root number per explant. A lower root number per explant was obtained by addition of a high concentration of AC to the medium. Activated charcoal is known to adsorb a number of compounds which is normally incorporated in the culture medium such as auxins, cytokinins, abscisic acid (JOHANSSON *et al.* 1982), vitamins (WEATHERHEAD *et al.* 1979), and iron chelates (HEBERLE-BORS 1980). It is possible that the addition of high concentrations of AC may induce nutrient deficiencies in culture media. Such a deficiency will affect explant growth.

CHAPTER FIVE

The effect of activated charcoal on the production and development of somatic embryos in cultures of carrot *Daucus carota*

Abstract

The effect of AC on somatic embryogenesis in cultures of *Daucus carota* L. Cape Market was investigated. The addition of activated charcoal to Murashige and Skoog medium containing 2,4-D resulted in somatic embryogenesis in cultures of *Daucus carota*. Somatic embryos were not formed in the absence of AC. In suspension culture, the incorporation of 0.01 to 1.0% AC to the MS medium, irrespective of 2,4-D, increased the number of somatic embryos produced. The maximum number of somatic embryos were produced with 1.0% AC-containing MS medium. Further development of the embryos occurred in the presence of AC, and the embryos could be regenerated into normal plantlets. Addition of 3.0% AC to the medium resulted in the formation of abnormal somatic embryos. These embryos were subsequently regenerated but produced abnormal plantlets.

5.1 Introduction

The carrot (*Daucus carota* L.) is widely used for studies of somatic embryogenesis (HANAI *et al.* 2000). Morphological and physiological studies demonstrated that the somatic embryogenesis process consists of at least two phases. Firstly, the induction of embryogenic competence in the cells with high concentrations of auxins. Secondly, the development of the embryogenic masses into embryos in the absence of, or in the presence of, a lowered concentration of auxins (NOMURA and KOMAMINE 1985). Somatic embryos develop from embryogenic cells of somatic tissue to form globular proembryos, and progress through the heart-, and torpedo-shaped stages with morphology similar to zygotic counterparts (MICHLER and LINEBERGER 1987). Abnormal forms of somatic embryos are known to arise from populations of callus cells (AMMIRATO 1977). Some of the anomalies described include lateral root development, multiple cotyledons, multiple embryos attached as a single unit, and secondary embryos associated with cotyledons and hypocotyls (AMMIRATO and STEWARD 1971). Several factors influence the second phase of the process and the media components, auxin and nitrogen, play crucial roles in manipulating embryogenesis (KOHLENBACH 1978). The importance of auxin was first recognized by Halperin and Wetherell (1964). Today, 2,4-D is most commonly used to bring about somatic embryogenesis. The process is often initiated in media containing high levels of auxin. Embryos development is optimized only once the auxin concentration is reduced. After initial induction the development of carrot somatic embryos is generally be enhanced by transferring the embryogenic cells to an auxin-free medium (GEORGE 1993).

Activated charcoal is commonly used in plant tissue culture to improve cell growth and development. Activated charcoal may enhance shoot formation and rooting (FRIDBORG and ERIKSSON 1975; PATEL and THORPE 1986; OMURA and HIDAKA 1992; NOUR and THORPE 1993), prevent the development of abnormal plantlets (ZIV and GADASI 1986) and promote embryogenesis (FRIDBORG *et al.* 1978; DREW 1979; JOHANSSON 1983; JONES and PETILINO 1988; DETHIER-ROGERS *et al.* 1993). It also restored the embryogenetic potential of long-term cultured *Festuca rubra* callus with a declining regeneration potential (ZAGHMOUT and TORELLO 1988). The effects of AC on *in vitro* culture may be attributed to establishing a dark environment, adsorption of undesirable/inhibitory substances, adsorption of plant growth regulators and other organic compounds, the release of growth promoting substances present in or adsorbed by AC or the influence of AC in combination with autoclaving and culture media on sucrose hydrolysis (PAN and VAN STADEN 1998,1999). A major effect of AC on somatic embryogenesis could well be to adsorp supra optimal level of auxins from the culture medium or preferential requirement of the explants for sucrose hydrolytic products, glucose and fructose.

This chapter of study investigated the effects of AC on the somatic embryogenic process of *Daucus carota.*

5.2 Materials and methods

5.2.1 Material and culture conditions

Seeds of carrot (*Daucus carota* L. Cape Market) were surface decontaminated in 70% (v/v) ethanol for 2 min and 1.05% (v/v) sodium hypochlorite for 30 min. The seeds were then thoroughly rinsed with sterile distilled water and placed in sterile petri dishes containing moist filter paper for germination at 25 ± 1°C. When seven days old the seedlings were surface decontaminated again with 1.05% sodium hypochlorite solution for 20 min and rinsed five times with sterile distilled water. Ten-mm-long segments were cut from the hypocotyls of the decontaminated seedlings and each of the segments placed on medium.

