National Library of Canada

Bibliothèque nationale du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, tests publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

NL-339 (r. 88/04)

Canada

In-vitro Propagation of *Capsicum annuum* cv. Early Prolific Hybrid Pepper and Evaluation of Regenerated Plants

Margaret Anne Cooke

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada

December 1987

© Margaret Anne Cooke, 1987

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-41628-9

ABSTRACT

IN-VITRO PROPAGATION OF CAPSICUM ANNUUM CV. EARLY PROLIFIC HYBRID PEPPER AND EVALUATION OF REGENERATED PLANTS

Margaret Anne Cooke

Cotyledon, hypocotyl, cotyledonary axil, and apical meristem explants of *Capsicum annuum* cv. Early Prolific Hybrid Pepper were excised from aseptically germinated seedlings and cultured on Murashige and Skoog (MS) basal nutrient medium supplemented with auxins, cytokinins and auxin-cytokinin combinations. Shoots were formed on cotyledons cultured on media containing BAP (2-20 mg/l) and IAA (0-4 mg/l), while media containing NAA alone, or in combination with equal concentrations of BAP, induced root formation in this explant. Roots were also produced on hypocotyl explants cultured on media containing IAA or NAA alone, or when BAP was present with the auxin. Shoot formation, followed by root development, was observed when cotyledonary axil explants were cultured in medium containing 1.0 mg/l IAA. When cultures were incubated

at 18, 22 or 28°c, and 16 or 24-hour photoperiods, enhanced organogenesis was observed when explants were cultured under a 16-hour photoperiod at 28°c. When multiple shoots were obtained on cotyledon and apical meristem explants, the addition of GA_3 to growth media, or culturing explants in the dark, failed to promote elongation of multiple shoots. Plantlets obtained from cotyledonary axil explants produced earlier, and greater, yields than parent stock under both greenhouse and field conditions.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to the following people who assisted in the realization of this thesis:

My supervisor, Dr. B.S. Mangat, and committee members, Dr. R. Ibrahim and Dr. P. Widden, for their guidance and encouragement throughout the course of this research; Dr. Paul Widden, for his invaluable time and direction in statistical analysis; Dr. N. Kapoor, for his assistance in photography; Mr. Hervé De La Fouchardière, for his assistance in the greenhouse; Ferme Yvon Prud'homme, St. Eustache, Que., for allowing field trials to be realized; My colleagues, Ann Greer and Mihir Roy for their constant moral support; John, for his ever-present love and support throughout this project; My mother, for her enduring love and understanding; My uncle, H. Stuart Cooke, for instilling in me a wonderment and respect for nature.

This research was supported in part by a graduate fellowship awarded by Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR).

To my Father (1900-1985)

TREES ARE MY BROTHERS

Trees are my brothers
Created by the same Master Hand.
When I am weak,
I stand among them.
Beholding their strength
Bred through resistance
In a thousand storms,
I, too, feel strong.
Yet, lean they with the wind,
As I must learn to lean
Without a broken wound,
When winds of fate breathe strong.
Hearing the proud songs they sing
Through twisted branches,
My heart sings;
Sings for the sense of beauty,
Beauty of rock, and green, and sky,
Beauty of resilience.
I feel my soul stretching upward
Even as the trees -
My brothers - reach toward God.

Ione W. Lyall

TABLE OF CONTENTS

ABSTRACT .... (iii)

ACKNOWLEDGMENTS .... (v)

DEDICATION .... (vi)

LIST OF TABLES .... (x)

LIST OF FIGURES .... (xii)

ABBREVIATIONS .... (xiv)

INTRODUCTION 1

MATERIALS AND METHODS 27

Experimental Plants and Culture Techniques.

In-vitro Regeneration of *Capsicum annuum*.

Determination of growth regulator requirements for morphogenesis

Effect of temperature, photoperiod, and media manipulations on the morphogenesis of cultured explants

Regeneration from cotyledonary axil explants

Greenhouse and Field Evaluations of Regenerated and Parent Stock.

Micropropagation:

Elongation of multiple shoots

Organogenesis from callus tissue

RESULTS..45

In-vitro Regeneration of *Capsicum annuum*.

Determination of growth regulator requirements for morphogenesis
Effect of temperature, photoperiod, and media manipulations on the morphogenesis of cultured explants.

Regeneration from cotyledonary axil explants

Greenhouse and Field Evaluations of Regenerated and Parent Stock.

Micropropagation.

Elongation of multiple shoots

Organogenesis from callus tissue

DISCUSSION.....................................107

SUMMARY..129

REFERENCES.....................................132

APPENDIX I.....................................135

A partial list of basal nutrient media utilized in studies of plant tissue culture.

APPENDIX II....................................137

Morphological response intensity and % frequency of response of cotyledon, hypocotyl and cotyledonary axil explants to different media formulations, temperatures and photoperiods.

APPENDIX III..144

Results of stepwise multiple regression analysis performed on seperate treatment morphological response data of cotyledon, hypocotyl and cotyledonary axil explants cultured under different media, temperature and photoperiod conditions.

APPENDIX IV...148

Results of ONEWAY analyses indicating differences in greenhouse and field trials of individual plant types for parameters measured.

LIST OF TABLES

TABLE		PAGE
1	Formulation and stock solution preparation of MS basal nutrient medium	31
2	Media formulations used to determine growth regulator requirements for morphogenesis	33
3	Media formulations used to determine effects of temperature and photoperiod on the morphogenesis of cotyledon, hypocotyl and cotyledonary axil explants	34
4	Morphological response of cotyledons and hypocotyls to various media formulations	48
5	Results of HILOGLINEAR analysis of temperature and photoperiod manipulation experiments	69
6	Results of regression analyses of temperature and photoperiod manipulation experiments	72
7	Results of regression analyses of seperate responses in temperature and photoperiod manipulation experiments	73
8	Comparison of media formulation effects on cotyledonary axil response	75
9	Plantlet formation from cotyledonary axil explants	76
10	Success of plantlet formation and survival of regenerated plants	78
11	Morphological response of apical meristem explants	99

12	Shoot elongation induction in cotyledon explants	100
13	Shoot elongation induction in apical meristem explants	101
14	Callus culture of explants	106
15	A partial list of basal media utilized in plant tissue culture	135
16-21	Morphological response frequency and intensity of explants cultured under different media, temperature and photoperiod regimes	137
22-24	Results of regression analyses of treatment conditions; temperature and photoperiod manipulation experiments	144
25	Results of ONEWAY analyses indicating differences in greenhouse and field trials of individual plant types for parameters measured.	148

LIST OF FIGURES

FIGURE		PAGE
1	Molecular structures of commonly used Auxins	5
2	Molecular structures of commonly used Cytokinins	6
3	Infuser for surface sterilizing seeds	28
4-12	Graphs of morphological response of explants in temperature and photoperiod manipulation experiments	52
13	Effects of temperature and photoperiod on the shooting response of cotyledon explants	63
14	Effects of temperature on the rooting response of cotyledon and hypocotyl explants	65
15	Effects of temperature on organogenesis in cotyledonary axil explants	67
16-22	Graphs of growth and fruit yield data obtained from 2 generations of regenerated and seed plants	82
23	Comparison of F-1 seed and F-1 regenerated plant height in greenhouse conditions	90

24	Plants grown under field conditions	92
25	Comparison of F-1 seed and F-1 regenerated plants grown under field conditions	94
26	Organogenesis in apical meristem explant	102

ABBREVIATIONS

BAP	6-benzylaminopurine
GA_3	gibberellic acid
IAA	indole acetic acid
Kin	kinetin
NAA	napthalene acetic acid
Z	zeatin
2,4-D	2,4-dichlorophenoxyacetic acid

INTRODUCTION

Haberlandt (1902), as well as White (1934), Gautheret (1942) and others, first introduced individual plant parts to an *in-vitro* environment in an attempt to answer some questions related to plant growth and development. Since their pioneering efforts, plant tissue culture has evolved to become a major tool in studies involving the physiology of plant growth and differentiation, genetic manipulation, as well as a means for obtaining rapid plant regeneration, multiple plantlet formation (micropropagation) and disease-free stocks from a large number of plant species (Bhojwani and Razdan, 1983). Today the use of *in-vitro* techniques, and their application to important plant species may potentially lead to an increase in crop production, development of new crops and allow for manipulations which could enhance desirable characteristics and/or eliminate undesirable characteristics.

Generally, *in-vitro* propagation studies are carried out using explants (e.g. cotyledon, hypocotyl, petiole, leaf, stem and root segments, apical meristem

sections), callus tissue or protoplasts, which are aseptically cultured on media in which nutrient and/or growth regulator constituents have been manipulated. The liquid medium is most often solidified by the addition of agar, to provide a support for the explant (George and Sherrington, 1984; Bhojwani and Razdan, 1983).

The nutritional requirements of plant tissues *in-vitro* may vary with the species. While a simple medium containing only inorganic salts and a useable sugar may support some callus growth of blackberry and carrot (George and Sherrington, 1984), for example, most tissues require more complex nutrient mixtures. These may include vitamins, amino acids and plant growth regulators. The inorganic nutrients essential for plant growth and development are nitrogen, phosphorus, sulphur, calcium, potassium and magnesium, carbon, hydrogen and oxygen, which are required in relatively large amounts, and thus are often referred to as macro-elements. Other elements are essential in only small amounts and are thus termed micro-elements.

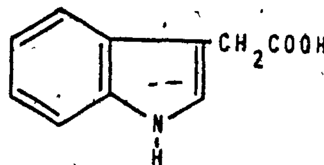
These include iron, manganese, copper, zinc, boron and molybdenum (George and Sherrington, 1984).

Although most cultured plant cells are able to synthesize all required vitamins, they tend to do so in sub-optimal quantities (Bhojwani and Razdan, 1983). Thus, to achieve optimum tissue growth it is often necessary to supplement the medium with one or more vitamins and amino-acids (George and Sherrington, 1984). Thiamine (Vitamin B_1), pyridoxine (Vitamin B_6), nicotinic acid (Vitamin B_3), and inositol are often added to fulfill this requirement. Casein hydrolysate, in most cases, is added to ensure that amino-acid requirements are satisfied (Bhojwani and Razdan, 1983; Linsmaier and Skoog, 1965).

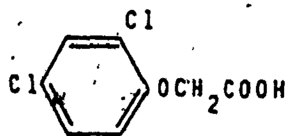
Since excised plant tissues do not photosynthesize, or do so to a limited extent, it is essential that a useable carbon source be supplied to the culture medium (George and Sherrington, 1984). Sucrose, the most commonly used carbon source, is supplied at a concentration of 2-5%. Glucose and fructose have also been utilized in the culture of some plant tissue (Bhojwani and Razdan, 1983; Van, 1976) (A

partial list of basal nutrient media utilized in studies of plant tissue culture is presented in Appendix I).

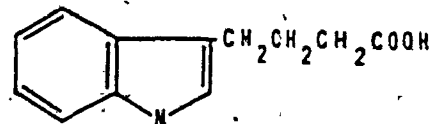
Notwithstanding the importance of nutritional requirements of cultured explants, Skoog and Miller (1957) showed the importance of plant hormones to organ formation on plant tissues cultured in-vitro. They reported that plant growth substances, such as auxins, cytokinins, and gibberellins, are required for organ formation and growth. Among the auxins most often used are indole acetic acid (IAA), a naturally occuring compound, and napthalene acetic acid (NAA), indole butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D) which are all synthetic compounds (Fig. 1). Some of the most common cytokinins include Zeatin (Z), 6-benzylaminopurine (BAP) and Kinetin (Kin) (Fig. 2). (Skoog and Miller, 1957). Auxins are generally administered if cell division and/or root differentiation is desired. Cytokinins are incorporated mainly for cell division and shoot differentiation (Bhojwani and Razdan, 1983).



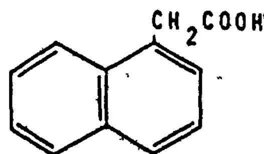
IAA MW 175.19



2,4-D MW 221.04

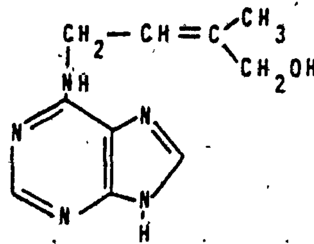


IBA MW 203.24

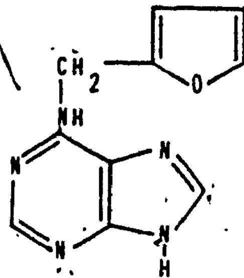


NAA MW 186.21

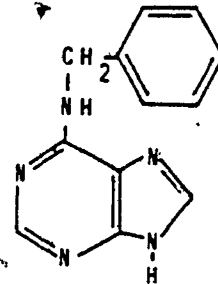
Figure 1. Molecular structure of commonly used auxins. Indole acetic acid (IAA) is a naturally occuring compound. 2,4-dichlorophenoxyacetic acid (2,4-D), indole butric acid (IBA) and napthalene acetic acid (NAA) are synthetic compounds.



Zeatin MW 219.25



Kinetin MW 215.22



BAP MW 225.26

Figure 2. Molecular structure of commonly used cytokinins. (BAP = 6-benzylaminopurine).

Of the over 20 known gibberellins, GA_3 is generally used, but compared to auxins and cytokinins, gibberellins are used only rarely, and usually only to stimulate the normal development of plantlets from embryos (George and Sherrington, 1984).

Apart from the requirements of individual growth regulators, Skoog and Miller (1957) reported that differentiation of roots and shoots in tobacco pith culture was a function of auxin/cytokinin ratios. These investigators showed that differentiation of shoots and roots could be regulated by changing the relative concentrations of auxin and cytokinin in the medium. Through a series of experiments it was determined that a high ratio of auxin to cytokinin promoted rooting, whereas a high ratio of cytokinin to auxin tended to stimulate the onset of shoot growth. When equal concentrations of auxin and cytokinin were utilized, the tissue tended to produce callus. (Skoog and Miller, 1957).

This concept, that organogenesis is controlled by hormonal regulation, is now applicable to the in-vitro culture of most plant species. However, since endogenous levels of growth regulators contribute to the ratio of auxin to cytokinin, the exogenous concentrations required will be different for each plant, as well as for each explant of the same plant. Depending on the plant and tissue being cultured, different media combinations may promote only callus tissue formation, root initiation, or induction of only shoots. It has been reported that a single medium sometimes may generate callus, roots and shoots. (George and Sherrington, 1984). For example, Wright et.al (1986) found that nodal explants of *Glycine max* (soybean) initiated multiple shoots when cultured on Murashige and Skoog's (1962) basal medium (MS basal medium) supplemented with 1 mg/l BAP. Subsequent rooting occured in basal medium containing no exogenous growth regulators. Kurtz and Lineberger (1983) studied the different culture requirements of leaf explants from genotypically different tomato cultivars. Optimum shooting response of 'Better Boy' and 'VC 134-1-2' was

obtained on MS basal medium supplemented with 5 mg/l BAP and 0.2 mg/l IAA. For the 'Starfire' cultivar, 2.5 mg/l BAP and 1.0 mg/l IAA proved the optimum combination for shoot induction. All cultivars formed roots when placed on a medium supplemented with only 0.2-2.0 mg/l IAA (Kurtz and Lineberger, 1983). An MS basal medium supplemented with 1.0 mg/l NAA and 0.01 mg/l BAP was found optimum for callus, root and shoot formation in lateral bud culture of the Elephant Yam (Arditti and Nyman, 1985). Leaf sections of African Violet produced adventitious shoots when cultured on MS basal medium supplemented with 1.01 mg/l NAA and 5 mg/l BAP while rooting occured on MS basal medium alone (Start and Cumming, 1976).

Apart from media formulations, temperature and light also play an important role in the organogenesis of plant tissue *in vitro*. (George and Sherrington, 1984). In the majority of the literature reviewed, a temperature of 25°c was generally used. In a study performed by Diamantoglou and Mitrakos (1979), 70% of olive embryos developed into plantlets at 25°c, whereas the success rate was more than halved at 15, 20 or

30°c. However other temperatures have also been used. Waikey and Woolfitt (1970) showed that the best plantlet growth from cauliflower floral meristems occured at 18°c as compared to 22°c. Favre (1977) demonstrated that leaf blade explants of _Vitis_ grew best at 29°c, did not grow at 20°c, and survived only a short while at 32 and 34°c.

Although most explants placed in culture are green, as are growing shoots derived from those explants, they do not photosynthesise. Instead, they grow as heterotrophs, deriving all their nourishment from the medium in which they are cultured. However, in most _in-vitro_ studies, light has been shown to be important. In plant tissue cultures, light is most important for its effects on photomorphogenesis, a process by which absorption of light energy effects morphogenesis. (George and Sherrington, 1984).

Basically, three qualities of light influence the growth and morphogenesis of plant tissues cultured _in-vitro_. These are wavelength, flux density (intensity), and photoperiod (the duration of light exposure). As with other factors, there is a marked

response difference with respect to light in tissues of different plant species. (George and Sherrington, 1984).

Seibert et al.(1975) have shown that the combined effect of both intensity and wavelength are important in callus growth and morphogenesis, and that of the wavelengths tested, near U.V. light and blue light can have a controlling effect on the rate of growth and morphogenesis of tobacco callus cultures. These investigators demonstrated that, although the callus tissue would grow in darkness, near U.V. light of 371nm at 90 lux stimulated growth and promoted shoot formation. This response was inhibited when intensity was increased above 540 lux. However, when blue light of 420nm or 467nm was employed, maximum tissue response was seen at 1080 and 2160 lux respectively. Seibert et al. (1975) have reported that growth and morphogenesis of their tobacco callus was stimulated by low irradiance of blue or near U.V. monochromatic light. But when fluorescent tubes emitting white light (thus light containing many different wavelengths) were compared, the best response was obtained when the light

source emitted a considerable portion of total light energy in the red portion of the spectrum (600-700nm). (George and Sherrington, 1984).

Light intensity takes into consideration the amount of light energy incident upon a plant tissue in culture. Veda and Torika (1972) found that green, photosynthetic Cymbidium protocorms cultured in darkness or in light of 1250 lux gave rise to shoots, while both roots and shoots were produced in light of 2200-2500 lux. Conversely, De Fossard et al. (1978) observed that high light intensity was inhibitory to rooting of shoots produced from Eucalyptus ficifolia cultures. In this case rooting was observed to be best in darkness. (George and Sherrington, 1984).

Photoperiod refers to the length of time the culture is exposed to light. For example, a 16-hour photoperiod would indicate a regime where, within a 24-hour period, the culture would be exposed to 16 hours of light and then 8 hours of darkness. Both callus growth and adventitious shoot formation may be influenced by the photoperiod under which tissue cultures are maintained. Shoot morphogenesis is

usually favored by light. Many callus tissue may grow equally as well in darkness as in light, but require light to initiate shoot buds. (George and Sherrington, 1984).

Callus obtained from shoot meristem cultures of lentil, after being incubated in darkness for 4 weeks, produced shoots when transferred to a 16-hour photoperiod regime (Williams and McHugen, 1986). Similarly, meristem tip cultures of red raspberry (Donnelly et al.,1985) and leaf segments of several tomato cultivars (Kurtz and Lineberger, 1983) produced adventitious shoots under a 16-hour light regime. A photoperiod of 12 hours of light was sufficient for shoot induction in cotyledonary nodes of soybean (Wright, et al.,1986), lateral buds of Elephant Yam (Arditti and Nyman, 1986), and leaf segments of African Violet (Start and Cumming, 1976).

By utilizing currently available information, methods and principles, criteria have been established for the successful in-vitro propagation of many plant species. Notwithstanding the importance of basic nutritional and environmental factors, the relationship

of auxin-cytokinin ratios to shoot and root initiation, remains at the foundation of *in-vitro* plant regeneration. However, success in the regeneration of other plant species by extension of established criteria may not be realized by simply duplicating previously successful methods (Murashige, 1976). Nevertheless, several investigators have reported varying degrees of success in the regeneration and micropropagation of *Capsicum*.

George and Narayanaswamy (1973) first attempted the *in-vitro* culture of *Capsicum annuum* using pollen grains of the chile pepper 'Sendt'. The pollen was cultured on Linsmaier and Skoog basal medium supplemented with coconut milk, yeast extract, casein hydrolysate, auxins and/or cytokinins, and incubated at 25°c under constant light conditions. Addition of auxin was necessary for the initiation of cell division and development of an embryoid-type of growth. Mature embryos capable of plantlet formation occurred in only one in a thousand anthers cultured, when myo-inositol and casein hydrolysate were added to basal medium (George and Narayanaswamy, 1973).