The media used comprised of Murashige and Skoog (1962) salts and vitamins (MS medium). Callus was initiated by placing the hypocotyl sections on MS medium supplemented with 30 g l^{-1} sucrose, 1.0 mg l^{-1} 2,4-D and 8.0 g l^{-1} agar. Callus that was produced was subcultured every 3 to 4 weeks on MS medium supplemented with 0.5 mg l^{-1} 2,4-D. Embryogenic suspension cultures formed four weeks after embryogenic callus was suspended in 50 ml Erlenmeyer flasks containing 20 ml liquid MS medium with 0.5 mg l^{-1} 2,4-D. These suspension cultures were kept on a rotatory shaker (100 rpm) and were transferred every 2 to 3 weeks to fresh MS medium with 0.5 mg l^{-1} 2,4-D. The pH of all media was adjusted to 5.6 to 5.8 with KOH or HCl prior to the addition of agar and after the addition of AC. Media were then autoclaved for 20 min at 121°C (118 kPa). All explants and callus were incubated at 25 ± 1°C with a 16:8 h/light:dark photoperiod

under photosynthetically active radiation of 50 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

5.2.2 Activated charcoal treatments

Different concentrations of AC (0, 0.01%, 0.05%, 0.1%, 0.5%, 1.0% and 3.0%) (w/v) were added to the MS medium containing 30 g l^{-1} sucrose with or without 0.5 mg l^{-1} 2,4-D. Solid media were gelled by adding 8.0 g l^{-1} agar. Callus was cultured on the MS media described above. Suspension cultures (5% v/v) were inoculated into medium with or without 0.5 mg l^{-1} 2,4-D in the presence or absence of AC.

5.2.3 Quantification of somatic embryo formation

Production of somatic embryos was determined microscopically. Somatic embryos progress through four stages of development while undergoing tissue differentiation: globular, heart, torpedo, and cotyledonary stages respectively. The stages are based on the overall embryo shape. In general, carrot somatic embryos remain in the torpedo stage for a few days before progressing to the cotyledonary stage. Cotyledonary-stage embryos have an elongated radicle and hypocotyl, which tends to exhibit hyperhydricity in liquid medium. With suspension cultures 1 ml samples were collected from suspensions every five days and the somatic embryos counted microscopically. Embryos were classified into the various developmental stages according to Shimazu and Kurata (1999).

5.3 Results

Callus of carrot was initiated by placing the hypocotyl sections on MS medium supplemented with 1.0 mg l^{-1} 2,4-D. To evaluate the effect of AC on embryogenesis callus was cultured on media supplemented with 2,4-D in the presence of various concentrations of AC. In both solid and liquid charcoal-treated media, addition of 0.01 to 1.0% AC induced somatic embryogenesis. After 7 days, globular embryos were observed from callus on all the media. After being grown for 14 days, a number of globular embryos were observed in the 2,4-D-free medium in the absence of AC (Figure 5.1A). Heart-shaped and torpedo shaped-embryos developed on all AC-containing media supplemented with 2,4-D (Figures 5.1B, C, D; 5.4A, B, C, D, E). Increasing the AC to 3.0% resulted in abnormal embryos, leaf and multiple cotyledons being produced (Figures 5.1E, F; 5.2F, G, H). In addition, the number of heart-stage embryos increased as the AC concentration used increased up to a level of 0.5% (data not shown). The heart-stage embryos developed subsequently developed. Roots elongated and they grew to normal plantlets (Figure 5.3A). Normal plantlets developed with the low concentrations (0.01 to 1.0%) of AC-treated media. However, abnormal plantlets occurred on the high concentration (3.0%) AC-containing media (Figures 5.3B, C, D).