Gunay and Rao (1978) examined the regeneration of Red Pepper from hypocotyl and cotyledon sections. The explants from two cultivars of Capsicum annuum (California Wonder and Pimento), and a hybrid variety of Capsicum frutescens (Barath) were cultured on MS basal medium supplemented with auxins (IAA, NAA, or 2,4-D), and cytokinins (BAP, Z, or Kin) or coconut milk, and incubated at 25°c under continuous light conditions. When 2,4-D was added to the medium vigorous callus growth occurred in the explants of all varieties tested. Root formation occurred upon the addition of IAA alone or NAA alone, with characteristic differences observed between the roots formed with IAA (long and thin) and NAA (short and thick, with fine network of root hairs). The explants of the three varieties tested also showed differences in shoot initiation with respect to the cytokinins incorporated into the medium. The addition of Kin (1 mg/l) produced only callus formation, while Z (1 mg/l) occasionally promoted shoot bud formation in the cotyledons of 'Pimento' and 'Barath'. BAP (1 mg/l) induced both callus and shoot formation in cotyledon explants of 'California Wonder' and 'Barath' and in hypocotyls of

'Pimento', but when the concentration of BAP was increased to 2 mg/l, shoot bud formation occurred in the cotyledons of all three varieties. (Gunay and Rao, 1978).

When auxin and cytokinin were used in combination with each other, Gunay and Rao (1978) obtained different results. Upon the addition of IAA (1 mg/l) and BAP (2 mg/l) shoot buds developed in cotyledons of Barath, and callus and root initiation was observed in hypocotyls of this hybrid variety. The shoot buds developed roots upon transfer to medium containing IAA (0.5 mg/l). California Wonder and Pimento developed shoot buds, followed by rooting when IAA (1 mg/l) and BAP (2 mg/l) were incorporated into the medium. A moderate amount of callus growth accompanied the morphogenic response in all three varieties. The shoot buds obtained on this medium grew into complete plantlets within 6 weeks after bud initiation. BAP was found to be more effective than other cytokinins tested for its shoot inducing capacity. When NAA was used in place of IAA, in combination with BAP, shoot bud

formation was completely suppressed. (Gunay and Rao, 1978).

Fari and Czako (1981) investigated the regenerative ability of hypocotyl segments of a *Capsicum annuum* variety, T. Hatvani, using a medium established by Gunay and Rao (1978), containing 2mg/l BAP and 1mg/l IAA. Hypocotyls were cut into 6 equal-length segments from apex to base, and monitored over a 28-day incubation period. Explants were cultured at 25°c with a 19-hour photoperiod of 7000 lux fluorescent light. The temperature of the darkness period was lowered to 21-22°c. Callus growth was observed on all segments, while rooting intensity showed an almost linear increase from apical segment to basal segment. Both shoots and roots were formed in the first three apical sections. Because root formation in these segments was accompanied by callus growth, the shoots were dissected from root and callus, and cultured on MS basal medium supplemented with 0.1 mg/l NAA and 0.05 mg/l BAP, where vigorous rooting and elongation of shoot buds occurred. The resulting

normal plantlets were successfully grown to maturity in greenhouse conditions (Fari and Czako, 1981).

Saxena, et al (1981) isolated and cultured protoplasts of Capsicum annuum. Protoplasts were isolated from mesophyll cells of the California Wonder cultivar, and cultured in Durand (1973) or Nagata and Takebe (1971) basal nutrient medium supplemented with 1 mg/l each, of 2,4-D, NAA and BAP, and 2% sucrose and 0.5 M mannitol. They were incubated at 25°c in the dark, for 15 days. The tiny cell clusters which subsequently developed, underwent rapid callus proliferation when transferred to 0.25 M mannitol and light (3,000 lux) conditions. Shoot morphogenesis occurred when calli were transferred to MS medium supplemented with IAA (4 mg/l) and Kinetin (2.56 mg/l). The shoots formed roots when they were transferred to MS medium supplemented with IAA (1 mg/l) and Kinetin (0.04 mg/l). Plantlets were formed which flowered after 5 months under a 16-hour photoperiod regime. (Saxena, et al, 1981).

Agrawal and Chandra (1983) reported success in the regeneration of Capsicum annuum from embryos cultured

in-vitro. Embryos of Mathania were excised and cultured on MS medium supplemented with Kinetin and BAP, alone, or in combination with IAA and 2,4-D. Cultures were incubated at 26°c in continuous light. Media containing 2,4-D (0.5-1 mg/l) alone or with Kinetin (0.5 mg/l) induced callus growth on the embryos. Roots were formed on a medium supplemented with Kinetin (0.5 mg/l) and IAA (3-5 mg/l). Numerous shoot buds formed on the margins of the expanded cotyledons when the media contained Kin or BAP (5 mg/l) alone, or in combination with IAA (0.5-1 mg/l). These buds proliferated furthur when subcultured to a medium supplemented with BAP (5 mg/l), but their size remained small. Upon subculture to a medium containing NAA (0.1 mg/l) roots formed and the growth of shoot buds was apparent. (Agrawal and Chandra, 1983).

Phillips and Hubstenberger (1985) performed an extensive study on the regenerative ability of four varieties of Capsicum annuum. These included California Wonder, Yolo Wonder (sweet bell-type peppers), New Mexico No.6-4 and NuMex r. Naky (long green chile types). Contrary to previous reports, all

cultivars were found to respond in the same manner in each treatment condition. Explant tissues included hypocotyl sections, distal cotyledon pieces (non-meristematic explants), shoot tips and cotyledonary nodes (meristematic explants). Explants were cultured in MS basal medium supplemented with IAA (0.01-10.0mg/l), IBA (0.01-1.0mg/l), BAP (0.02-50.0mg/l) and/or Kin (0.01-3.0mg/l). Incubation environments were varied with temperatures of 25 or 28.5°c and photoperiods of 12, 16 or 24-hours.

Meristematic explants showed a 2- to 10-fold greater capacity for shoot initiation than did non-meristematic explants, which exhibited a greater capacity for root organogenesis. Shooting was found to occur in the presence of BAP, with or without IAA or IBA, and in contrast to the findings of Gunay and Rao (1978), it was noted that cytokinin (BAP) was important for rooting. In this study, when cytokinin was absent organogenesis was not observed. While high BAP/low IAA combinations induced the highest frequency of adventitious bud formation, 0.05 mg/l each of IAA and BAP were required for shoot elongation. This latter

combination of growth regulators was also found to be optimum for root formation (Phillips and Hubstenberger, 1985). Thus, these investigators established that IAA and BAP were the best growth regulators for use in *Capsicum* tissue culture.

Phillips and Hubstenberger (1985) also evaluated environmental incubation variables for their effects on morphogenesis in long-term cultures of pepper (cultures were observed for 9 monthly passages). In general, shooting frequencies from meristematic explants increased as temperature and photoperiod increased. Non-meristematic explants showed the highest shooting responses at 25°c, 16-hour photoperiod, and 28.5°c, continuous light. Warmer temperatures (28.5°c) and continuous light were found to maintain shoot organogenesis over an extended time period compared to the other incubation environments tested. The highest frequency of root organogenesis in non-meristematic explants was observed under 16-hour photoperiod regimes at 25°c, and in meristematic explants under continuous light at either 25°c or 28.5°c (Phillips and Hubstenberger, 1985).

Campiotti, et al. (1986), attempted _in-vitro_ culture of shoot apices from three _Capsicum_ varieties (Golia, Quadrato d'Asti, and Cornodi Toro). After 30 days of culture in MS basal medium supplemented with 1 mg/l IAA, 100% of the apices regenerated roots. With 2.0-4.0 mg/l BAP and 0.05 mg/l IAA, the axillary buds began to grow and adventitious shoots, as well as callus, were generated. However, after the 30-day culture period, the adventitious shoots remained small in size. In an effort to promote shoot elongation the tissue was transferred either to fresh medium of the same concentration, or to a new medium supplemented with GA_3 (up to 1 mg/l). GA_3 has been regarded as a growth regulator involved in the promotion of cell elongation (Salisbury and Ross, 1978). This resulted in a large amount of callus being formed on the fresh medium as well as on the GA_3-containing medium, and elongation of established shoots was not achieved (Campiotti et al., 1986).

Detailed studies had been devoted to the determination of requirements for _in-vitro_ plantlet regeneration (Guany and Rao, 1987; Campiotti, et al,

1986; Wright, et al, 1986; Williams and McHugen, 1986; Arditti and Nyman, 1985; Donnelly, et al, 1985; Phillips and Hubstenberger, 1985; Agrawal and Chandra, 1983; Kurtz and Lineberger, 1983; Fari and Czako, 1981; Saxena, et al, 1981; Diamantoglou and Mitrakos, 1979; De Fossard, et al, 1978; Favre, 1977; Start and Cumming, 1976; Seibert, et al, 1975; George and Narayanaswamy, 1973; Walkey and Woolfit, 1970; Murashige and Skoog, 1962; Skoog and Miller, 1957;), but few authors have monitored plantlet development beyond the culturing environment, other than to report that plants grew to maturity in greenhouse conditions.

Following plantlet development beyond the in-vitro stage would be beneficial for the following reasons:

1) To determine whether any differences may exist between the regenerated plant and the parent plant.

2) Second generations may be evaluated to determine if traits are carried from one generation to another.

3) A comparison of regenerated and parent plant development in a greenhouse environment and in field

trials would allow for economic assessment of growth patterns and fruit yield potential for industry.

Although growth regulator requirements and growth conditions for *Capsicum* established by previous investigators have been successful, there have been conflicting reports regarding the response of various explants and cultivars to media formulations and environmental conditions. Notwithstanding, research to date indicates that IAA may be the best auxin, and BAP the best cytokinin for use in *Capsicum* regeneration. Temperatures of 25°c, and photoperiods of 16-hours or 24-hours have most often been employed, but few attempts have been made to establish the effects of varying temperature and photoperiod on the morphogenesis of *Capsicum* plant tissue. Additionally, no extensive study has been done to evaluate both media formulations and growth conditions at the same time.

The aim of the present research was to successfully regenerate a hybrid cultivar of *Capsicum annuum* cv. Early Prolific Hybrid Pepper (a sweet, bell-type pepper) using tissue culture methods. To date, there have been no reports concerning the

regeneration or micropropagation of this cultivar. Hybrid seed of <u>Capsicum</u> are expensive as compared to regular, open-pollinated seeds because these seeds are produced by manual pollination. The hybrid plants are vigorous, very productive and produce an early crop. Thus, successful regeneration of a hybrid species could lead to economical advantages. An additional aim of this study was to add to the present knowledge of the culture requirements of <u>Capsicum</u>, through environmental, coupled with growth regulator, manipulations.

It was considered of interest to attempt to regenerate a single hybrid cultivar of <u>Capsicum</u>, allowing for extensive experimentation with growth regulator manipulations and environmental factors, and their effects upon the morphogenesis of different explants. Additionally, although investigators generally tend to employ the use of statistics to a limited extent, statistical anaylsis of results obtained in the present experiments was utilized not only to establish the significance of differences in observations, but also to attempt to show differences

in the effects of growth regulators upon different explants cultured under various incubation environments. To date, there has been no documentation in this regard. Plantlets formed <u>in-vitro</u> were then grown to maturity under greenhouse, as well as field conditions, where evaluation (through statistical analysis) of qualitative differences from parent stock, and potential economic value could be determined.

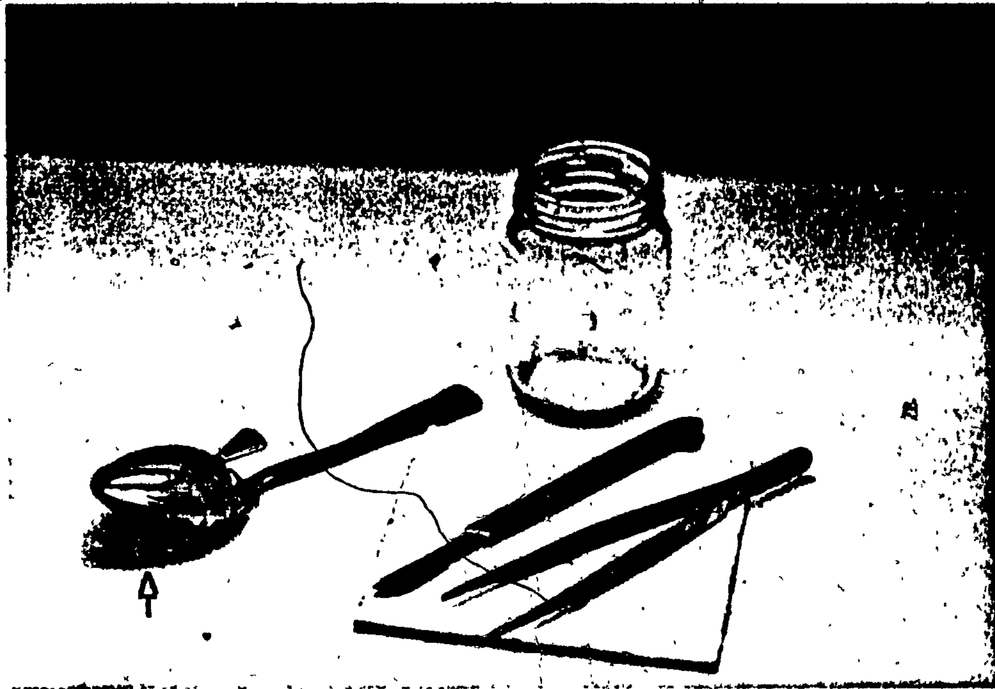
MATERIALS AND METHODS

Experimental Plants and Culture Techniques

Seeds of *Capsicum annuum*, cv. Early Prolific Hybrid Pepper, were purchased from Dominion Seed House (Georgetown, Ontario). All seed and explant manipulations, as well as sub-culturing, were carried out aseptically in a laminar flow hood (Forma Scientific), equipped with an ultra-violet (U.V.) light and a bunsen burner.

Seeds were surface sterilized by placing between 50 to 150 seeds in a sterilized infuser (Fig. 3), and immersion for 2 minutes in 70% ethanol, followed by 10 minutes in a 6% sodium hypochlorite (pure javex) solution. The sterilized seeds were then washed three seperate times in sterile distilled water. The washed seeds were transferred to a sterile cutting tile before placing 3-4 seeds in 125 ml jars containing MS basal medium. The seeds were germinated at 22°c ± 2°c under a 16-hour photoperiod regime. Explants were removed 24 to 27 days after sowing, from 10 to 13 day-old seedlings.

Figure 3. Infuser (indicated by arrow) for surface sterilizating *Capsicum* seeds.



Cotyledons were excised taking care that no part of the petiole was removed, and small perforations were made along the mid-rib and lamina before placing them in culture vials. Hypocotyl sections were excised and small longitudinal slits were made along the entire length before they were placed in culture medium. Cotyledonary axil and apical meristem sections were excised and placed directly into culture vials.

Explants in all experiments were cultured on Murashige and Skoog (1962) basal medium (MS). Stock solutions (Table 1) were made and stored in the refrigerator or freezer for all ingredients except casein-hydrolysate, myo-inositol, sucrose and agar, which were weighed out directly as required. One litre of MS basal medium contained 10 mls of each stock solution, 0.1g myo-inositol, 1.0g casein-hydrolysate, and 30.0g sucrose. Before adding 6.0g agar (Oxoid, Bacteriological, Agar No.1) the pH of the solution was adjusted to 5.7 ± 0.1 with 1N NaOH or 3N HCl. The medium was heated on a magnetic stirrer until clear. Growth regulators, when required, were then added. Stock solutions of growth regulators, at appropriate concentrations, were prepared by dissolving them in absolute ethanol and were stored in the refrigerator. Approximately 20mls of the nutrient solution were placed in each culture jar. The jars were then capped and autoclaved for 20 minutes at 121°c.

Table 1: Formulation of MS basal nutrient medium. Stock Solutions (X100 concentration) were stored in the cold. (From Bhojwani and Razdan, 1983)

Macro-Elements	g/100ml
Stock Solution I:	
$CaCl_2.2h_2O$	4.4
Stock Solution II:	
KH_2PO_4	1.7
KNO_3	19.0
$MgSO_4.7H_2O$	3.7
NH_4NO_3	16.5
Micro-Elements	
Stock Solution III:	
KI	0.0083
Stock Solution IV:	
$FeSO_4.7H_2O$	0.278
Na-EDTA	0.373
Stock Solution V:	
$CoCl_2.6H_2O$	0.00025
$CuSO_4.5H_2O$	0.00025
H_3BO_3	0.0629
$MnSO_4.4H_2O$	0.2230
$Na_2MoO_4.2H_2O$	0.0025
$ZnSO_4.7H_2O$	0.0860
Vitamins	
Stock Solution VI:	
glycin	0.02
nicotinic acid	0.005
pyridoxine-HCl	0.005
thiamin-HCl	0.01

In-vitro Regeneration of Capsicum annuum

Determination of growth regulator requirements for morphogenesis.

Experiments were initiated to determine growth regulator combinations required for the induction of shoots, roots and callus in cotyledon and hypocotyl explants.

In total, 32 different media formulations were tested (Table 2). All cultures were incubated at 22°c ± 1°c and 16-hour photoperiod, under a combination of flourescent and incandescent lighting at an intensity of 520 ft. candles. Five cotyledons and five hypocotyls were cultured for 30 days on each medium. Tissue response was monitored once per week over a 30-day culture period, at the end of which the extent of morphological change was scored.

Effect of temperature, photoperiod and media manipulations on the morphogenesis of cultured explants.

For this investigation, nine different media formulations and three different explants (cotyledons, hypocotyls, cotyledonary axils) were used (Table 3).

TABLE 2: Formulations of media used to determine growth regulator requirements for morphogenesis in cotyledon and hypocotyl explants.*

	AUXIN			CYTOKININ		GA
MEDIUM #	IAA	NAA	2,4-D	BAP	KIN	GA3
1	-	1.00	-	-	-	-
9	1.00	-	-	-	-	-
10	-	-	-	1.00	-	-
12	-	-	-	2.00	-	-
18	-	-	-	5.00	-	-
7	.05	-	-	.05	-	-
6*	2.00	-	-	2.00	-	-
6	.05	-	-	2.00	-	-
5	1.00	-	-	2.00	-	-
8	.05	-	-	10.00	-	-
13	.10	-	-	.10	-	-
14	.10	-	-	.50	-	-
15	.10	-	-	1.00	-	-
16	.10	-	-	5.00	-	-
17	.10	-	-	10.00	-	-
11	4.00	-	-	10.00	-	-
3	-	.01	-	.10	-	-
24	-	.10	-	.10	-	-
25	-	.10	-	.50	-	-
26	-	.10	-	1.00	-	-
27	-	.10	-	5.00	-	-
28	-	.10	-	10.00	-	-
23	-	.05	-	10.00	-	-
21	.10	-	-	1.00	.10	-
2	-	-	-	.10	-	1.00
4	.10	-	-	-	.01	-
22	-	1.00	-	-	-	.10
29	-	.02	-	-	2.00	-
30	-	.02	-	2.00	-	-
19	.02	-	-	-	2.00	-
20	.02	-	-	2.00	-	-
0	.35	-	.22	-	.02	-

* All media contained MS basal nutrient solution, except media 2 and 4, which contained half-strength MS basal nutrient solution. Concentrations are in mg/l.

Table 3: Growth regulator constituents of MS medium used to determine effects of temperature and photoperiod on the morphogenesis of cotyledon, hypocotyl and cotyledonary axil explants.

MEDIUM #	GROWTH REGULATOR CONSTITUENTS (mg/l) IAA	NAA	BAP	Kin
1	-	1.00	-	-
6*	2.00	-	2.00	-
7	0.05	-	0.05	-
8	0.05	-	10.00	-
9	1.00	-	-	-
11	4.00	-	10.00	-
12	-	-	2.00	-
21	0.10	-	1.00	0.10
23	-	0.05	10.00	-

Environmental treatment conditions consisted of three temperatures (18 ± 2°c, 22 ± 2°c, 28 ± 2°c) and two photoperiods (16- and 24-hour). Three different incubators were used which varied in light intensity from 520 to 600 ft. candles.

Three to five explants of each tissue type were cultured for each condition (i.e. medium, temperature, photoperiod). For each condition, experiments were replicated two or three times. Thus, a total of ten explants were cultured per treatment condition.

Observations of morphological change were recorded at least once per week throughout the 30-day culture period, at which time the extent of morphological change was noted in terms of percent frequency and intensity of response. This method of reporting response is best described by use of the following example:

Cotyledons cultured in 22°c, 16-hr. photoperiod responded with callus growth of 2/10(++++)6/10(+).

Thus, since 8 out of the 10 explants cultured responded with callus growth,

% Frequency = 80

However, 2 responded with much callus growth, and 6 responded with little callus growth, thus

Intensity = [2 X 4(+'s)] + [6 X 1(+'s)]

= 8 + 6

= 14(+'s)

and

14/10 = 1.40

Thus, the average intensity of callus growth is 1.40.

With this method of reporting, we can see that 80% of the cotyledons responded to the treatment condition and that the average callus intensity of all the cultured explants (10) was 1.40.

Statistical analysis was performed on the results of these experiments, using the computer program

entitled Statistical Package for the Social Sciences (SPSS) (Norusis, 1985; Nie, et al, 1975) available for use at the Concordia University Computer Science Department. A multiple analysis of variance (ANOVA) was performed with media formulation, explant, temperature and photoperiod as independent variables, shooting, rooting and callus intensity as dependent variables. A hierarchical log-linear (HILOGLINEAR) analysis was also performed on these data.

Additional analysis was performed which consisted of a stepwise multiple linear regression analysis. Concentrations of IAA, NAA, BAP, and Kin, type of explant, temperature, and photoperiod were independent variables, while shooting, rooting and callus intensities were dependent variables. A series of stepwise multiple regressions were performed with explant, temperature and photoperiod held constant, IAA, NAA, BAP and Kin as independent variables, and shooting, rooting and callus intensities as dependent variables.

Regeneration from cotyledonary axil explants.

To compare the effects of several promissing media from the two previous experiments, eight cotyledonary axil explants were cultured on each of six different media formulations, at 28°c and 16-hour photoperiod, and morphological response was scored after the 30-day culture period. Explants which had produced shoots, but not roots, were placed in basal medium supplemented with IAA (1.0 mg/l) to induce a rooting response.

As many as 73 cotyledonary axils were subsequently cultured, in several experiments, at 28°c, and 16-hour photoperiod in medium 9 (IAA 1.0mg/l). When explants formed both shoots and roots, and when these shoots began to elongate, plantlets were transplanted (soil pH=5.7) and transferred to greenhouse conditions. Supplemental lighting (both fluorescent and incandescent) was provided on a 16-hour photoperiod regime.

Greenhouse and Field Trials

Plantlets which had been regenerated from cotyledonary axil explants cultured in medium 9 (1.0 mg/l IAA) (F-1 regenerated plants) and transferred to

greenhouse conditions, were monitored once per week, and the plant height, number of leaves, age of fruit set and harvest, number and weight of fruit were recorded. Similarly, parent stock seeds (F-1 seed), second generation parent stock seeds (F-2 seed) (from fruit produced by F-1 seed plants), and second generation regenerated seeds (F-2 regenerated) (from fruit produced by F-1 regenerated plants) were planted in the greenhouse and the same parameters monitored once per week. The plants were monitored for two fruit yields. To ensure that the fruit were fully mature when picked, they were harvested only when they began to turn a slight red color. Except for F-2 regenerated plants, greenhouse trials were repeated to obtain data from 20 plants of each of the four plant types.

In the spring and summer of 1987, a field trial was performed at Ferme Prud'homme, located in the St.Eustache region of Quebec, a region well renowned for its vegetable production (soil pH=6.4). No fertilization, or soil adjustments were made to the trial plots. A minimum of 20 plants from each plant type were planted when they were between 35 and 42 days

old, and bore between 8 and 15 leaves. F-1 seed plants were one week younger when planted out (June 3) than either F-2 seed or F-2 regenerated plants. F-1 regenerated plants were four weeks younger, and were transferred to field conditions four weeks later (June 24) than F-2 seed and F-2 regenerated plants. As in greenhouse trials, the plants were monitored once per week, and the plant height, number of leaves, age at fruit set and harvest, number and weight of fruit were recorded. Fruit from all plants were harvested on September 16.

Statistical analyses were performed on all data obtained from both the greenhouse and field trials. Initially, a 2-way analysis of variance (ANOVA) was performed to determine whether any differences existed between the greenhouse and/or the field trials and plant type (F-1 seed, F-2 seed, F-1 regenerated, F-2 regenerated) upon measured parameters. Two seperate greenhouse experiments had been performed for each plant type, except F-2 regenerated plants where only one was performed, and one field experiment was performed for each plant type. Parameters upon which

plant type was found to have no effect were eliminated from furthur analysis. The data from parameters not found to be significantly different between individual greenhouse trials were subsequently pooled and further ANOVA analysis was performed to determine the significance of differences between greenhouse trials and the field trial for individual plant types. Alternatively, the data from those parameters found to be significantly different in greenhouse trials of individual plant types were kept seperate in subsequent analyses. To this end, a series of ONEWAY (oneway ANOVA) analyses were performed on data for individual plant types. As well, a series of ONEWAY analyses were performed on all data to determine whether differences existed between plant types in greenhouse and/or field trials. Postpriori tests (Scheffe) were employed to establish whether significant differences ($P<0.05$) existed.

Micropropagation

Elongation of multiple shoots.

In this series of experiments, an attempt was made to obtain multiple plantlets *in-vitro* from cotyledon and apical meristem culture.

An initial experiment was performed to determine the potential of apical meristems to form shoots in culture. Four media (medium numbers 11, 18, 31 and 32) which had been previously found to induce a shooting response in cotyledons, were utilized, and 10 apical meristems were cultured in each medium, at 28°c and 16-hour photoperiod, for 30 days.

Shoot elongation experimentation consisted of placing established shoots in GA_3 (0.1mg/l), or placing them in rooting media, or by either of these two methods coupled with placing them in the dark to induce etiolation.

Subsequently, at least 10 cotyledons and 10 apical meristem sections were cultured, at 28°c and 16-hour photoperiod, on media containing BAP (20.0mg/l) and

from 0.1 to 5.0 mg/l GA_3. Additionally, the optimum shooting medium established in previous experimentation (M32: BAP 20.0mg/l) was employed, and 10 apical meristems were cultured at 28°c and 16-hour photoperiod for 30 days. Multiple shoots that were formed on apical meristem explants cultured in BAP (20.0mg/l) alone, were sub-cultured in media which contained from 0.1 to 5.0 mg/l GA_3. Seven meristems which had the most profuse shooting response, were transferred to MS basal medium (containing no growth regulators) in an attempt to induce elongation in already established shoots.

Organogenesis from callus tissue.

Callus proliferation was induced in cotyledon and hypocotyl explants by culture at 28°c, or 22°c, 16-hour photoperiod, in callus-inducing medium prescribed by Murashige and Skoog (1962) containing IAA (0.35 mg/l), 2,4-D (0.22 mg/l) and Kin (0.02 mg/l). The callus was allowed to proliferate for 30, 60, or 90 days, at which times sections were placed into previously established shoot- or root-inducing media, at 28 or 22°c, and

16-hour photoperiod conditions, where tissue response was monitored on a weekly basis.

RESULTS

In-vitro Regeneration of Capsicum annuum

Determination of growth regulator requirments for morphogenesis.

Cotyledon explants tended to respond with callus, shoot and/or root growth, whereas hypocotyl explants generally did not tend to produce shoots (Table 4). Callus growth was promoted in all but three cases [i.e. cotyledons cultured on medium 8 (0.05 mg/l IAA + 10.0 mg/l BAP) and medium 11 (4.0 mg/l IAA + 10.0 mg/l BAP); hypocotyls cultured on medium 9 (1.0 mg/l IAA)]. When IAA was held constant (at 0.1 mg/l), and BAP varied from 0.1 to 10.0 mg/l (media 13 - 17), the shooting intensity of cotyledons increased with increasing concentrations of BAP, while the rooting intensity of hypocotyls decreased. However, when NAA was substituted for IAA (media 24 - 28), cotyledons produced roots instead of shoots, and rooting intensity in both explants decreased as the concentration of BAP was increased (Table 4).

In all cases, the roots which were produced from both cotyledons and hypocotyls cultured on media supplemented with NAA (1.0 mg/l) were short and thick, and developed in a mass-like fashion. Conversely, roots which developed from hypocotyl explants cultured on media 7 [IAA (0.05 mg/l) + BAP (0.05 mg/l)] and 9 [IAA (1.0 mg/l)] were long and thin and developed singly. Shoots (actually small leaves) arising from cotyledons cultured on media 6*, 8, 11, 16, 17 and 18, varied in the extent of proliferation but appeared to develop in the same fashion. Masses of shoots often formed at the cut base of the cotyledon, whereas single shoots tended to form elsewhere (i.e. from the center of the cotyledon at the midrib, upper or lower cotyledon surface, and from the tip of the cotyledon). Growth regulator concentrations of the various media tested did not appear to have an effect on the area of organogenesis within the cotyledon itself.

Overall results of this experiment indicated that the best shoot-inducing media for cotyledon explants were media numbers 17, 18, 8, 11, 23 and 6*, containing from 2 - 10 mg/l BAP and from 0 - 4 mg/l IAA. None of

the media tested in this experiment induced a good shooting response in hypocotyls. The best root-inducing media for cotyledons were media numbers 1 (1.0 mg/l NAA) and 24 (0.1 mg/l NAA + 0.1 mg/l BAP), while media 1, 7, 9, 13 and 24, containing from 0.1 - 1.0 mg/l NAA, or 0.05 - 1.0 mg/l IAA and from 0 - 0.5 mg/l BAP, seemed best for rooting of hypocotyls. The best callus-inducing medium for both explants was found to be medium 0 [IAA (0.35mg/l) + 2,4-D (0.22mg/l) + Kin (0.02 mg/l)] (Table 4).

TABLE 4[a]: Media constituents (mg/l) and morphological response of explants cultured for 30 days at 22°c, 16-hour photoperiod. A minimum of 5 explants were cultured per treatment.

	AUXIN			CYTOKININ		GA	RESPONSE **	
MEDIUM #*	IAA	NAA	2,4-D	BAP	KIN	GA_3	COTYLEDONS	HYPOCOTYLS
1	-	1.00	-	-	-	-	C(++)R(++++)	C(+)R(++)
9	1.00	-	-	-	-	-	C(+)	R(+++)
10	-	-	-	1.00	-	-	C(+)	C(+)
12	-	-	-	2.00	-	-	C(+)	C(+)
18	-	-	-	5.00	-	-	C(+)S(+++)	C(++)
7	.05	-	-	.05	-	-	C(+)	C(+)R(+++)
6*	2.00	-	-	2.00	-	-	C(+)S(+)	C(++)
6	.05	-	-	2.00	-	-	C(+++)	C(+)
5	1.00	-	-	2.00	-	-	C(+++)	C(++)R(+)
8	.05	-	-	10.00	-	-	S(+)	C(++)
13	.10	-	-	.10	-	-	C(++)	C(++)R(+++
14	.10	-	-	.50	-	-	C(+++)	C(+++)R(+)
15	.10	-	-	1.00	-	-	C(++)	C(+++)R(+)
16	.10	-	-	5.00	-	-	C(++)S(+)	C(++)R(+)
17	.10	-	-	10.00	-	-	C(+)S(+++)	C(+)
11	4.00	-	-	10.00	-	-	S(+)	C(+)
3	-	.01	-	.10	-	-	C(+++)	C(+)
24	-	.10	-	.10	-	-	C(++)R(++++)	C(++)R(+++
25	-	.10	-	.50	-	-	C(++)R(+)	C(++)R(+)
26	-	.10	-	1.00	-	-	C(+++)	C(++)R(+)
27	-	.10	-	5.00	-	-	-	-
28	-	.10	-	10.00	-	-	C(+++)	C(++)
23	-	.05	-	10.00	-	-	C(++)S(+)	C(+)S(+)
21	.10	-	-	1.00	.10	-	C(++)	C(++)
2	-	-	-	.10	-	1.00	C(+)	-
4	.10	-	-	-	.01	-	C(++)	-
22	-	1.00	-	-	-	.10	C(++)	C(+)
29	-	.02	-	-	2.00	-	C(+++)	C(+++)R(+)
30	-	.02	-	2.00	-	-	C(++)	C(+)
19	.02	-	-	-	2.00	-	C(++)	C(+++)R(+)
20	.02	-	-	2.00	-	-	C(++)S(+)	C(+++)R(+)
0	.35	-	.22	-	.02	-	C(+++)	C(+++)

[a] All media contained MS basal nutrient solution, except medium numbers 2 and 4 which contained half-strength MS basal nutrient solution.

** C = callus; R = roots; S = shoots
(++++) extensive response; (+++) much response;
(++) moderate response; (+) little response; (-) no response.

Effect of temperature, photoperiod and media formulations on the morphogenesis of cultured explants.

The morphological response was observed to vary with the explant, media formulation, temperature, and photoperiod (Appendix II). The shooting response of cotyledons (Fig. 4) cultured on medium 11 (4.0 mg/l IAA + 10.0 mg/l BAP), for example, was seen to increase as temperature increased from 18°c to 28°c. Additionally, while these explants responded at all three temperature conditions under the 16-hour photoperiod regime, a limited response was only seen at 22°c under the 24-hour photoperiod regime. While 4.0 mg/l IAA + 10.0 mg/l BAP induced a shooting response, no response was observed under any condition when IAA and BAP were present in equal concentrations (1.0 mg/l each IAA and BAP).

Alternatively, while medium 11 induced shooting in cotyledon explants, only a slight shooting response was induced in hypocotyls, at 22°c and 16-hour photoperiod (Fig. 5). Cotyledonary axils (Fig. 6) were seen to

respond to this medium in a similar manner to cotyledons, however the shooting response of axils was more intense, and these explants responded at all temperatures under the 24-hour photoperiod regime.

Explant rooting response was also found to vary with treatment condition. For example, cotyledons (Fig. 7) cultured on medium 1 (1.0 mg/l NAA) responded with a rooting response which was greater at 28°c than at 22°c, and although response was observed at the 24-hour photoperiod, it was considerably less than that observed at the 16-hour photoperiod. While NAA induced a rooting response in cotyledons, the same concentration of IAA (medium 9) induced only limited rooting in this explant, and only at 28°c, 16-hour photoperiod.

Alternatively, while NAA (1.0 mg/l) induced the greatest rooting response in cotyledons, IAA (1.0 mg/l) induced the greatest rooting response in hypocotyl explants (Fig. 8), and cotyledonary axils (Fig. 9). However, 1.0 mg/l IAA induced a more intense rooting response in cotyledonary axil explants than in hypocotyls.

While callus was seen to proliferate under most conditions, explants tended to produce more callus at 28°c than at 18°c, and at the 16-hour photoperiod than at the 24-hour photoperiod (Figs. 10-12). Hypocotyls (Fig. 11) tended to produce more callus than other explants, and cotyledonary axis (Fig. 12) tended to produce the least (with the exception of that produced in medium containing 1.0 mg/l NAA).

The effect of temperature and photoperiod on the shooting response of cotyledons can be clearly seen in figure 13. No response was observed at either photoperiod at 18°c. At 22°c, some shoot buds were visible at the 16-hour photoperiod, while at 28°c, shoots were present on explants cultured in both photoperiods. However, shoots produced at the 16-hour photoperiod were more extensive than those produced at the 24-hour photoperiod.

Figures 4 - 12 represent the morphological response of explants to different temperatures and photoperiods. Data is presented along decreasing ratios of auxin to cytokinin. Error bars represent 95% confidence intervals - their absence indicates identical response intensity of 10 explants. (Ten explants were cultured per treatment). (A = 16-hour photoperiod; B = 24-hour photoperiod).