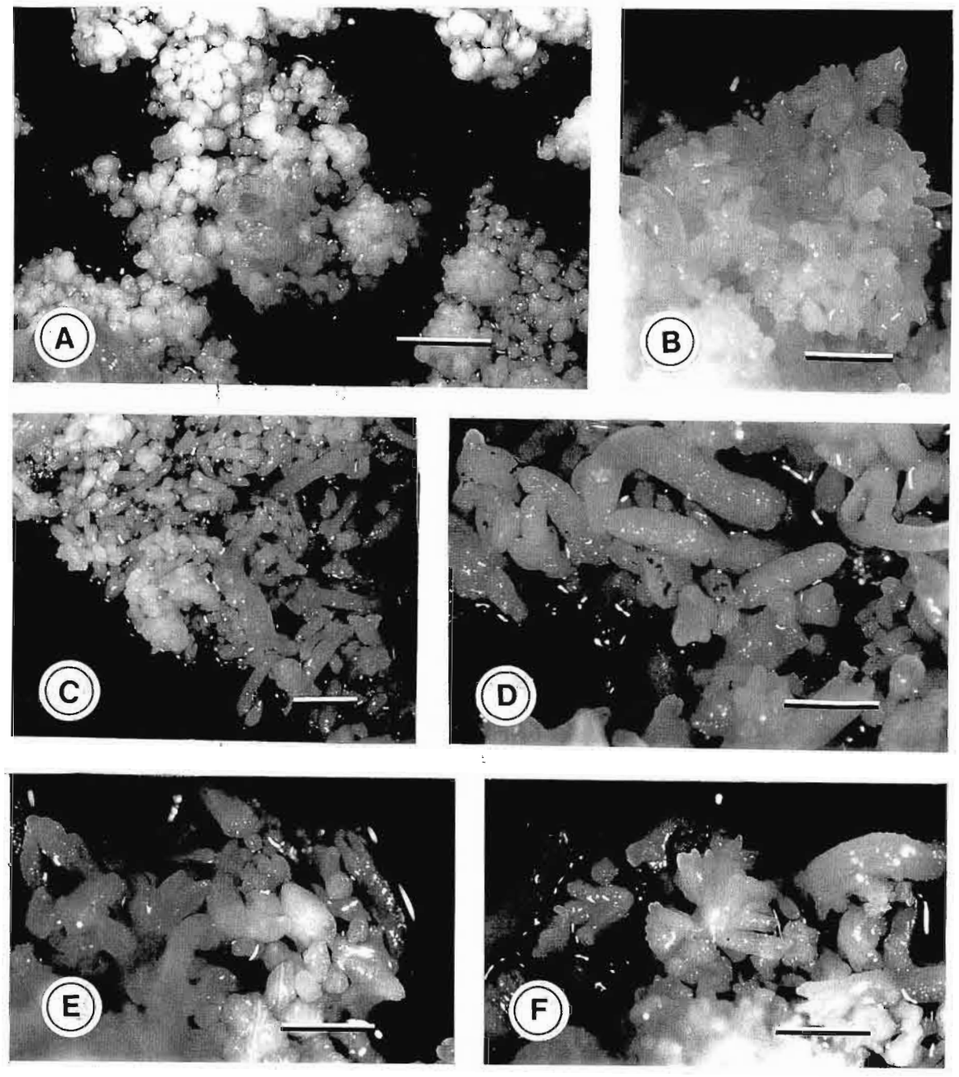

Figure 5.1: Somatic embryogenesis of *Daucus carota* induced with various concentrations of activated charcoal. (A): In 2,4-D-free medium in the absence of AC; (B, C, D): In 2,4-D-containing medium in the presence of 0.5% AC; (E, F): In 2,4-D-containing medium in the presence of 3.0% AC. Bar = 200 µM.