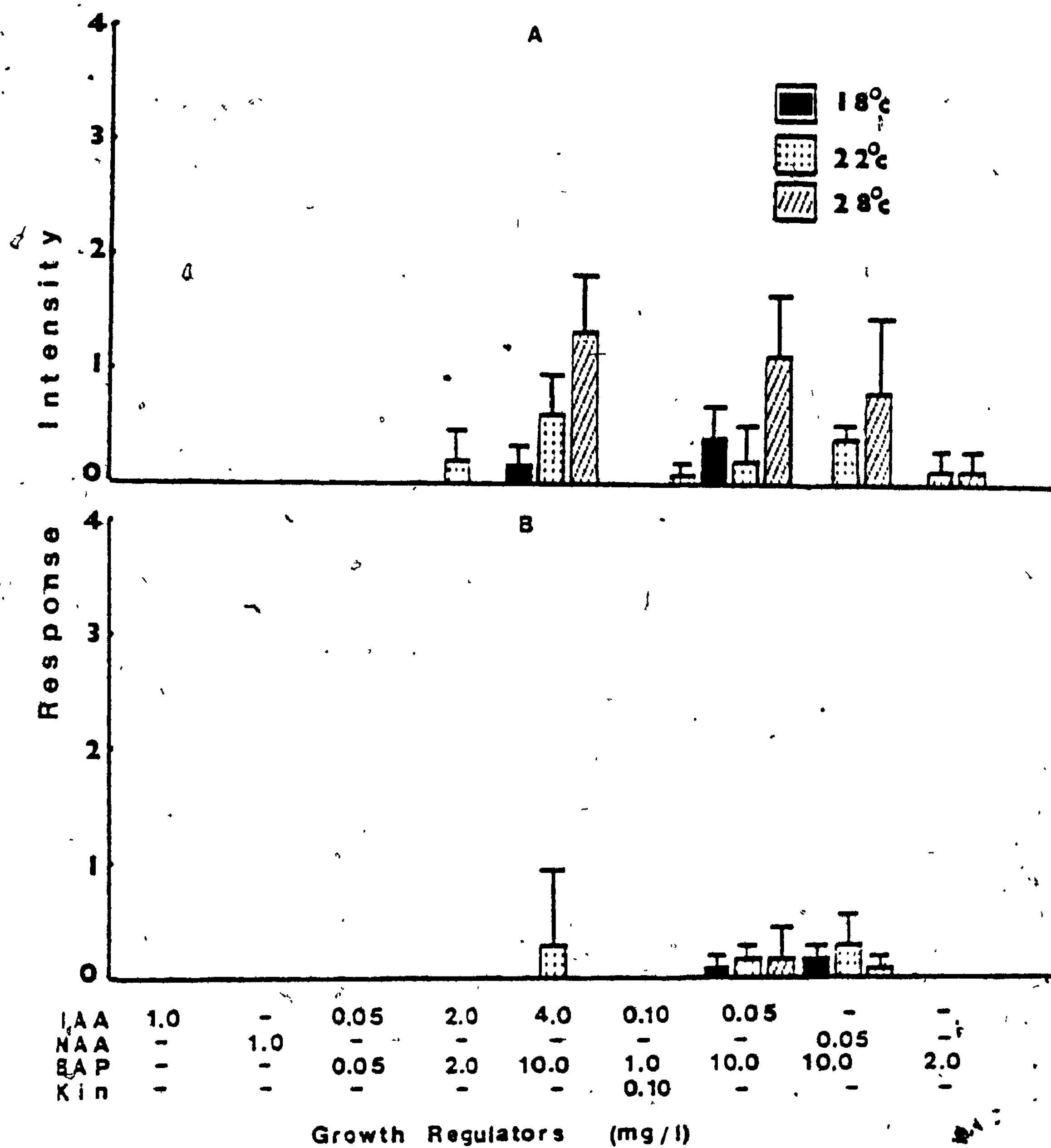


Fig. 4: Cotyledons - Shooting Intensity

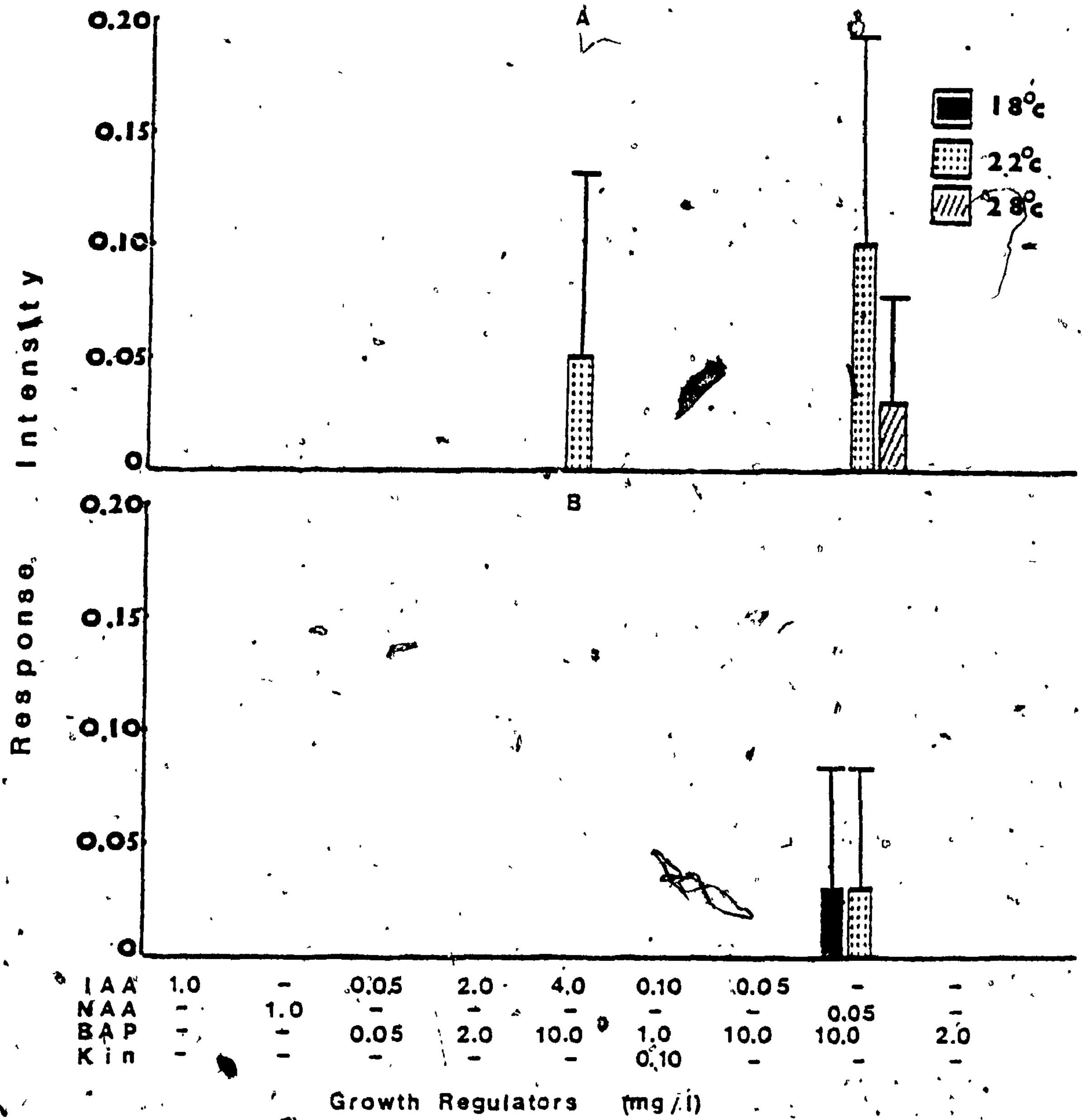


Fig. 5: Hypocotyls - Shooting Intensity

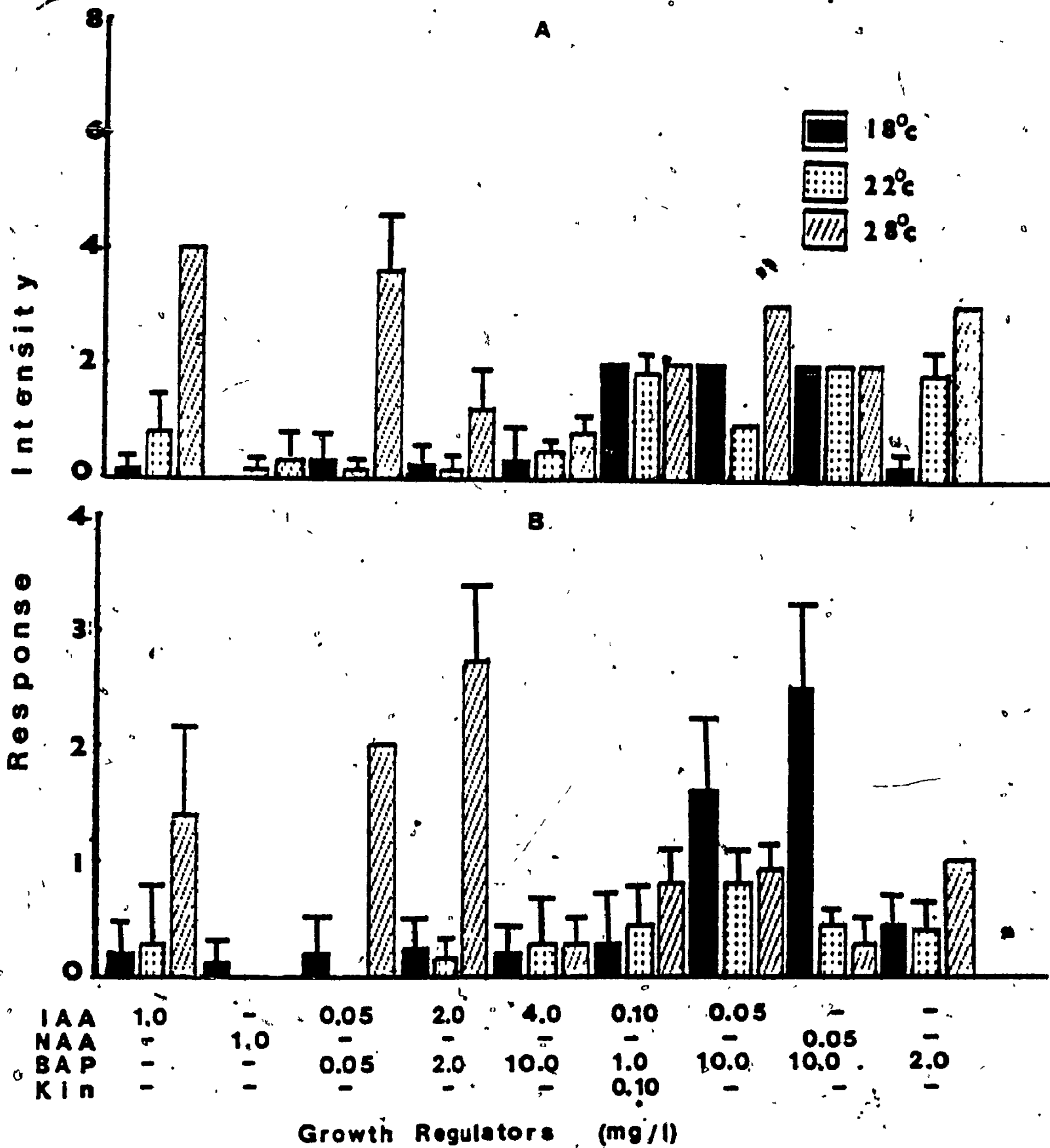


Fig. 6: Cotyledonary Axils - Shooting Intensity

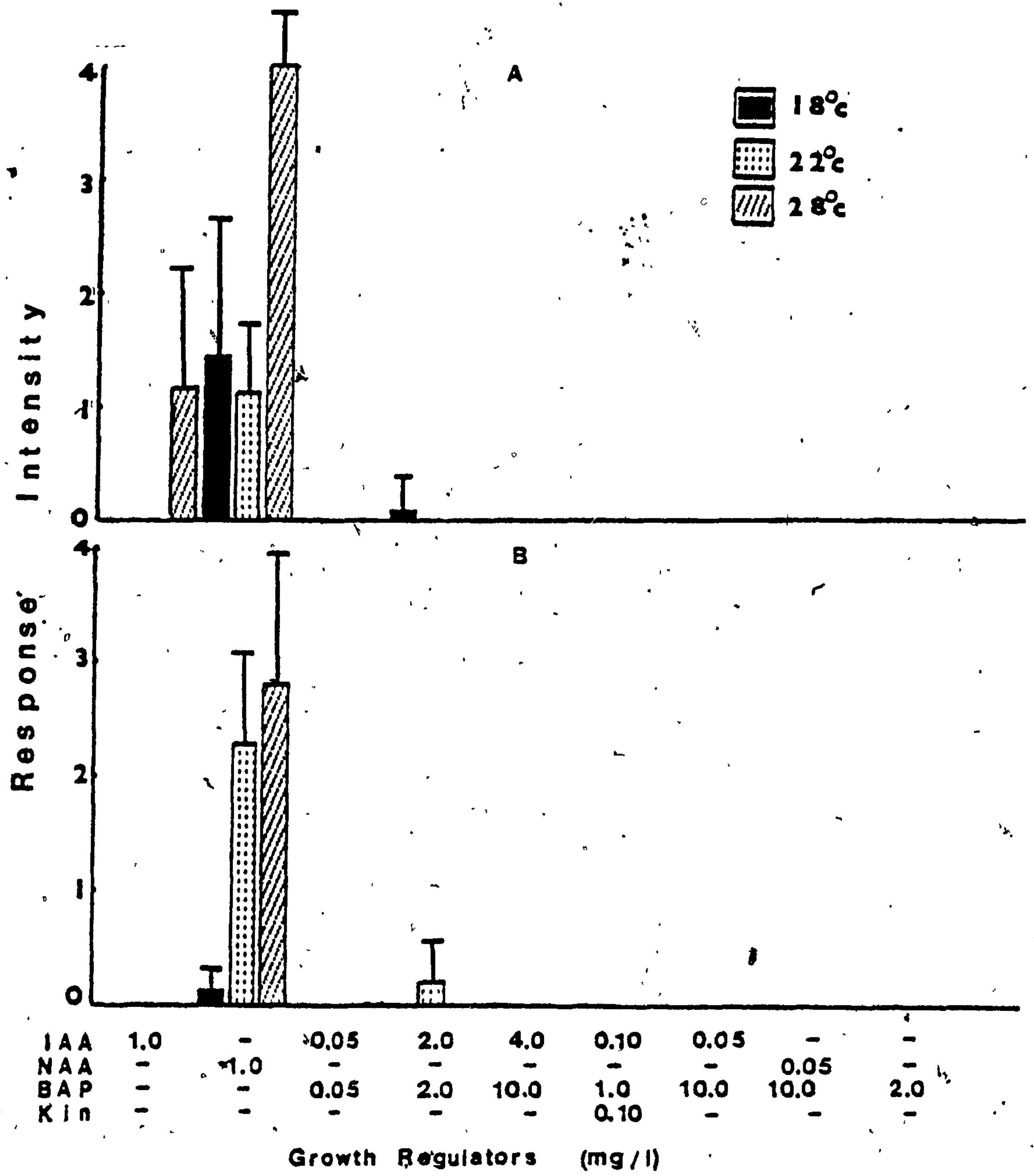


Fig. 7: Cotyledons - Rooting Intensity

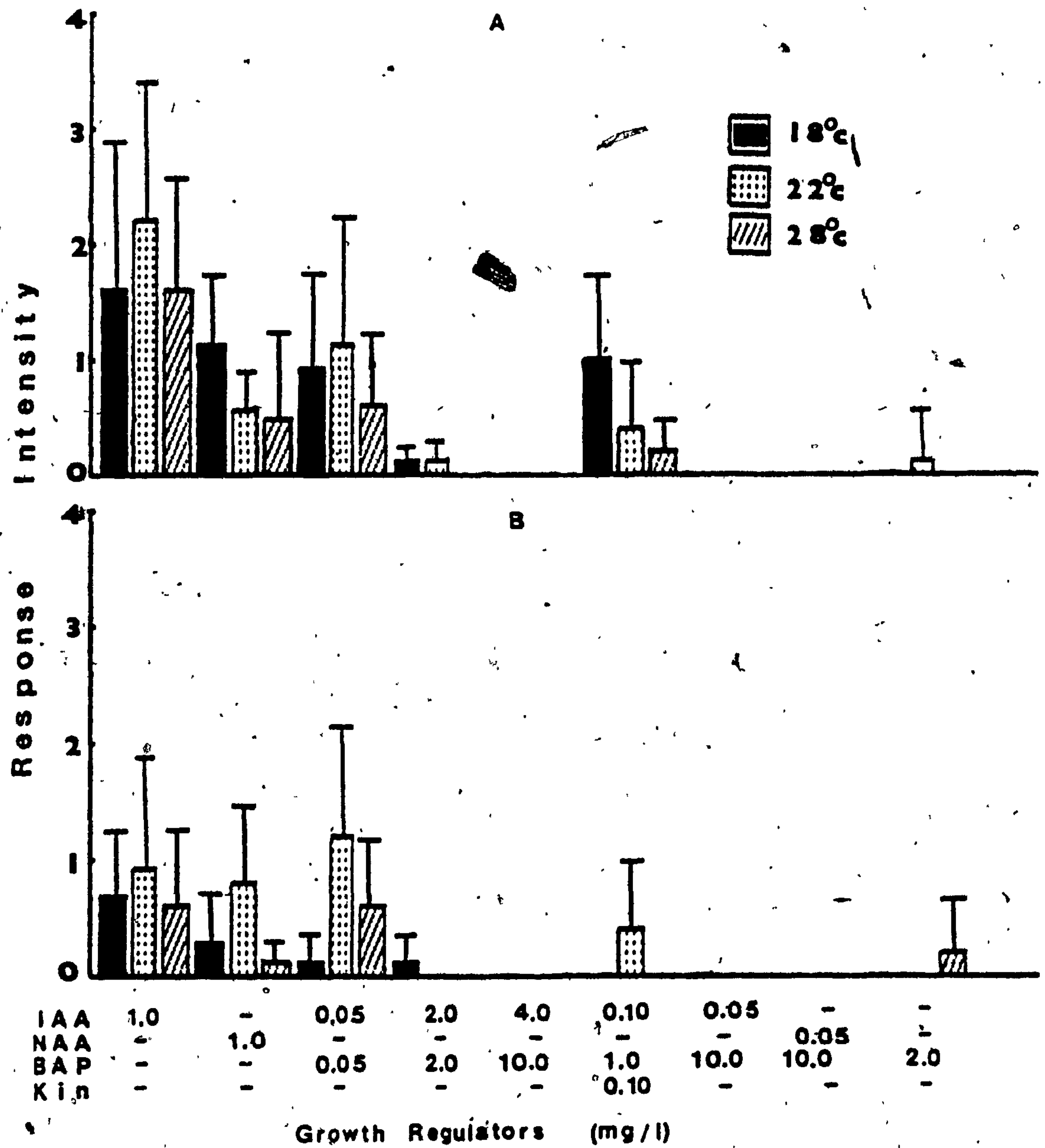


Fig: 8: Hypocotyls - Rooting Intensity

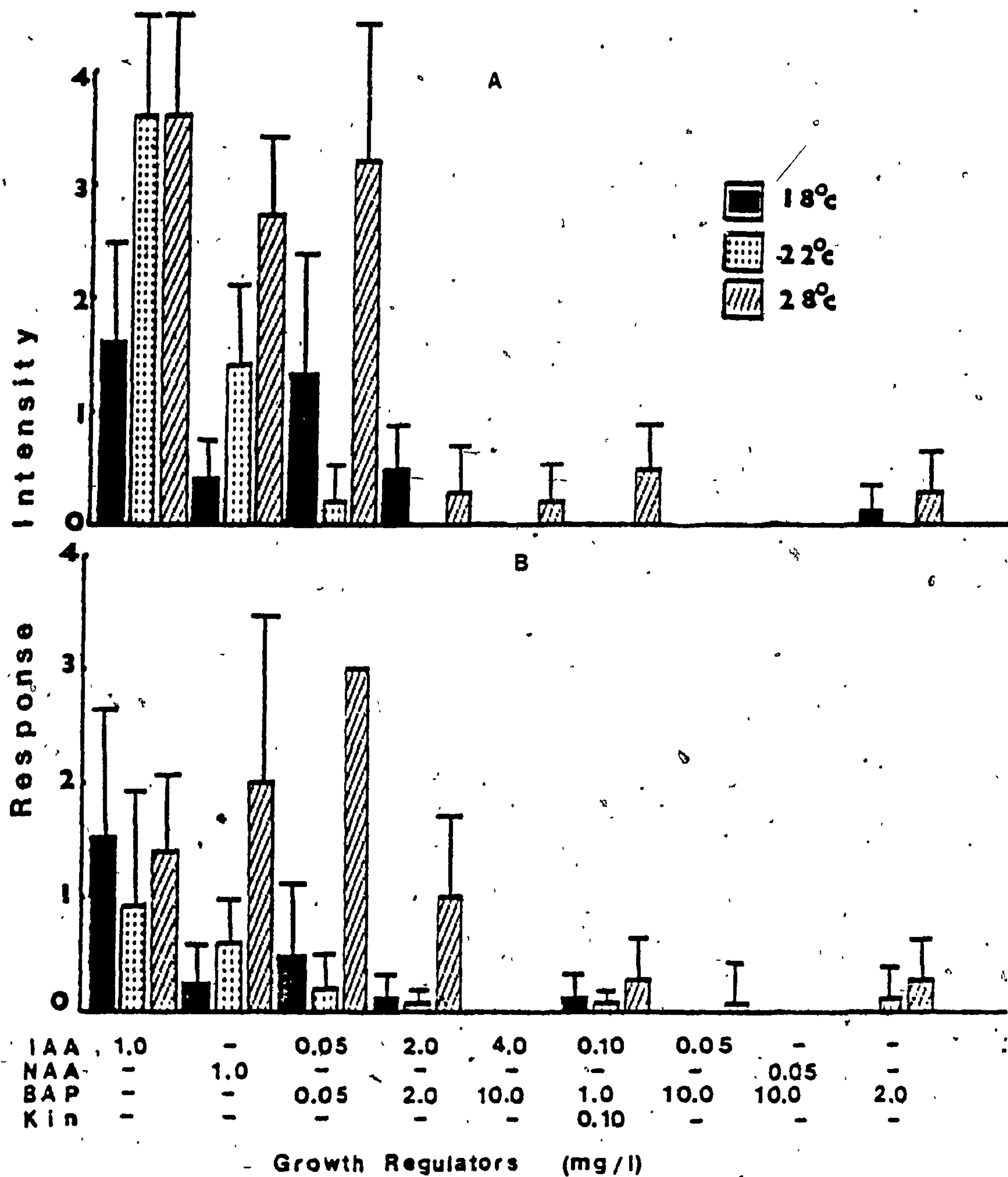


Fig. 9: Cotyledonary Axils - Rooting Intensity

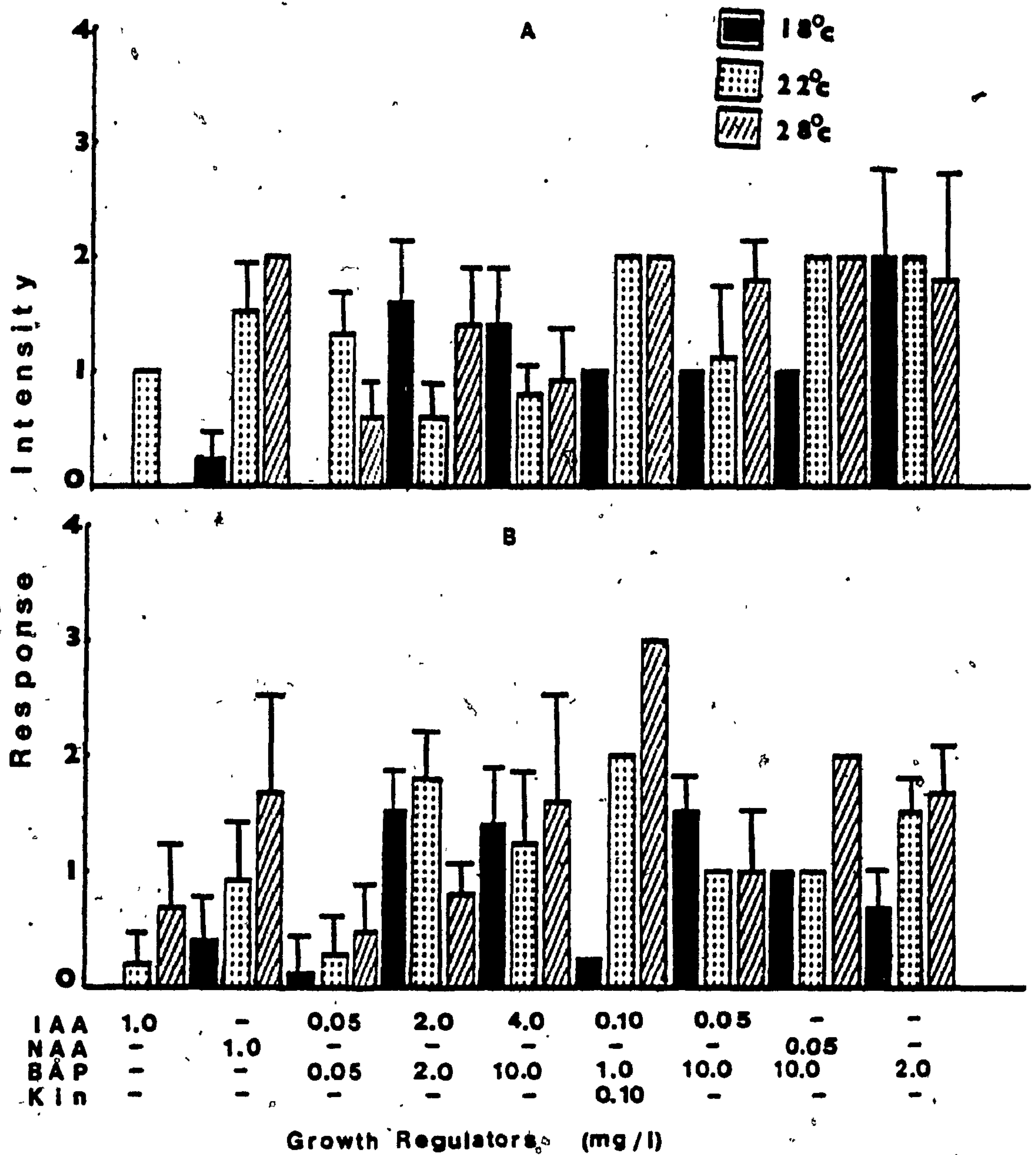


Fig. 10: Cotyledons - Callus Intensity

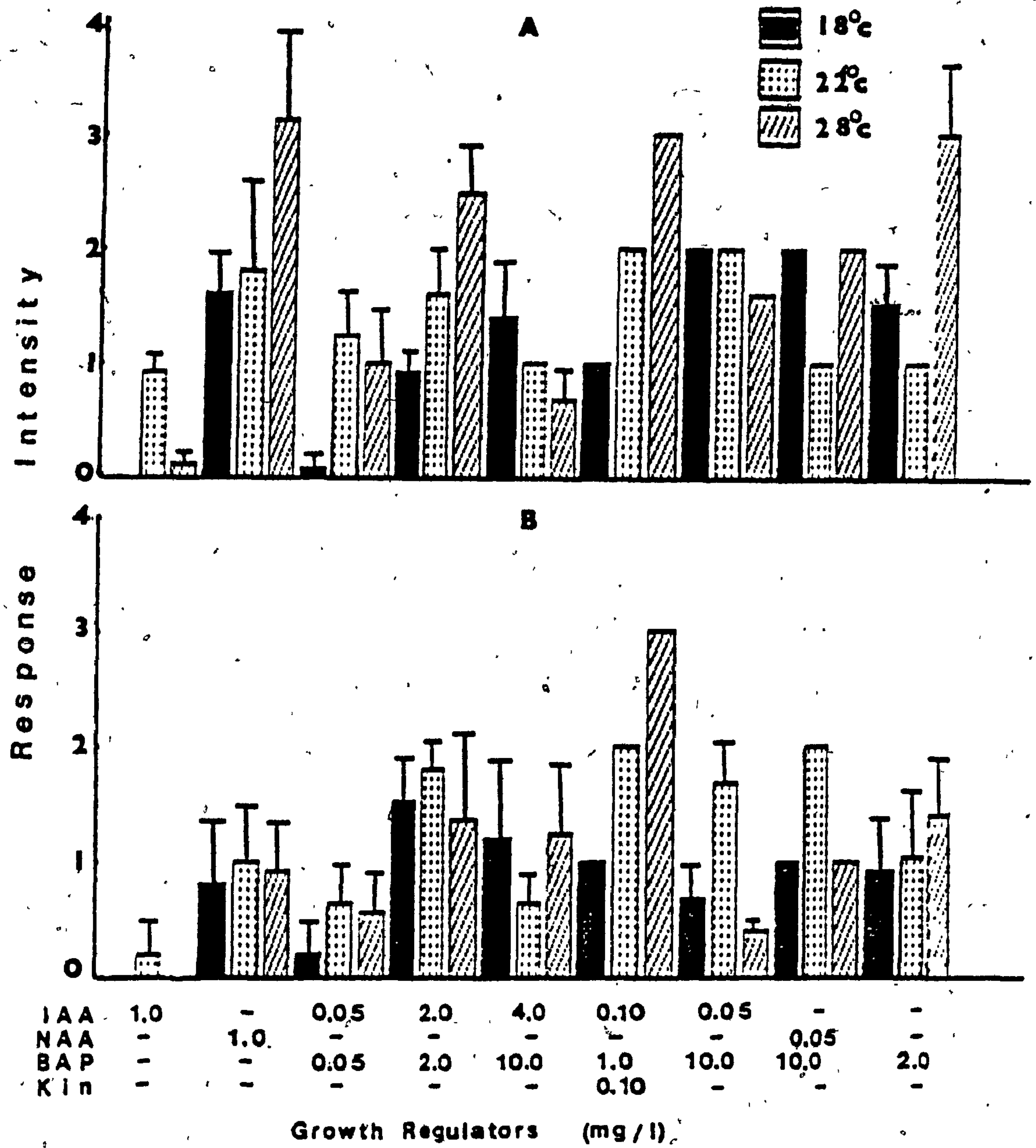


Fig. 11: Hypocotyls - Callus Intensity

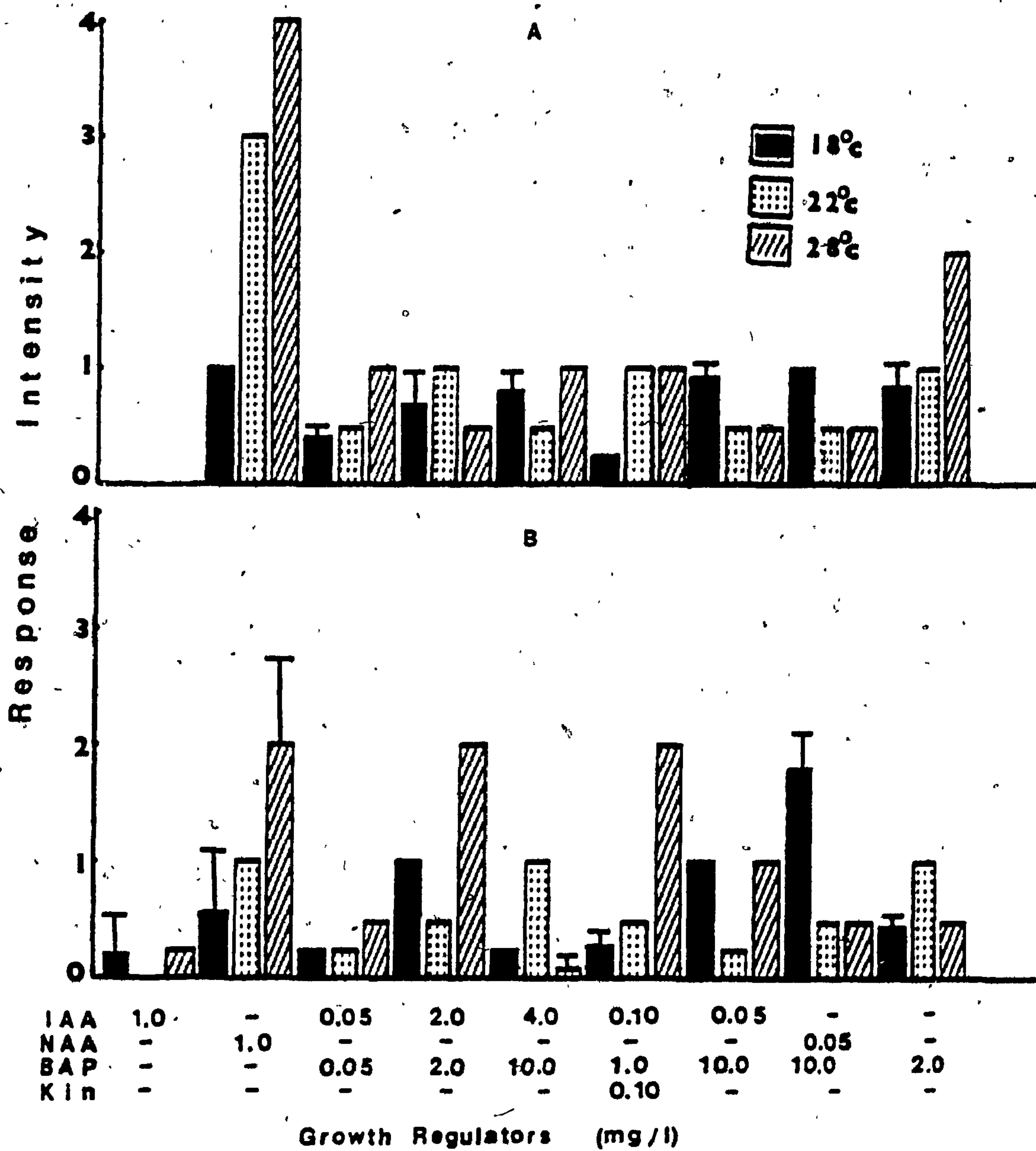


Fig. 12: Cotyledonary Axils - Callus Intensity

The effect of temperature on the rooting response of both cotyledon and hypocotyl explants was also clearly seen (Fig. 14). No response was observed in either explant at 18°c, while at 22°c limited root growth was observed. At 28°c, root growth was extensive on both explants, and substantial lateral root growth was seen on hypocotyl explants.

Cotyledonary axil explants (Fig. 15) exhibited greater response at 22°c than at 18°c, and, while a photograph is not available, more extensive shoot and root growth was observed at 28°c than at 22°c.

Analysis of variance (ANOVA) indicated that media formulation, temperature, and photoperiod all had a significant effect, at $P<0.05$, on all dependent variables (callus, rooting and shooting intensity).

Hierarchical log linear analysis (HILOGLINEAR) showed that cotyledonary axil explants responded to multiple interactions of environmental conditions, while a shooting or rooting response of cotyledon and hypocotyl explants was found to be governed by effects of individual conditions (Table 5). The induction of

Figure 13. The effect of temperature and photoperiod on cotyledon shoot formation. Explants were cultured in medium supplemented with 0.05 mg/l IAA + 10.0 mg/l BAP. (Arrows indicate masses of leaf-like shoots; c = callus growth) (A, B, C - 16-hour photoperiod; D, E, F - 24-hour photoperiod; A, D - 18°c; B, E - 22°c; C, F - 28°c).

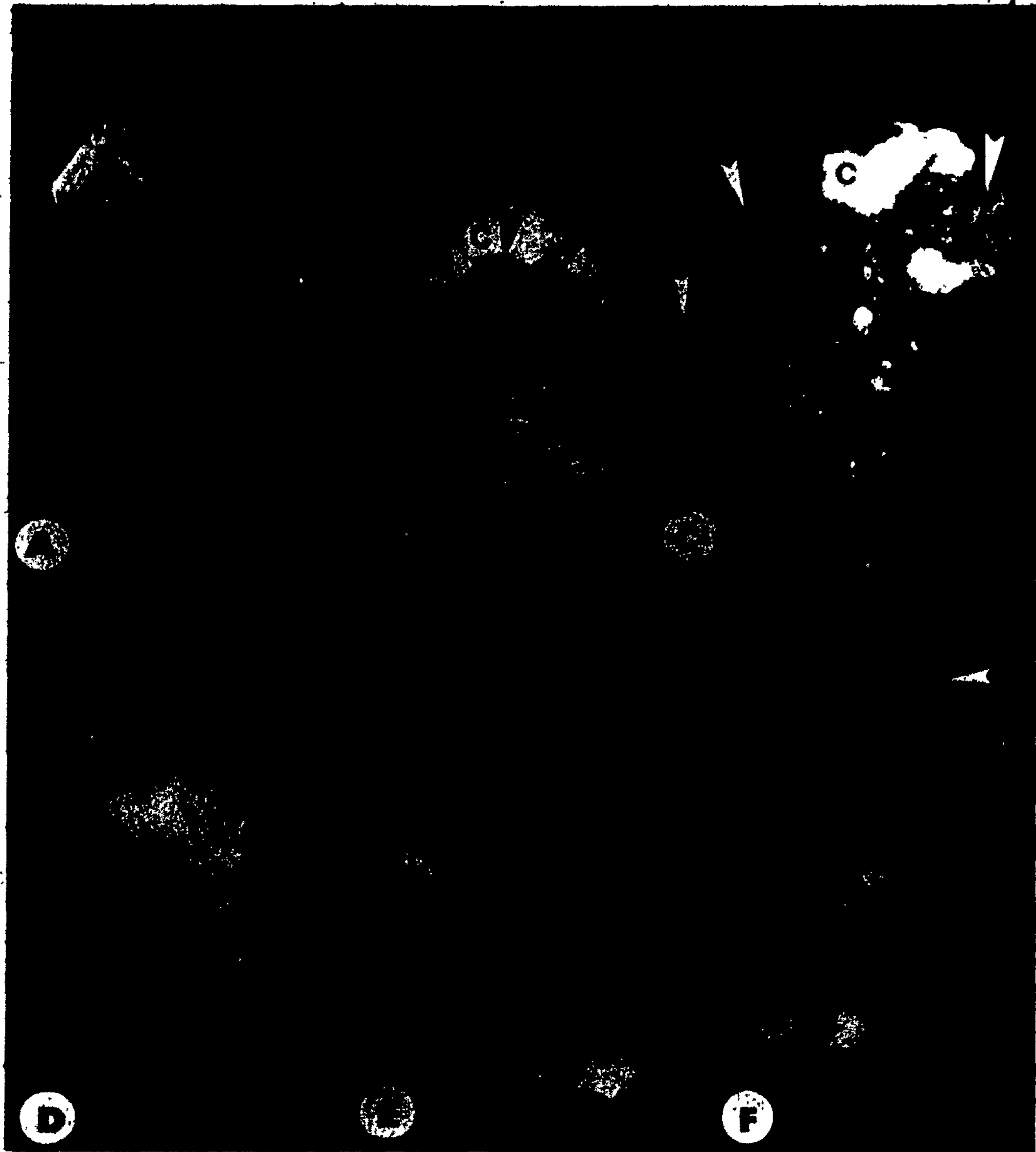

C
A
D
F

Figure 14. The effect of temperature on root formation in cotyledon (A,B,C) and hypocotyl (D,E,F) explants cultured at 16-hour photoperiod. Cotyledons were cultured in media containing 1.0 mg/l NAA (A,B) and 0.1 mg/l NAA + 0.1 mg/l BAP (C). Hypocotyls were cultured in medium containing 1.0 mg/l IAA. (c = callus growth) (18°c - A, D; 22°c - B, E; 28°c - C, F).

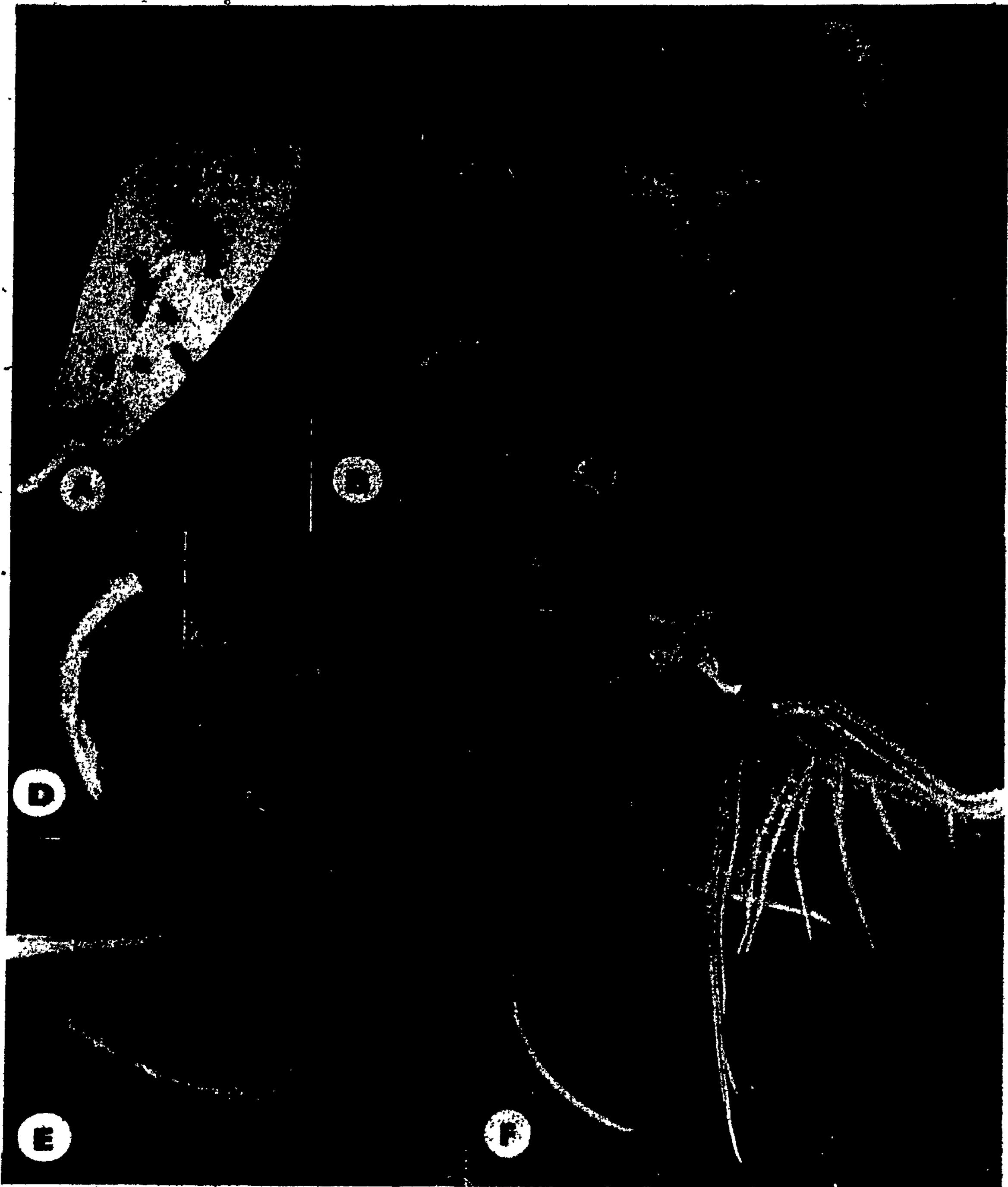

D
E
F

Figure 15. The effect of temperature on organogenesis in cotyledonary axil explants cultured at 16-hour photoperiod, in medium supplemented with 1.0 mg/l IAA. (c = callus growth) (A = 18°c; B = 22°c).

A
C
B

TABLE 5 : Results of HILOGLINEAR analysis indicating individual independent variables and/or multiple interactions of independent variables contributing significantly to response intensity of explants.* ($P<0.05$).
(a) Cotyledons; (b) Hypocotyls; (c) Cotyledonary Axils.

(a)

RESPONSE	MEDIUM	TEMP.	PHOTO.	M x T	M x P	T x P	M x T x P
Shooting	***	**	**				
Rooting	***	***					
Callus							***

(b)

RESPONSE	MEDIUM	TEMP.	PHOTO.	M x T	M x P	T x P	M x T x P
Shooting							
Rooting	***		**				
Callus							***

(c)

RESPONSE	MEDIUM	TEMP.	PHOTO.	M x T	M x P	T x P	M x T x P
Shooting				***	***		***
Rooting				**	***	***	
Callus				***	***	**	***

* Interactions are indicated by M (medium number); T (temperature); P (photoperiod).
* = $P < 0.05 - 0.01$; ** = $P < 0.01 - 0.001$;
*** = $P < 0.001$.

callus growth in all explants was found to be governed by multiple interactions of independent variables (Table 5).

Stepwise multiple regression analysis showed that the most frequent variables making significant contributions to the combined treatment regressions were temperature, BAP and photoperiod, whereas IAA was rarely found to be an important variable (only twice in combined treatment analysis) (Table 6).

BAP was found to contribute positively to the shooting response of all three explants. Additionally, cotyledons and cotyledonary axils showed a positive correlation with temperature and a negative correlation with photoperiod (Table 7).

Rooting response was found to vary considerably with the explant, however this response was generally negatively correlated with BAP and positively correlated with temperature (Table 7). The rooting of all three explants was found to be negatively correlated with photoperiod. Rooting of hypocotyls and cotyledonary axils was negatively correlated with both

cytokinins (BAP and Kin), but auxins (NAA and IAA) produced no significant effect upon the rooting of these explants.