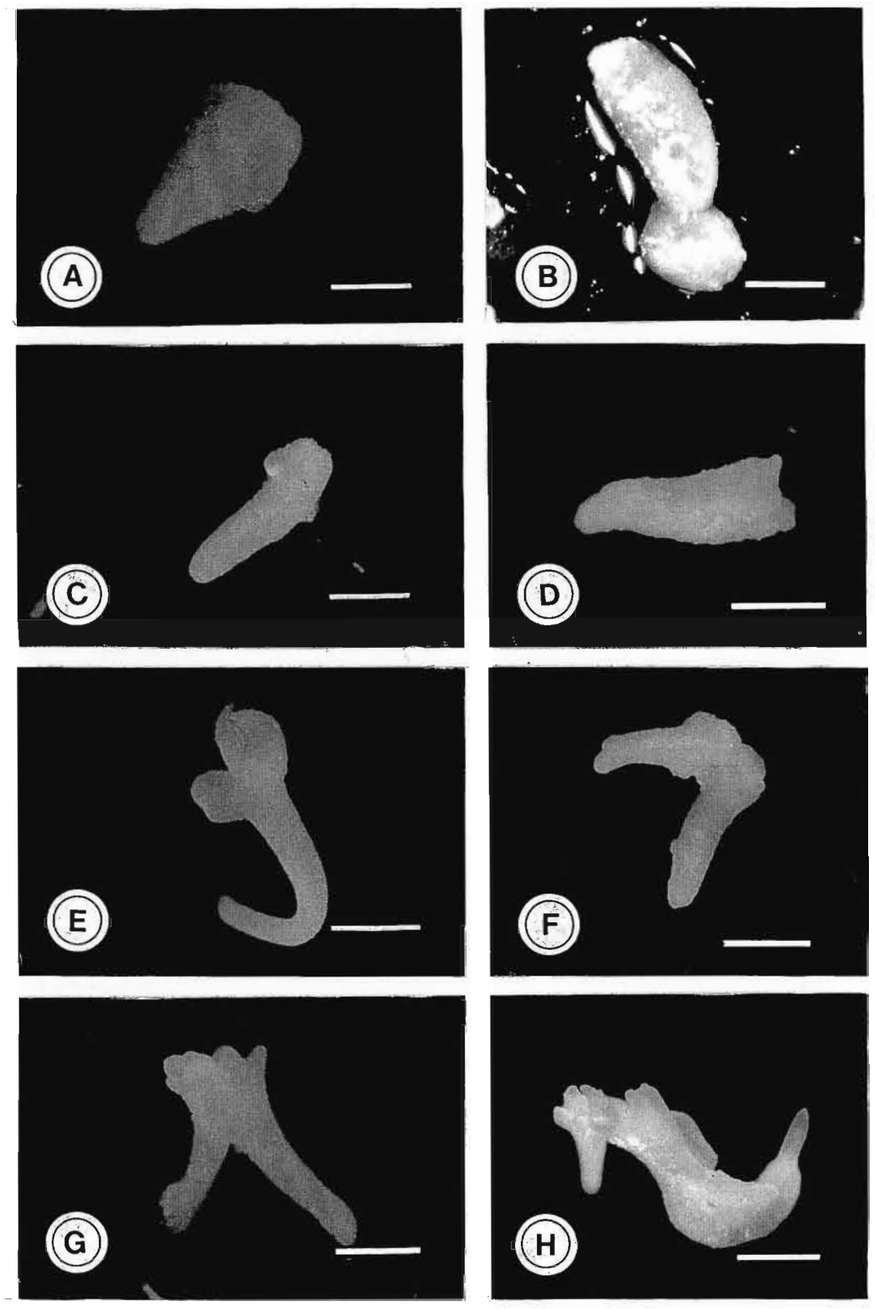

Figure 5.2: Somatic embryos of *Daucus carota* after culturing on media with various concentrations of AC showing normal and abnormal embryo development. On 0.01% AC-containing medium, globular embryos were observed but formation of the cotyledons did not occur (A,B,C). On the 0.1% AC-containing medium, cotyledons did develop but the radicles and hypocotyls did not elongate (D). Elongation of the embryos did occur with 0.5% AC in the medium (E). Abnormal embryos with multiple cotyledons developed on the 3.0% AC-containing medium (F,G,H). Bar = 350 μM.