The variable most correlated with callus formation of pepper explants was temperature - all three explants exhibited a positive correlation with temperature at $P<0.001$. NAA, BAP and Kin were also found to contribute significantly to this response. (Tables 22-24, Appendix III, present the results of the separate treatment regression analysis of morphological response).

Although regression equations were significant ($P<0.001$), R^2 values were fairly low for most equations (Table 7). The highest R^2 value (0.54) was seen in the rooting response of cotyledons, indicating that 54% of the variation was accounted for by the variables NAA, temperature and photoperiod.

Table 6: Summary of results of stepwise multiple regression analysis on combined treatment (morphological) response data. Explants and morphological response are listed in decreasing order of the R^2-value. Only those regression coefficients (R-values) for individual variables in the equation that were found to be statistically significant ($P<0.05$) are reported. Independent variables are in sequence according to how often they were significant in regression equations.*

EXPLANT	RESPONSE	TEMP	BAP	PHOTO	NAA	KIN	IAA	R^2	P
Coty	rooting	0.17***		-0.07*	0.71***			0.54	0.000
C.Axil	shooting	0.37***	0.14***	-0.29***	-0.28***		-0.20***	0.40	0.000
C.Axil	callus	0.23***	-0.10*	-0.12**	0.52***	0.01*		0.35	0.000
C.Axil	rooting	0.26***	-0.41***	-0.12**		-0.13***		0.30	0.000
Hyp	callus	0.24***	0.08***	-0.21***	0.10***	0.27***	-0.12*	0.24	0.000
Coty	shooting	0.16***	0.39***	-0.21***				0.22	0.000
Coty	callus	0.27***	0.18***		-0.01**	0.22***		0.19	0.000
Hyp	rooting		-0.34***	-0.14**		-0.01*		0.14	0.000
Hyp	shooting		0.18***					0.03	0.000

* P = overall probability of the equation.

* = $P < 0.05 - 0.01$
** = $P < 0.01 - 0.001$
*** = $P < 0.001$

Table 7 : Summary of results of stepwise multiple regression analysis on seperate responses of combined treatment morphological data. Only those regression coefficients (R-values) for individual variables in the equation that were found to be statistically significant (P<0.05) are reported. Independent variables are in sequence according to how often they were significant in regression equations.* [(a) shooting (b) rooting (c) callus formation].

(a)

EXPLANT	BAP	TEMP	PHOTO	NAA	IAA	KIN	R^2	P
COTY.	0.39***	0.16***	-0.21***				0.22	0.000
HYP.	0.18***						0.03	0.000
C.AXILS	0.14***	0.37***	-0.29***	-0.28***	-0.20***		0.40	0.000

(b)

EXPLANT	BAP	TEMP	PHOTO	KIN	NAA	IAA	R^2	P
COTY.		0.17***	-0.07*		0.71***		0.54	0.000
HYP.	-0.34***		-0.14**	-0.01*			0.14	0.000
C.AXILS	-0.41***	0.26***	-0.12**	- 0.13***			0.30	0.000

(c)

EXPLANT	TEMP	NAA	BAP	KIN	PHOTO	IAA	R^2	P
COTY.	0.27***	-0.01**	-0.18***	0.22***			0.19	0.000
HYP.	0.24***	0.10***	0.08***	0.27***	-0.21***	-0.12*	0.24	0.000
C.AXILS	0.23***	0.52***	-0.10*	0.01*	-0.12**		0.35	0.000

* Data = R-values for variables in equation significant at P<0.05.
P = Overall probability of equation
* = P < 0.05 - 0.01
** = P < 0.01 - 0.001
*** = P < 0.001

Regeneration from cotyledonary axil explants.

Medium containing IAA (0.1 mg/l) and BAP (10.0 mg/l) (medium 17) induced callus proliferation, root and shoot formation in cotyledonary axil explants, whereas the medium containing IAA (1.0 mg/l) alone (medium 9) induced the desired shooting and rooting response, without callus proliferation (Table 8).

Successful plantlet formation (Table 9) occurred in only 3 of the 5 media tested (media 9, 17 and 18). Callus formation was absent in media containing IAA alone or BAP alone (media 9 and 18). Explants cultured in media 8 (0.05 mg/l IAA + 10.0 mg/l BAP) and 16 (0.1 mg/l IAA + 5.0 mg/l BAP) formed shoots, but not roots. When these shoots were placed in rooting medium (1.0 mg/l IAA) much callus proliferation was apparent, and while rooting was essentially successful, plantlets failed to form since the callus growth tended to engulf the small shoots that had formed previously.

Medium 9, containing 1.0 mg/l IAA, proved to be the most successful in _in-vitro_ plantlet formation.

TABLE 8 : Comparison of media from experiments 1 and 2, reporting morphological response of cotyledonary axils cultured for 30 days at 28°c and 16-hour photoperiod in various modified media. Eight explants were cultured per treatment .

MEDIUM* #	GROWTH REGULATORS	MORPHOLOGICAL** RESPONSE
1	NAA 1.0mg/l	R(++) C(+++)
8	IAA 0.05mg/l BAP 10.0mg/l	S(+)
9	IAA 1.0mg/l	R(++) S(++)
16	IAA 0.1mg/l BAP 5.0mg/l	S(+++) C(+++)
17	IAA 0.1mg/l BAP 10.0mg/l	R(+) S(+++) C(+++)
18	BAP 5.0mg/l	S(++)

* Medium numbers 1, 8 and 9 were taken from Exp.1
Medium numbers 16, 17 and 18 were taken from Exp.2

** C = callus; R = roots; S = shoots
(+++) much response; (++) moderate response; (+) little response.

TABLE 9 : Results of plantlet formation from cotledonaryary axil explants cultured at 28°c and 16-hour photoperiod, in various modified media. To elicit a rooting response, regenerated shoots which had not developed roots on original medium were placed in 1.0 mg/l IAA.

MEDIUM #	GROWTH REGULATORS	# EXPLANTS CULTURED	DAYS IN CULTURE	RESPONSE	SUCCESSFUL R	SUCCESSFUL P*
8	IAA 0.05mg/l BAP 10.0mg/l	12	26	S-11(+)** C-1(+)	3	-
9	IAA 1.0mg/l	12	20	S-10(++) R-9(++)	N.A.	9
16	IAA 0.1mg/l BAP 5.0mg/l	8	67	S-6(+++) C-8(++++)	1	-
17	IAA 0.1mg/l BAP 10.0mg/l	8	67	S-8(+++) R-4(+) C-8(+++)	N.A.	4
18	BAP 5.0mg/l	11	26	S-11(++)	7	5

* R = rooting success; P = plantlet formation

** - S = shoots; R = roots; C = callus
- Numbers indicate the number of explants responding.
- (+++) extensive response; (+++) much response; (++) moderate response; (+) little response.

The morphological response of cotyledonary axils to this medium was very fast, when compared to responses induced by other media (plantlets formed in less than 20 days), and plantlets could be easily transferred to greenhouse conditions where they were grown to maturity. Table 10 presents combined results of several experiments which monitored the success rate of plantlet formation from cotyledonary axils cultured in medium 9 and survival (i.e. growth to maturity) rate of plantlets transferred to greenhouse conditions.

Greenhouse and Field Trials

Results of ANOVA showed that greenhouse trial number had no effect on plant height, weight of fruit (both yields), nor on age of fruit set and age of fruit harvest of the second yield. Greenhouse trial number was found to effect leaf number, age of fruit set and harvest as well as number of fruit in yield 1, and number of fruit harvested in yield 2. Plant type had a significant effect on all parameters except number and weight of fruit in the second yield.

TABLE 1Q : Success rate of plantlet formation and survival rate of plants produced from *in-vitro* culture of Cotyledonary Axil explants cultured in medium supplemented with 1.0 mg/l IAA (medium 9)*

# EXPLANTS CULTURED MEDIUM 9	# PLANTLETS PRODUCED *IN-VITRO*	# PLANTS GROWN TO MATURITY	% SUCCESS	% SURVIVAL
73	54	47	74	87

* Results from five experiments

In spite of differences observed between trials of individual plant types (Appendix IV), overall evaluation of greenhouse and field trials combined indicate that the first and second generations of the seed plant grew significantly taller (P=0.0000) than the first and second generations of the regenerated plant (Figs. 16 and 28).

Although the manner in which plants produced new leaf growth was very different in greenhouse and field trials (Figs. 23-25), overall evaluation of combined greenhouse and field trials indicated that there were no significant differences in maximum leaf number (P>0.05) between plant type (Fig. 16). However, it was observed that leaves produced on F-1 regenerated plants grown in the field were a deeper green color than those of F-1 seed plants under the same conditions (Fig. 25).

Notwithstanding differences found between greenhouse trials and field trials the data indicated that the first generation regenerated plants set fruit significantly earlier (P=0.0000) than other plant types (Fig. 17). F-1 regenerated plants also had an earlier

harvest age than other plant types (P=0.0000) (Fig. 17). For time between fruit set and harvest (yield 1), no significant differences (P>0.05) were found between plant type even though some differences were observed between greenhouse and field trials (Fig. 18).

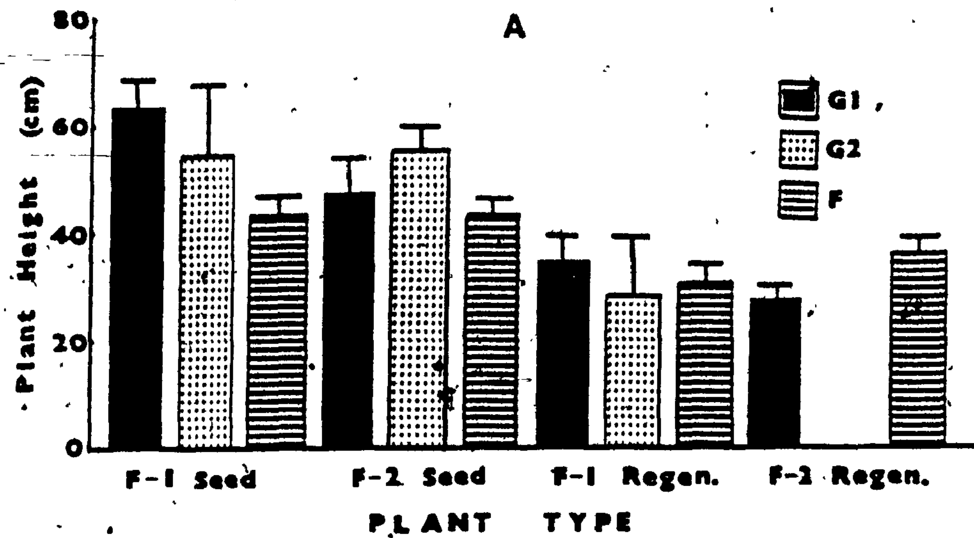
Plants grown in the field produced consistently more fruit than their greenhouse counterparts, (P=0.0000) in all cases. Evaluation of combined treatments showed that F-2 seed plants produced significantly (P=0.002) more fruit than either F-1 seed or F-2 regenerated plants (Fig. 19). F-1 regenerated plants produced significantly (P=0.0000) heavier fruit than other plant types, while F-1 seed plants produced significantly (P=0.0000) heavier fruit than F-2 seed plants (Fig. 19).

Parameters measured for the second fruit yield were evaluated only for greenhouse trials since a second yield was not obtained from plants grown in the field. F-1 regenerated plants set a second crop of fruit significantly (P=0.0000) earlier than other plants (Fig. 20). Similarly, F-1 regenerated plants had a significantly (P=0.0000) earlier harvest age than

both F-2 seed and F-2 regenerated plants. (Fig. 20). F-1 seed plants took significantly (P=0.0001) less time to produce a second yield of mature fruit than all other plant types (Fig. 21). In the second yield, no significant differences (P>0.05) were found between plant types for fruit number or weight of fruit. (Fig. 22).

Figures 16 - 22 represent growth and fruit yield data obtained from F-1 seed, F-2 seed, F-1 regenerated and F-2 regenerated plants grown in the greenhouse and in field conditions. Error bars represent 95% confidence intervals. Forty plants of each plant type were monitored (20 plants through two greenhouse trials, except for F-2 regenerated plants where 20 plants were observed in one greenhouse trial, and 20 in the field trial).

G1 = greenhouse trial #1
G2 = greenhouse trial #2
F = field trial



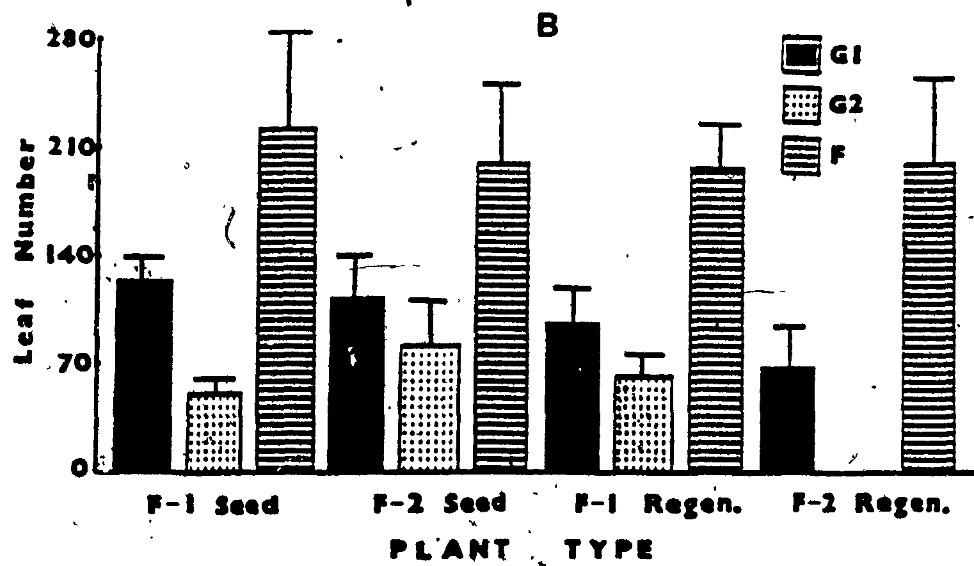
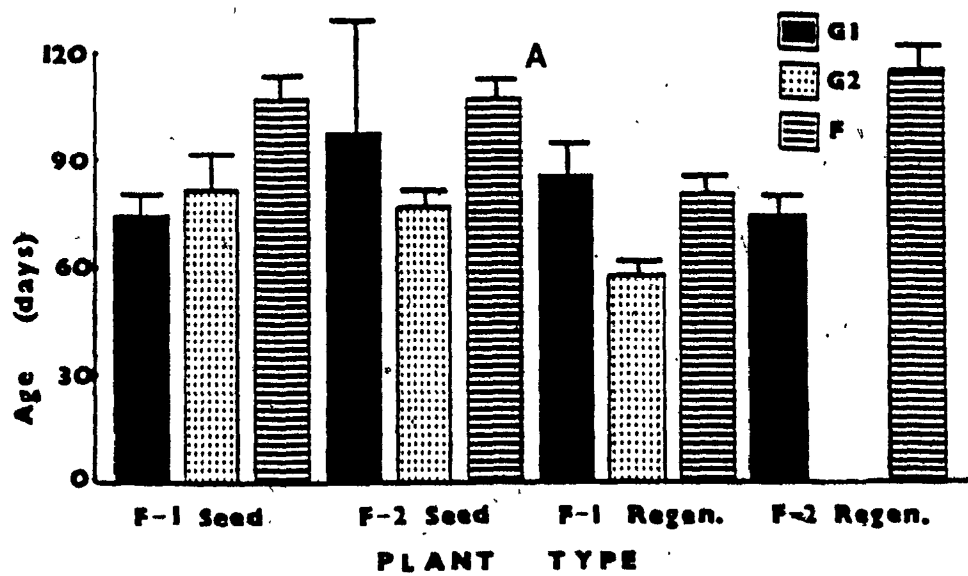


Fig. 16: Average maximum plant height (cm) (A) observed throughout experimental period, and average maximum leaf number (B) observed throughout experimental period.



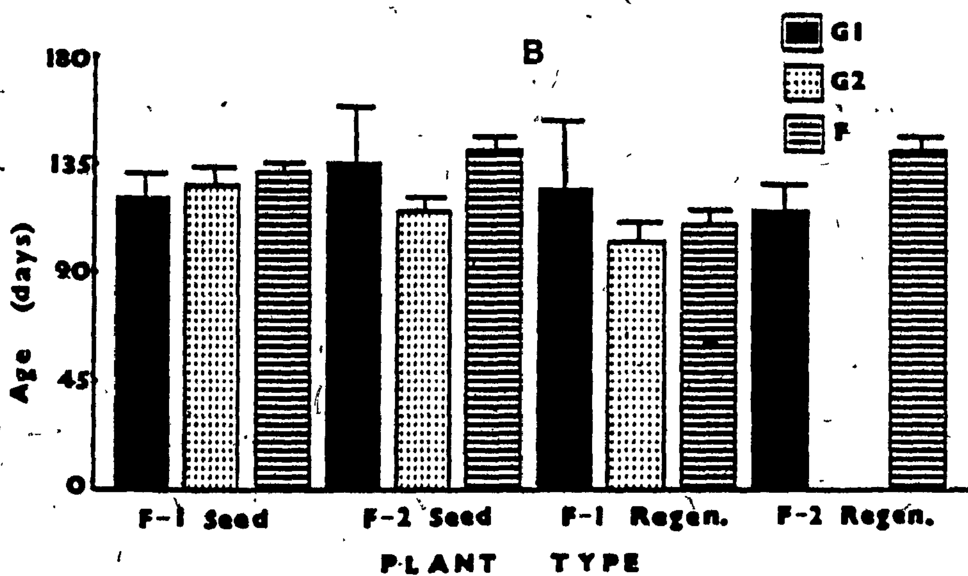


Fig. 17: Average age (days old) of plants at first fruit set (A), and average age (days old) of plants at fruit harvest (B). (Yield 1).

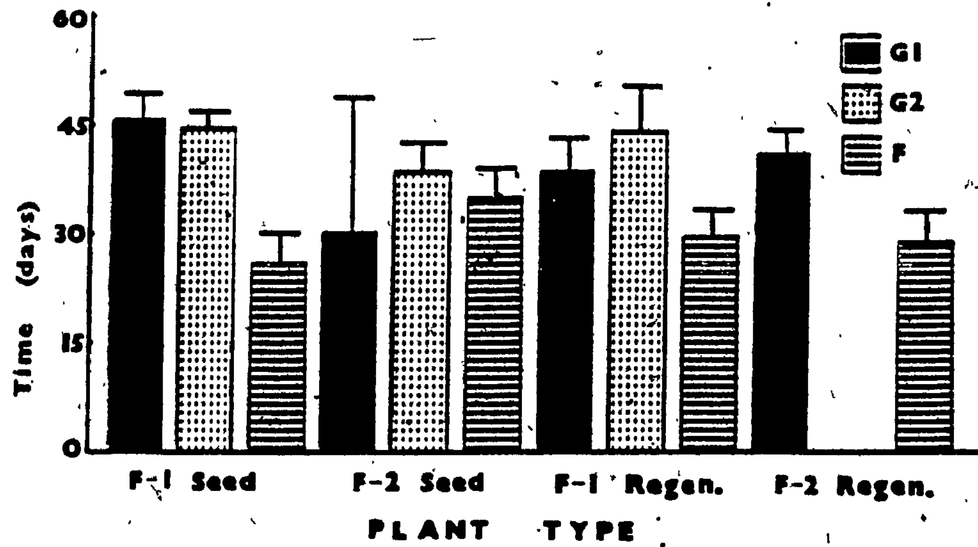
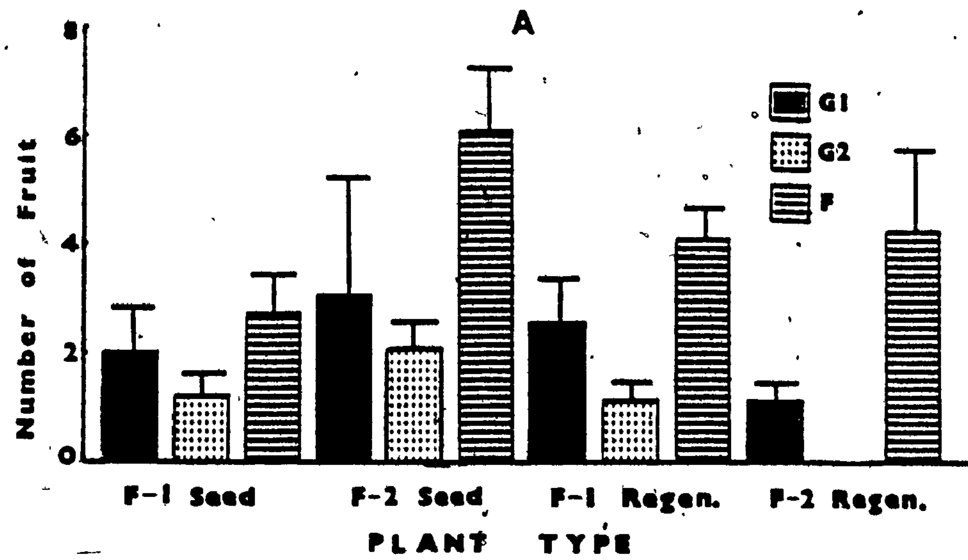


Fig. 18: Average time (days) between fruit set and fruit harvest. (Yield 1).