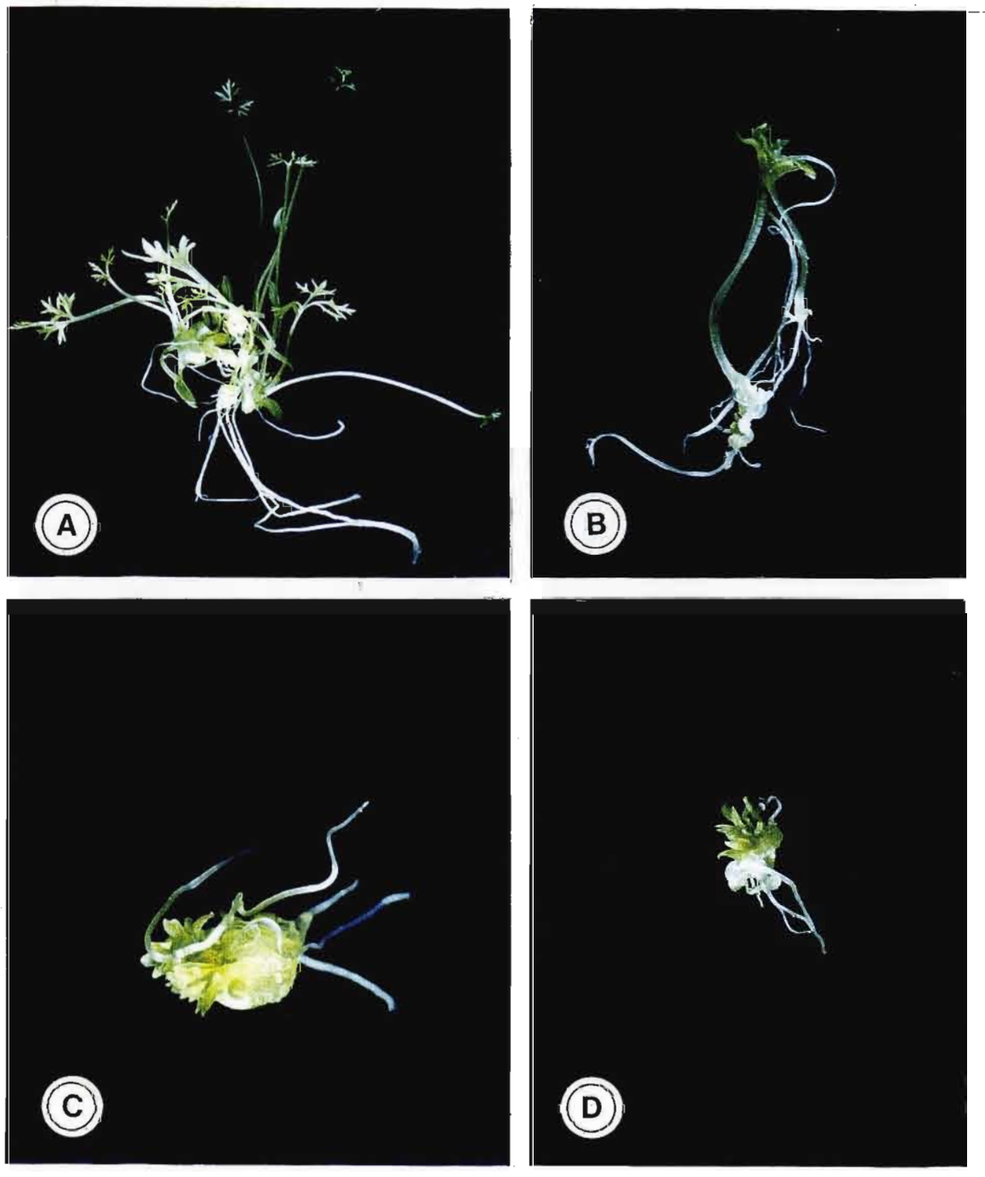

Figure 5.3: Regenerated *Daucus carota* plantlets from somatic embryos grown on different concentrations of activated charcoal. A: 0.5% AC treatment; B, C, and D: 3.0% AC treatment.

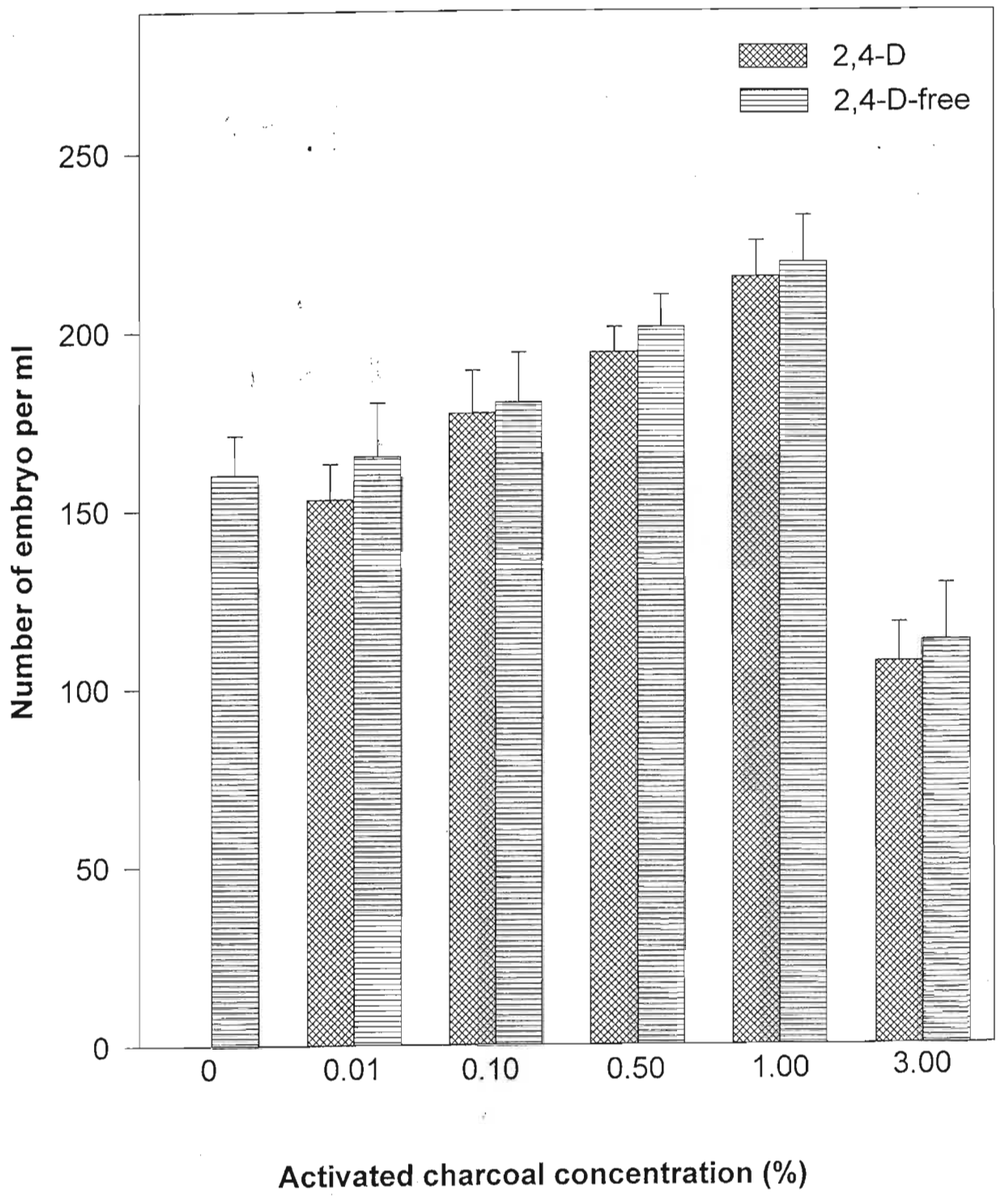

Figure 5.4: Number of *Daucus carota* somatic embryos produced from tissue in MS liquid media with various concentrations of activated charcoal in the presence or absence of 2,4-D.

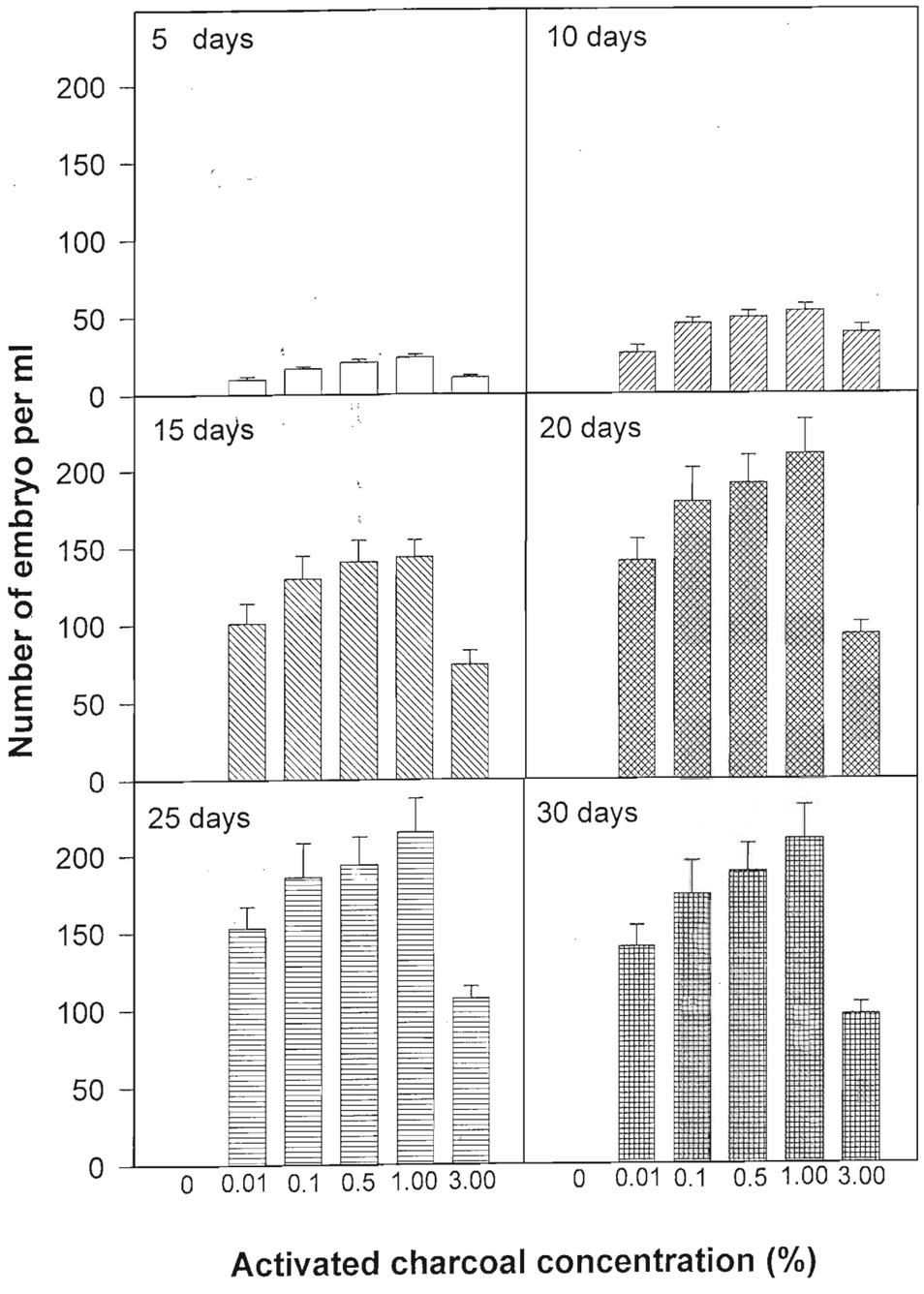

Figure 5.5: Mean number of *Daucus carota* somatic embryos produced over time course per unit volume of the activated charcoal-containing culture medium.

Figure 5.4 show the number of total somatic embryos produced in suspension culture when the embryogenic callus was suspended in MS medium supplemented with 0.5 mg l^{-1} 2,4-D in the presence of various concentrations of AC. Within the lower range of AC concentrations used (up to 1.0%) the number of total embryos, which included globular, heart and torpedo shape-embryos, increased with increasing AC concentration used. However, addition of 1.0% AC to the medium resulted in a decrease in the total number of embryos. Production of somatic embryos also resulted when the callus was culture on a 2,4-D-free MS media in the presence or absence of AC (Figure 5.4). There was no significant differences in the total number of somatic embryos between in 2,4-D-free MS media in the presence or absence of AC (Figure 5.4).