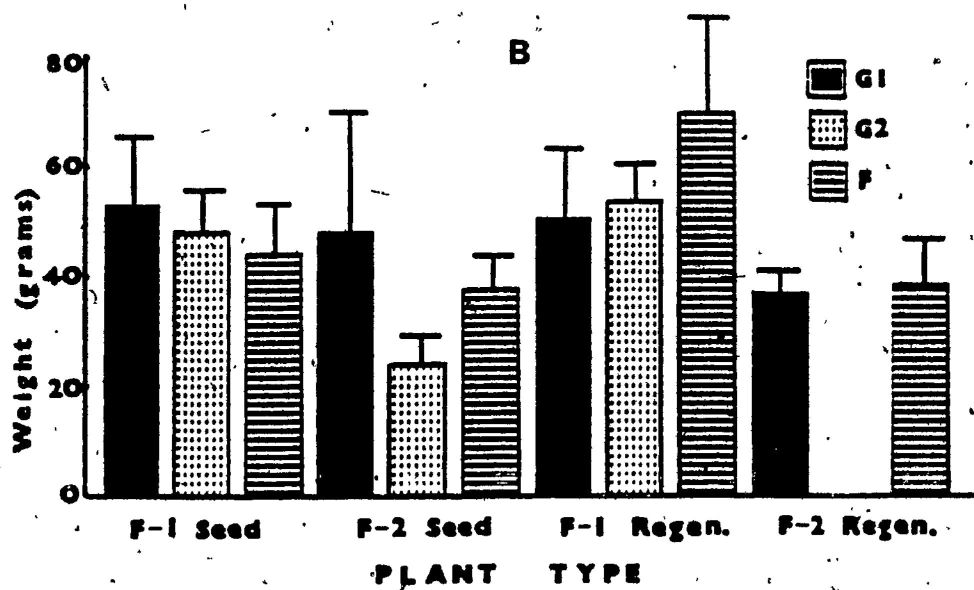
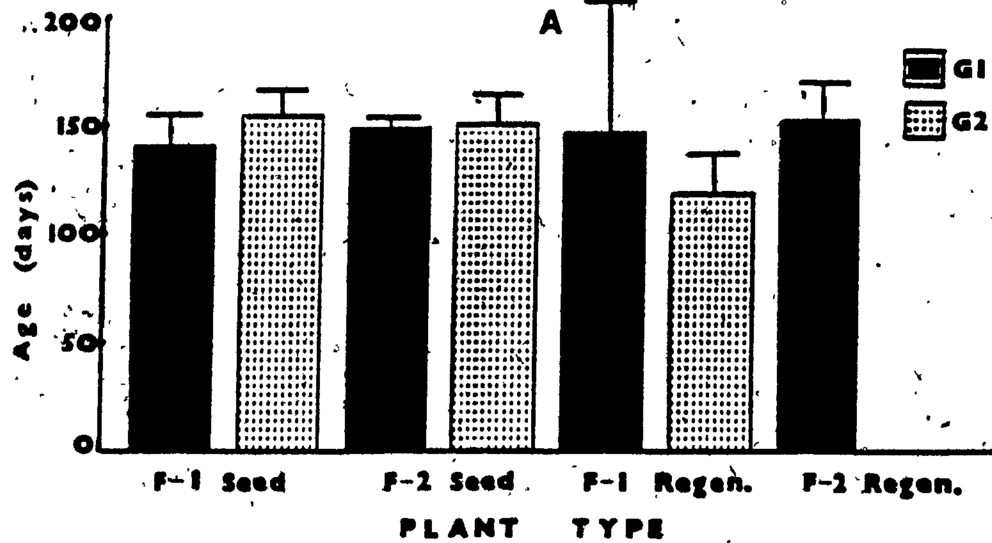


Fig. 19: Average number of fruit harvested (A), and average weight (grams) of fruit (B). (Yield 1).



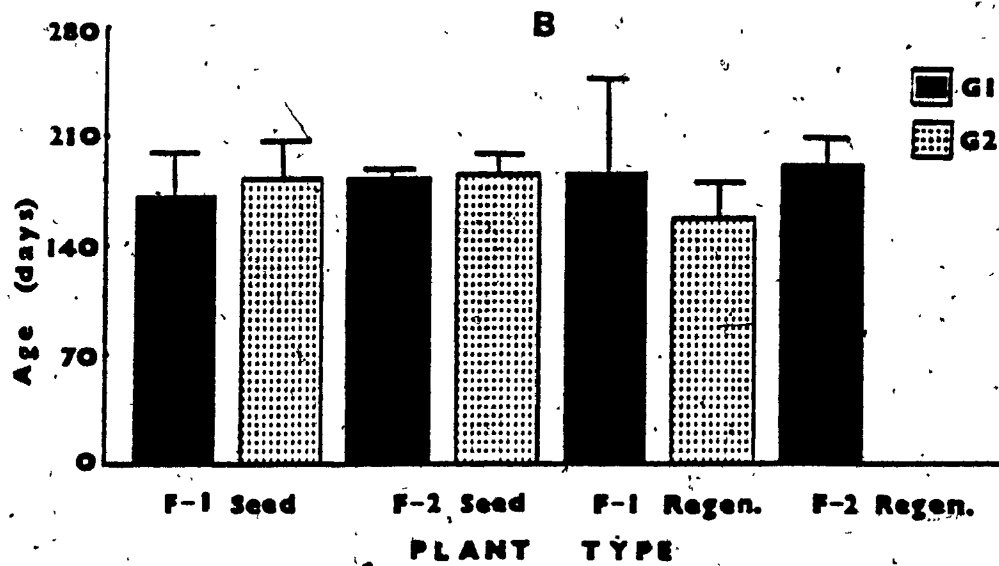


Fig. 20: Average age (days old) of plants at second fruit set (A), and average age (days old) of plants at second fruit harvest (B). (Yield 2).

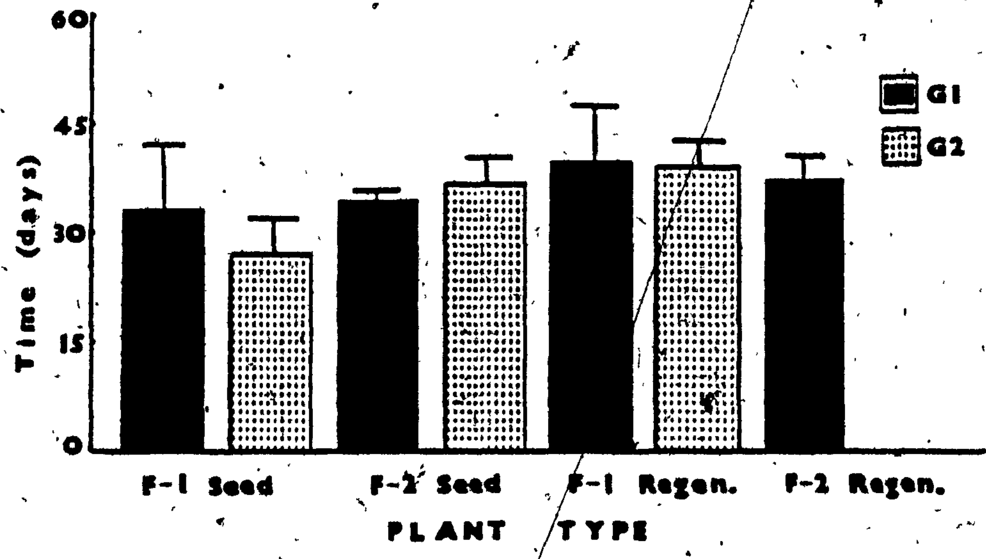
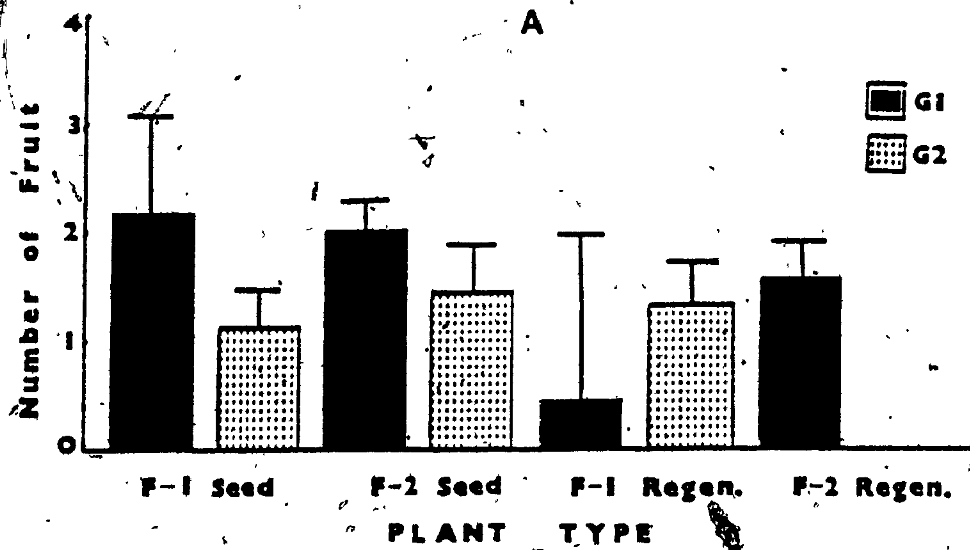


Fig. 21: Average time (days) between fruit set and fruit harvest. (Yield 2).



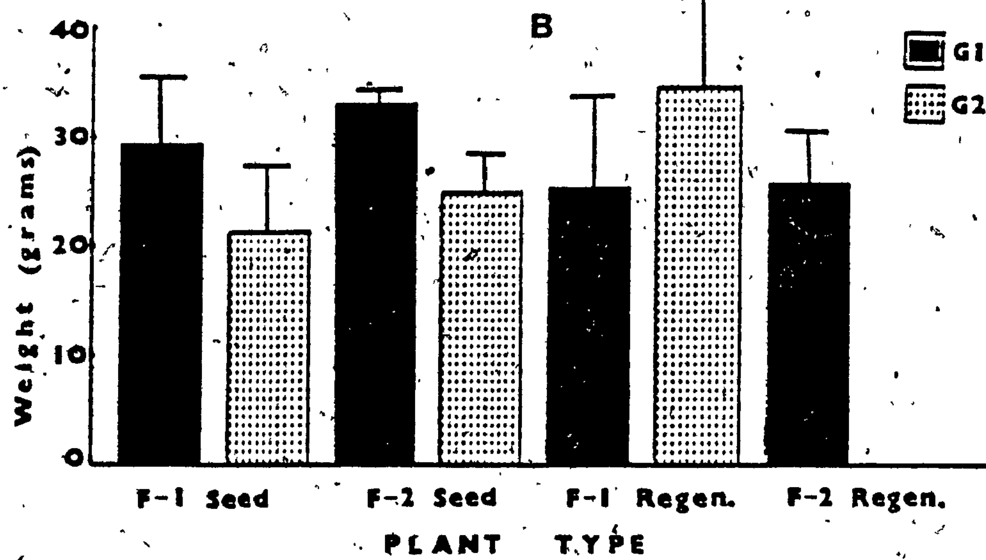


Fig. 22: Average number of fruit harvested (A), and average weight (grams) of fruit (B). (Yield 2).

Figure 23. Comparison of height of F-1 regenerated (A) and F-1 seed (B) plants grown under greenhouse conditions. (A is approximately 15 days younger than B). Notice that both plants have set fruit.

A
B

Figure 24. Plants grown under field conditions. (a = F-1 seed plants; b = F-1 regenerated plants; c = F-2 seed plants; d = F-2 regenerated plants).

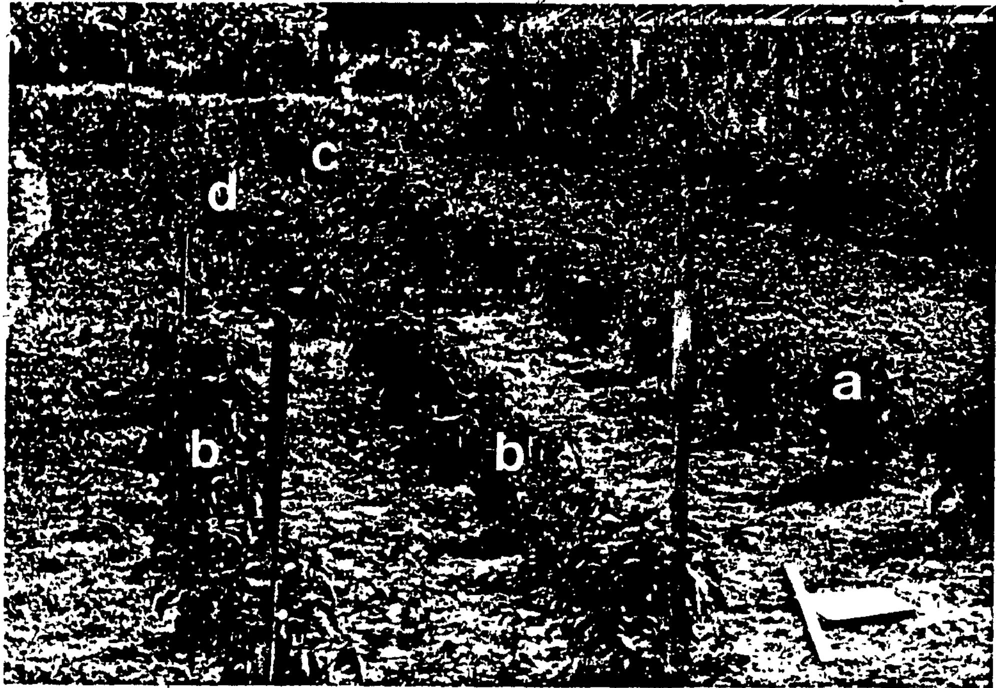

c
d
a
b
b

Figure 25. Comparison of F-1 seed (A) and F-1 regenerated (B) plants grown under field conditions. Notice the differences in height and leaf color.

A

B

Micropropagation

Elongation of multiple shoots.

Four different media (11, 18, 31 and 32), which had the ability to induce shooting in cotyledons, were also found to elicit the same response in apical meristem explants (Table 11). Shooting occured in all apical meristems in all media, while the amount of callus proliferation varied according to culture medium. All media induced shooting in a similar manner - masses of compact leaves developed from the apex, as well as from the cut base of the meristem. After 30 days of culture, no shoot elongation was apparent, and upon subculture to the same medium, callus proliferation continued, masses of leaves increased somewhat, but elongation was not observed.

After 30 days of culture, those shoots which were placed in GA_3 (0.1 mg/l) showed increased callus proliferation. Those placed in rooting medium (0.1 mg/l NAA + 00.1 mg/l BAP) also showed increased callus proliferation, although some rooting also occurred. Shoots placed in either GA_3 or rooting medium and

placed in darkness became pale brown or yellow in color, and also showed increased callus growth. Elongation of shoots was not apparent, and in most cases, the callus growth was so extensive, that it tended to engulf the already existing shoots.

In the combined growth regulator [BAP (20.0 mg/l) + GA_3 (0.1 - 5.0 mg/l)] experiment, as the concentration of GA_3 in the medium increased, the callus response of cotyledon explants also increased, while the shooting response decreased (Table 12). Although shooting of apical meristems (Table 13) remained fairly stable, with increasing concentrations of GA_3, elongation of shoots was not observed. In the separate growth regulator experiment (i.e. explants cultured in 20 mg/l BAP for 30 days, then shoots transferred to 0.1 - 5.0 mg/l GA_3), results were similar in all media, although shooting of apical meristems tended to be hindered by 5.0 mg/l GA_3 (Table 13). A rooting response was observed when meristems were cultured in 0.5 mg/l GA_3 (Table 13, Fig. 26). Although a limited amount of elongation was observed [one meristem cultured in medium 43 (0.5 mg/l GA_3), and

one in medium 46 (5.0 mg/l GA_3)], this appeared to occur from only one shoot within the masses of compact leaves present on the tissue. Thus, elongation of multiple shoots was not achieved in these experiments.

Subsequently, seven meristems which had the most profuse shooting response, were transferred to MS basal medium (containing no growth regulators) and cultured for another 3 weeks. Roots developed on five of the seven meristems cultured, and some elongation was apparent on one. These responses were coupled with limited callus growth. Five resulting plantlets were transferred to greenhouse conditions, where only two survived. After four months one plant had grown to approximately 20 cm. in height and had borne two very small fruit, while the other had grown to approximately 6 cm. and did not bear fruit.

Table 11: Morphological response of apical meristem explants cultured for 30 days at 28°c and 16-hour photoperiod, in media previously shown to induce shoots in cotyledon explants. Ten explants were cultured per treatment.

MEDIUM #	GROWTH REGULATORS	RESPONSE Callus	Shoots	Roots
11	IAA (4.0mg/l) BAP (10.0mg/l)	4(+)*	10(++++)	-
18	BAP (5.0mg/l)	6(++)	10(++++)	-
31	BAP (10.0mg/l)	2(+)	10(++++)	-
32	BAP (20.0mg/l)	-	10(++++)	-

* Numbers indicate the number of explants responding

(++++) extensive response; (+++) much response; (++) moderate response; (+) little response; (-) no response.

Table 12: Results of experiment designed to induce shooting, and a shoot elongation response in cotyledon explants. Ten explants were cultured per treatment, under 28°c, 16-hour photoperiod condition.

MEDIUM #	BAP (mg/l)	GA_3 (mg/l)	RESPONSE* Callus	Roots	Shoots	Elongation
37	20.0	0.1	10(+)	-	7(++)	-
38	20.0	0.5	10(+)	-	5(+)	-
39	20.0	1.0	4(++)	-	2(+)	-
40	20.0	2.0	10(+++)	-	2(+)	-
41	20.0	5.0	9(+++)	-	-	-

* Numbers indicate number of explants responding; (+++) much response; (++) moderate response; (+) little response; (-) no response.

Table 13: Results of experiment designed to induce a shooting, and a shoot elongation response in apical meristem explants (BAP + GA_3), and to induce shoot elongation after a shooting response had been obtained in 20.0 mg/l BAP alone (GA_3 alone). Ten explants were cultured per treatment, under 28°c, 16-hour photoperiod condition.

MEDIUM #	BAP (mg/l)	GA_3 (mg/l)	RESPONSE* Callus	Roots	Shoots	Elongation
37	20.0	0.1	10(+)	-	2(+++)	-
38	20.0	0.5	10(+)	-	1(+++)	-
39	20.0	1.0	10(+)	-	3(+++)	-
40	20.0	2.0	10(Death of tissue)			
41	20.0	5.0	10(Death of tissue)			
42	-	0.1	10(+)	-	10(+++)	-
43	-	0.5	10(++)	4(+)	10(+++)	1
44	-	1.0	10(++)	-	10(+++)	-
45	-	2.0	10(Death of tissue)			
46	-	5.0	10(++)	-	10(+)	1

* Numbers indicate number of explants responding
(+++) much response; (++) some response
(+) little response; (-) no response

Figure 26. Organogenesis in an apical meristem explant cultured in medium containing BAP (20.0 mg/l) for 30 days and transferred to medium supplemented with 0.5 mg/l GA_3 for 30 days. This explant was cultured at 28°c, 16-hour photoperiod.

Organogenesis from callus tissue.

Callus growth was successfully induced in cotyledon, hypocotyl and cotyledonary axil explants cultured in medium containing IAA (0.35 mg/l), 2,4-D (0.22 mg/l) and Kin (0.02 mg/l). In all cases callus grew over the entire surface of the tissue, and proliferation was extensive within a 30-day culture period at 28°c, 16-hour photoperiod. Proliferation was similar, although less extensive, when explants were cultured at 22°c, 16-hour photoperiod.