Figure 5.5 shows the effect of AC concentration on carrot somatic embryogenesis in terms of the number of somatic embryos in suspension culture with time. The rate of increase in the total number of somatic embryos was affected by the AC concentration, as shown by the similarity of the curves for all treatments. The total number of somatic embryos reached a constant level after day 20 for all treatments, with the exception of the control treatment. The day at which somatic embryos began to appear also was not affected by the AC treatment. The number of somatic embryos increased with increased AC concentration when the range of AC concentration was raised upwards from 0.01% to 1.0% in the medium. The number of somatic embryos increased clearly at high AC concentration (0.05% and 1.0% AC). However, The number of embryos decreased with the 3.0% AC treatment . Only half the number of embryos were observed in the suspension treated with 3.0% AC compared to 1.0% AC.

5.4 Discussion

The differentiation of carrot cells into somatic embryos occurred when carrot callus was cultured on a 2,4-D-containing medium in the presence of AC, but not on the 2,4-D-containing medium in the absence of AC. This seems to be in agreement with the fact that embryogenesis in carrot cell cultures is inhibited by high levels of auxin (REINERT 1959). It seems that the effects of AC is mainly due to the removal of substances such as 2,4-D from the medium. It was confirmed that 2,4-D is effectively adsorbed by AC from aqueous solutions (Chapter 3). Activated charcoal is known to have the ability to adsorb certain growth regulators that are added to the medium such as NAA, cytokinins (WEATHERHEAD *et al.* 1978) and ABA (JOHANSSON *et al.* 1982). The process of carrot somatic embryogenesis is initiated in media containing high levels of 2,4-D, the embryos do not develop until the 2,4-D concentration is reduced. Thus, if the embryos produce one or several hormones that are unnecessary for embryo growth and these substances diffuse into the medium the added AC could adsorb and remove these substances from medium.

The results presented here also showed that addition of 0.01 to 1.0% AC increased the total number of carrot somatic embryos in suspension cultures in both 2,4-D-containing and 2,4-D-free media. This suggested that embryogenesis of *Daucus carota* was dependent on the AC concentration used in the medium. This is in agreement in that AC concentration was an important factor for embryogenesis in *Datura innoxia* and *Solanum tuberosum* (SOPORY *et al.* 1978;

TYAGI *et al.* 1980). The present results are in contrast to those obtained by Wernicke and Kohlenbach (1976) and Johansson and Eriksson (1977) which showed that embryogenesis in *Nicotiana tabacum* and *Anemone virginiana* was relatively independent of AC concentration in the culture medium. However, addition of a high concentration of AC (3.0%) to the medium caused negative effects on embryogenesis. A high concentration of AC inhibited somatic embryogenesis. The somatic embryo response to AC concentration may be dependent on plant species, cell density, cell cluster size and cell line used and it also may be related to the fact that AC was able to adsorb nutrient components in the culture medium. This could resulted in a nutrient deficiency. The nutrient deficiency could stop further embryogenesis if not compensated for by transfer of the cultures to fresh medium. Many materials are adsorbed or desorped to colloidal soil particles and AC acts similarly (PROSKAUER and BERMAN 1970). Desorption from AC is generally a very slow process depending on solvent and solution conditions.

A number of late-stage embryos appeared on the AC-containing media with or without 2,4-D. It seems that AC also provided a major benefit in the progression of later embryo stages in *Daucus carota* culture. Several factors can influence somatic embryo formation such as cell density and chemical substances which may be secreted into the culture medium from the cells (HANAI *et al.* 2000). Activated charcoal is able to remove 2,4-D from the medium which is either added to the medium or produced by the cells and excreted to the medium. It also adsorb inhibitory substances such as 5-hydroxymethyl-2-furaldehyde, ethylene and

phenolic compounds (BON *et al* 1988; EBERT and TAYLOR 1990; FRIDBORG *et al.* 1978; HORNER *et al.* 1977; THEANDER and NELSON 1988; WEATHERHEAD *et al.* 1978, 1979) produced by medium and cultures/embryos, that may inhibit embryogenesis and further embryo development. Addition of an appropriate concentration of AC to the culture medium may result in adsorption of substances produced by the cultures and thus promote somatic embryogenesis.

CHAPTER SIX

General conclusions

Activated charcoal (AC) is prepared by the controlled carbonisation of wood in steam or air and has been used as an adsorbent for many years. The adsorbent properties of different AC has been exploited in many research fields such as pharmacy and engineering. Activated charcoal is used in tissue culture media to improve *in vitro* growth. Addition of AC to plant tissue culture media may be either beneficial or harmful, especially on organogenesis and embryogenesis. These effects may be attributed to (a) provision of a dark environment in the medium; (b) adsorption of certain inhibitory substances in culture, produced by either media or explants; (c) adsorption of plant growth regulators and other organic compounds; (d) the release of substances naturally present in or adsorbed by AC, which are beneficial to growth of *in vitro* culture; and (e) improving aeration of the culture medium. Activated charcoal is not a growth regulator, but it is commonly used *in vitro* culture, and addition of an effective concentration of AC to media improves or regulates plant growth *in vitro*.

Many grades of AC are available from chemical suppliers. An important factor of AC is the surface area per gram of charcoal. Grades with large surface areas are more promising for culture media. As many of the beneficial effects of AC depends on its ability to adsorb a wide range of compounds, results from even the

most effective brands are liable to be unpredictable. Effects of charcoal on tissue response in *in vitro* culture appear to be dependent not only on the kind of AC and their degree of activation, but also on the plant species cultured. The addition of AC to tissue culture media may have either a beneficial or an adverse effect on growth and development, depending upon the medium, tissue used, and/or objective(s) of the researcher.

It was noticed that addition of AC to the medium resulted in altering the media pH and this subsequently increased sucrose hydrolysis in culture medium upon autoclaving. The pH can influence culture growth and morphogenesis. It is probable that at least some of the effects of AC on plant cultures can be attributed to the changes related to pH of the medium and the sugar content (degree of hydrolysis) of the culture medium.

There is evidence for the adsorption of 2,4-D from methanol or aqueous solutions. The levels of the added 2,4-D decreased in both methanol and aqueous solutions in the presence of activated charcoal, compared with those in the absence of AC. Addition of AC to MS-salts solutions resulted in changes of the concentrations of inorganic elements. The results suggested that AC adsorbed magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn) and released potassium (K), copper (Cu), manganese (Mn), phosphorus (P) and sulphur (S).

Addition of AC to culture medium improved the potential for *in vitro* rooting and somatic embryogenesis of *Daucus carota*. The present results suggest that the stimulatory effect of AC on *in vitro* rooting and somatic embryogenesis is

particularly associated with the AC concentrations used. The beneficial effect of AC on root and somatic embryo production is mainly, but probably not entirely, due to the adsorption of 2,4-D by AC. Darkness which can be provided by addition of AC to culture medium may be beneficial to *in vitro* rooting, especially the root inductive phase. The present results showed that the hypocotyls of *Daucus carota* produced roots polarly on the NAA/IAA- containing media in the presence of AC and no-polarity of root formation was observed on media supplemented with NAA/IAA in the absence of AC. This suggested that AC might have an affect on auxin transport. It requires further investigation.

Although further investigations of the mechanism(s) of the effect of AC on *in vitro* culture are required for a better understanding of the roles of AC on interactions with growth regulators, the physical, chemical and biochemical properties of AC may well be of primary importance in rooting and embryogenesis of explants. The utilization of AC in tissue culture is of major importance for purposes of micropropagation, the production of phytochemicals and the establishment of transgenic plants.

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APPENDIX 1. NUTRITIONAL COMPONENTS OF PLANT TISSUE CULTURE MEDIA

B5 (GAMBORG *et al.* 1968) medium composition and preparation.

Constituent	Concentration of Stock solution (mg/litre)	Volume of stock per litre of medium (ml)
Major inorganic		
$MgSO_4.7H_2O$	5 000 mg	
$NaH_2PO4.H_2O$	3 000 mg	
KNO_3	50 000 mg	50
$CaCl_2.2H_2O$	3 000mg	
$(NH4)_2SO_4$	2 680 mg	
Trace elements		
H_3BO_3	600 mg	
$MnSO_4.H_2O$	20 000 mg	
$ZnSO_4.7H_2O$	400 mg	
$Na_2MoO_4.2H_2O$	50 mg	5
$CuSO_4.5H_2O$	5 mg	
$CoCl_2.6H_2O$	5 mg	
KI	150 mg	
Iron source		
$Na_2EDTA.2H_2O$	7 460 mg	5
EDTA Na ferric salt	8 600 mg	
Organic supplement		
Thiamine HCl	2 000 mg	
Pyridoxine HCl	200 mg	5
Nicotinic acid	200 mg	
Myo-inositol	20 000 mg	
Carbon source		
Sucrose	30 g	

adjusted pH to 5.5

Murashige and Skoog (1962) medium composition and preparation.

Constituent	Concentration of Stock solution (mg/litre)	Volume of stock per litre of medium (ml)
Major inorganic nutrients		
$MgSO_4.7H_2O$	7 400 mg	
KH_2PO_4	3 400 mg	
KNO_3	3 800 mg	50
NH_4NO_3	33 000 mg	
$CaCl_2.2H_2O$	8 800 mg	
Trace elements		
H_3BO_3	1 240 mg	
$MnSO_4.4H_2O$	4 460 mg	
$ZnSO_4.7H_2O$	1 720 mg	
$Na_2MoO_4.2H_2O$	50 mg	5
$CuSO_4.5H_2O$	5 mg	
$CoCl_2.6H_2O$	5 mg	
KI	166 mg	
Iron source		
$FeSO_4.7H_2O$	5 560 mg	5
$Na_2EDTA.2H_2O$	7 460 mg	
Organic supplement		
Thiamine HCl	100 mg	
Pyridoxine HCl	100 mg	
Nicotinic acid	100 mg	5
Myo-inositol	20 000 mg	
Glycine	400 mg	
Carbon source		
Sucrose	30 g	

adjusted pH to 5.8

All media were autoclaved prior to use.

B5 and MS media were solidified with 10 g bacteriological agar per liter.