Results were consistent, whether explants were cultured in callus-inducing medium for 30, 60 or 90 days before being subcultured into shoot-inducing or root-inducing media. (Table 14). At 28°c, the tissue slowly turned from a green-compact callus tissue, to a brown-friable tissue within the first 30 days of culture in shooting or rooting media. After subculture on the same medium for another 30 days, the tissue became darker brown in color, and remained in this state. At 22°c, the tissue tended to remain green and compact for an extended period of time. Darker green

globules were apparent on some tissue in this condition, but after culture in shooting or rooting medium for up to 90 days, no organogenesis was observed.

Table 14: Results of callus culture of three different explants cultured at 16-hour photoperiod from 30 to 90 days in callus producing medium, before being placed in shoot-inducing or root-inducing medium for up to 90 days.

TEMP	# OF EXPLANTS IN CALLUS-INDUCING MEDIUM *		# OF DIFFERENT MEDIA		OBSERVATIONS
			Shooting	Rooting	
28°c	Cotyledons	44	7	2	white/green (compact) ->brown(compact) -> Death
	Hypocotyls	42	7	2	white/green/ brown (friable) ->brown (very friable) ->Death
	C. Axils	10	7	2	white/ green/brown (friable/compact) ->brown (friable) ->Death
22°c	Cotyledons	6	3	1	white/green (compact) ->white/green/brown (compact)
	Hypocotyls	6	3	1	white/green (compact) ->white/brown/green (compact)

* Callus medium : 0.35mg/l IAA + 0.22mg/l 2,4-D + 0.02mg/l Kin.

** Shooting media: media numbers 8, 11, 17, 18, 23, 31, 32 (consituents listed in materials & methods).

*** Rooting media: media numbers 1, 9 and 24 (constituents listed in materials & methods).

DISCUSSION

Fundamental to the induction of organogenesis in plant tissues *in-vitro* is the receptivity of plant cells to external stimuli, especially plant growth regulators. Auxin-cytokinin balances exert a controlling influence on shoot and root induction from cultured explants.

Experimental results showed that cotyledon, hypocotyl and cotyledonary axil explants of Early Prolific Hybrid Pepper responded differently to the same medium conditions. Both cotyledon and cotyledonary axil explants responded with shoot, root and/or callus growth, whereas hypocotyls rarely produced shoots (Table 4, Figs. 4-12). That different explants of *Capsicum* respond differently to the same growth regulator combinations has also been shown by Phillips and Hubstenberger (1985) and Gunay and Rao (1978), and since response to exogenous applications of growth regulators has been found to be dependent upon

their endogenous concentrations, different explants of most plant species cultured *in-vitro* tend to respond differently to the same medium conditions (George and Sherrington, 1984).

The results of present experiments showed that cotyledonary axil explants exhibited a greater capacity for shoot formation than did hypocotyls or cotyledons (Figs. 4-6). Phillips and Hubstenberger (1985) also found that cotyledonary axils exhibited a greater capacity for shoot organogenesis than hypocotyl sections and distal cotyledon pieces, and in general, it has been found that meristematic explants (eg. cotyledonary axil segments) show a greater potential for shoot formation than do non-meristematic explants (eg. hypocotyl) (George and Sherrington, 1984).

A high ratio of cytokinin to auxin tended to induce a shooting response in cotyledons of Early Prolific Hybrid Pepper, a finding consistent with the observations of Phillips and Hubstenberger (1985) and Gunay and Rao (1978). Further, explants of most plant species cultured *in-vitro* have also been shown to require a high ratio of cytokinin to auxin for shoot induction (George and Sherrington, 1984). On determining the best auxin to cytokinin ratio for shoot

formation, it was found that low IAA concentrations (0 - 4.0 mg/l), coupled with high BAP concentrations (2.0 - 20.0 mg/l) induced the best shooting response in cotyledons. However, it is interesting to note that when NAA was used in place of IAA, shoot formation tended to be inhibited.(Table 4, Fig. 4). This has also been observed in other varieties of Capsicum (Phillips and Hubstenberger, 1985). In other plant explants, for expample, elephant yam (Arditti and Nyman, 1985) and african violet (Start and Cumming, 1976), the presence of low concentrations of NAA together with BAP stimulated shoot formation.

When BAP alone was present in the medium, shoot formation was also induced in cotyledons. However, a concentration of 5.0 mg/l was required to induce a shooting response in cotyledons of Early Prolific Hybrid Pepper, whereas Gunay and Rao (1978) had reported that only 2 mg/l BAP was required to induce shoot bud formation in cotyledons of California Wonder, Pimento, and Barath. It is possible that endogenous BAP concentrations were lower, or that auxin levels were higher in Early Prolific Hybrid Pepper as compared to those varieties tested by Gunay and Rao (1978).

Cotyledonary axils exhibited a greater capacity for root formation than hypocotyl explants, while cotyledons tended to show fewer roots (Figs. 7-9). These results contradict those obtained by Phillips and Hubstenberger (1985), who observed that hypocotyl sections and distal cotyledon pieces showed a greater capacity for root organogenesis than did cotyledonary axils. In the present experiments, roots formed on cotyledonary axil explants in media supplemented with auxin and cytokinin, as well as when cytokinin alone was present (Fig. 9). However, media containing 1.0 mg/l IAA, or 0.05 mg/l each of IAA and BAP induced a significant rooting response in cotyledonary axil explants (Fig. 9).

In agreement with the findings of Gunay and Rao (1978), rooting was found to occur in cotyledons and hypocotyls (Figs. 7 and 8), in the presence of auxin alone, or with BAP. Formation of roots was also induced in these explants when auxin:cytokinin ratios were equal, as well as when media contained a higher concentration of cytokinin than auxin (Table 4, Figs. 7-8). Phillips and Hubstenberger (1985) also found that equal concentrations (0.05 mg/l) of both IAA and BAP induced the best rooting response in hypocotyl and

cotyledon segments of *Capsicum*. However, it is generally accepted that a high ratio of auxin to cytokinin will stimulate the onset of root growth in cultured explants of most plant species (Bhojwani and Razdan, 1983). Therefore, as observed earlier, this may indicate that explants of *Capsicum* contained high endogenous concentrations of auxin. The best root inducing media for cotyledons contained 0.10 and 1.0 mg/l NAA coupled with 0.10 and 0 mg/l BAP respectively. In addition to these formulations, equal concentrations of IAA and BAP (0.05 and 0.10 mg/l) and 1.0 mg/l IAA alone were found to induce the best rooting response in hypocotyl explants (Table 4, Fig. 8).

Roots produced from cotyledon and hypocotyl explants were different depending on whether IAA or NAA was included in the medium. Those produced by both explants in the presence of NAA alone were short and thick and accompanied by much callus growth, while roots produced on hypocotyl explants when IAA alone was present in the medium, were long and thin with lateral root development, and callus growth was completely surpressed. This is in agreement with the findings of Gunay and Rao (1978). However, when BAP was present with NAA or IAA, cotyledons and hypocotyls produced

long, thin roots, with lateral root development. It thus appears, that while BAP is not required to induce a rooting response, as indicated by Gunay and Rao (1978), its presence tends to produce a better root system than when NAA is used alone, and this tends to support the observation of Phillips and Hubstenberger (1985) that BAP is important in rooting.

Notwithstanding the importance of media formulation to organogenesis, Phillips and Hubstenberger (1985) showed that a variation in temperature and photoperiod also had an effect on morphogenesis of Capsicum. It was considered of interest, therefore, to determine the effect of varying media conditions on cotyledonary axil explants of Early Prolific Hybrid Pepper, and to determine the effects of varying temperatures and photoperiods on all three explants (cotyledons, hypocotyls, cotyledonary axils). However, where Phillips and Hubstenberger (1985) used only two temperature conditions (25 and 28.5°c), the present experiment employed three temperature conditions (18, 22 and 28°c). Where these investigators utilized three photoperiod regimes (12, 16 and 24-hours), the present experiment used only two (16 and 24-hours). The shorter photoperiod was not taken into consideration,

as Phillips and Hubstenberger (1985) had shown that a 12-hour photoperiod inhibited morphogenic response considerably.

Results of these experiments have shown that morphological response differs depending upon the explant, temperature, photoperiod, and growth regulator constituents utilized. All explants tended to respond best with root and/or shoot formation at 28°c and 16-hour photoperiod. Consistent with initial results, cytokinins tended to promote a shooting response, and the presence of auxin tended to promote rooting in all three explants tested. (Figures 4 to 12) (Appendix II).

Generally, shooting intensity of cotyledons and cotyledonary axils was greatest at high temperatures and 16-hour photoperiods (Figs. 4 and 6) (Appendix II). Phillips and Hubstenberger (1985) also observed that cotyledonary nodes exhibited increased shooting as both temperature and photoperiod increased, but, contrary to results of the present experiment, these investigators found that hypocotyl sections and distal cotyledon pieces exhibited the highest shooting response at 25°c, continuous light. Other investigators, for example, have shown that a 16-hour photoperiod stimulates organogenesis in lentil (Williams and McHugen, 1986),

raspberry (Donnelly et al, 1985) and tomato (Kurtz and Lineberger, 1983). Also, explants of different plant species exhibit different temperature requirements for shoot formation [eg. 25°c for olive (Diamantoglou and Mitrakos, 1979); 18°c for cauliflower (Walkey and Woolfitt, 1970)].

Depending on media conditions, root formation was significantly higher in all explants at 16-hour photoperiod. The best temperature for rooting of cotyledons and cotyledonary axils was 28°c and that of hypocotyls generally tended to be best at 22°c (Figs. 7-9) (Appendix II). These results are in contradiction to results obtained by Phillips and Hubstenberger (1985), who found that root organogenesis was greatest in cotyledonary nodes under continuous light, at 25 or 28.5°c, and in hypocotyl and cotyledon explants at 25°c and 16-hour photoperiod.

Results of ANOVA showed that media formulation, temperature, and photoperiod all had an effect on the morphological response of explants. These results reflect observations as seen in Figures 4 to 12, and support previous findings by Skoog and Miller (1957) that the induction of _in-vitro_ organogenesis is a function of auxin-to-cytokinin ratios, and that

temperature and light also play an important role in the organogenesis of plant tissue <u>in-vitro</u> (George and Sherrington, 1984). However, since growth regulator constituents utilized in this experiment were not increased or decreased in a systematic manner, it was necessary to determine which regulators were responsible for the effects observed. Similarly, furthur analysis was required to accurately determine whether temperature and photoperiod effects were positive or negative, as well as the manner in which these variables interacted with growth regulator and explant effects.

Observations of explant morphological response in the present experiment were initially reported using the "+++" method as in initial experiments. However, this became a cumbersome method of reporting response because of the number of treatment conditions involved in this experiment. Since not all explants in the same treatment condition responded to the same extent, this could also be misleading. It was thus considered pertinent to develop a more comprehensive method of reporting response, which would take into account the number of explants within a treatment condition that responded, as well as the extent of their response.

Since this method yielded data that was still cumbersome, involving two figures - % frequency and response intensity of explants responding - this data was converted to one value indicating average response intensity of the population.

Response intensity data did not consist of continuous variables, a basic assumption of multiple regression analysis, but were basically categorical variables, since initial evaluation of response consisted of subjective indicies [(i.e. very much (4), much (3), some (2), little (1), very little (0.50), almost no (0.25), or no (0) response)].

Although morphological data violated some basic asumptions of multiple regression analysis, results were fairly consistent with those obtained in HILOGLINEAR analysis, which assumes that data consist of categorical, or discrete, variables. For this reason, results of multiple regression analyses have been utilized to show the <u>direction</u> of the effect, as well as for the determination of the medium constituent responsible for the media formulation effect. However, R-values and R^2-values indicated by regression analysis were ignored, since they cannot be considered accurate. HILOGLINEAR analysis established degree of significance

at $P<0.05$, thus probability values obtained in regression analysis are considered redundant.

HILOGLINEAR analysis (Table 5) showed that the shooting response of cotyledons was governed by individual effects of media formulation, temperature and photoperiod. Multiple regression analysis indicated that temperature had a positive effect and photoperiod had a negative effect upon the shooting of cotyledons, and also indicated that, of the medium constituents, BAP effected this response in a positive manner (Table 7). However, when temperature and photoperiod were held constant in the regression analysis of cotyledon shooting response, IAA was also found to contribute to this response: negatively at 18°c, 16-hour photoperiod and 28°c, continuous light, and positively at 22°c, 16-hour photoperiod (APPENDIX III) These results appear to indicate that the different regulators operate differently under different environmental conditions, that is, at extreme conditions, auxin acted in a negative manner, while at the intermediate condition it had the opposite effect.

HILOGLINEAR analysis showed that the shooting response of cotyledonary axil explants was governed by multiple interactions of media formulation and

temperature, media formulation and photoperiod, and media formulation, temperature and photoperiod, indicating that a seperate equation could be formulated for each of these interactions - the resulting three equations, explaining the shooting response. Stepwise regression analysis indicated that temperature had a positive effect and photoperiod had a negative effect on the shooting of cotyledonary axils. This analysis also indicated that BAP had a positive effect, while both auxins (IAA and NAA) had a negative effect on this response. When temperature and photoperiod remained constant, BAP and Kin were found to contribute positively at 18 and 22°c, but negatively at 28°c. Depending on the environmental condition, it was again observed that growth regulators had opposing effects.

Rooting response of hypocotyls was found to be controlled by media formulation and photoperiod in HILOGLINEAR analysis. In regression analysis photoperiod was found to contribute negatively, as was BAP, and Kin was found to contribute negatively to a slight extent. Thus, temperature appears to have less effect upon the rooting of hypocotyls than of other explants.

It is clear that explants responded, by root formation, to growth regulators in a different manner. That cotyledons responded positively to NAA, while both hypocotyls and cotyledonary axils responded negatively to cytokinins, is interesting. As demonstrated by statistical analysis, these latter explants did not respond to auxin. However, one can see that rooting of cotyledons occurred in the one medium containing NAA alone, and that this response generally did not occur in any other media (Figure 7). Alternatively, rooting of hypocotyls and cotyledonary axils, occurred in various media (Figs. 8 and 9). Rooting was found to be much less intense, however, in media containing increasing amounts of cytokinin. Although auxins obviously had a positive effect on the rooting of these two explants, the presence of cytokinin drastically decreased this response, and thus cytokinin was found to have a very significant negative effect on this response. While regression analysis yields some very interesting information on the effect of growth regulators on the morphogenic response of explants, it must be interpreted in a cautious manner.

Plantlets were formed in-vitro from cotyledonary axils cultured at 28°c and 16-hour photoperiod, on MS

basal medium supplemented with only 1.0 mg/l IAA. When NAA was substituted for IAA, shooting was completely surpressed and callus proliferation was fairly extensive. This may indicate that NAA (a synthetic auxin) acts in a different manner than does IAA (a naturally occurring auxin). When IAA was held constant at 0.10 mg/l, and BAP increased from 5.0 to 10.0 mg/l, a rooting response was seen to accompany the induction of shooting and callus proliferation. These results appear to support previous observations that BAP is somehow involved in rooting. However, when BAP alone was present in the medium, shooting occurred in the absence of callus growth. Thus, a high concentration of BAP in the presence of a low concentration of IAA, or alone, tends to supress callus proliferation in cotyledonary axil explants of Early Prolific Hybrid Pepper.

Cotyledonary axils which had produced shoots, were induced to root in 1.0 mg/l IAA. Callus growth hindered plantlet formation, as these plantlets were not formed when callus was present.

In previous studies, many investigators were able to achieve plantlet formation after a 30-day culture period (Gunay and Rao, 1978; Fari and Czako, 1981;

Saxena, et al., 1981; Agrawal and Chandra, 1983). However, in the present study, the presence of 1.0 mg/l IAA in the media, and conditions of 16-hour photoperiod and 28.5°c, induced plantlet formation within 20 days, thus reducing culture time by 10 days.

Although success had been achieved in the regeneration of Early Prolific Hybrid Pepper through cotyledonary axil culture, only one plantlet was obtained from each axil cultured. Thus, it was considered of economic interest to attempt to obtain multiple plantlets from each cultured explant. Both cotyledon and apical meristem explants, cultured under similar conditions, were found to be capable of producing masses of leaf-like shoots _in-vitro_, and thus were candidates for multiple plantlet formation (micropropagation). Since GA_3, as well as etiolation, have been considered important in cell elongation (Salisbury and Ross, 1978), these factors were used in an attempt to induce elongation in established multiple shoots. Campiotti, et al. (1986) had also failed to induce elongation in apical sections of _Capsicum_. Although elongation was not achieved in these experiments, I believe that these explants, and especially apical meristems, are capable of producing

multiple plantlets. Furthur research is therefore required to determine conditions under which elongation may be induced.

The induction of callus growth in cotyledon, hypocotyl and cotyledonary axils was successful, but the induction of morphogenic responses (shoots and/or roots) was not accomplished from callus tissue. Notwithstanding, conditions of 22°c and 16-hour photoperiod seemed best for possible organogenesis since the callus remained compact and dark green globules, possibly resembling a commencement of embryoid-type growth, were present under these conditions. In an isolated study, Saxena, et at. (1981) reported success in obtaining organogenesis from callus tissue arising from protoplast culture of Capsicum cv. California Wonder in medium supplemented with IAA and Kin. Even so, it is felt that furthur experimentation is required to determine conditions under which organogenesis and subsequent multiple plantlet formation is induced in callus tissue derived from whole explants of Capsicum.

Plantlets obtained from in-vitro culture of cotyledonary axil explants were capable of growing to maturity under greenhouse conditions. Thus, it was of

interest to compare two generations of both regenerated and parent plants in greenhouse, as well as in field, conditions. In interpreting results of these experiments, it must be kept in mind that plant-types in the field trial were not the same age. F-1 seed plants were one week younger than both F-2 seed and F-2 regenerated plants, and F-1 regenerated plants were four weeks younger than F-2 seed and F-2 regenerated plants.

In spite of differences seen between greenhouse trials of individual plant types, experimental results obtained indicate that real differences existed between regenerated plants and parent (seed) plants. Both generations of the regenerated plants did not grow as tall, but developed the same number of leaves as did the seed plants (both generations). F-1 regenerated plants also set fruit earlier and had an earlier harvest age than other plant types, although all plants required the same amount of time to produce mature fruit once fruit had set (40 - 45 days). While F-2 seed plants produced more fruit, the first generation regenerated plants produced larger and heavier fruit than other plant types, even though they were four weeks younger than other plant types in field

conditions and thus had less time to produce fruit. Fruit from all plant types had been harvested on the same day. In results obtained from the second fruit yield, first generation regenerated plants again set fruit earlier and had an earlier harvest age than other plants.

It is interesting to note differences in plant responses to greenhouse and field conditions. Under greenhouse conditions, seed plants tended to be much taller than regenerated plants. In field conditions seed plants did not grow as tall, and regenerated plants grew taller, than in the greenhouse. Similarly, although no overall differences were found in maximum leaf number between plant types, addition of new leaves in the greenhouse was quite different from that in field conditions. Under greenhouse conditions, leaves tended to accumulate until the first fruit were set. Almost no leaf accumulation occurred until fruit were harvested, at which time rapid leaf accumulation was resumed until the second yield of fruit were set. In the field however, new leaf growth was continuous and rapid even after large fruit were produced. Many more leaves were accumulated in field-trial plants than in greenhouse grown plants. In fact, it was impossible to

accurately count leaves on field plants after 200 leaves had accumulated (this was attempted on two field grown plants where the leaf number was estimated at between 313 and 330 after two counts for each plant - and all plants appeared to have the same large number of leaves). Thus, field grown plants tended to accumulate approximately 200 more leaves than their greenhouse counterparts.

Fruit set tended to take longer in the field than in the greenhouse, except for F-1 regenerated plants, where it was approximately the same. This may have been due to adverse weather conditions which all plant types, except F-1 regenerated plants which were planted four weeks after the others, had been subjected to. Notwithstanding, the number of fruit produced in the field was much greater (about double) than in the greenhouse, and the weight of these fruit was comparable to those which had been allowed to fully mature under greenhouse conditions.

From results obtained, it appears that the first generation regenerated plant was of a superior quality compared to both generations of the seed plant, in terms of earlier fruit set and yield, and weight of fruit in the first yield. Thus this plant had a

shorter growing season in which it produced larger fruit. Other advantages of this plant were seen in physical terms. These plants, although shorter than seed plants, were able to produce the same number of leaves as the parent (both generations) plant. This resulted in a bushier plant, which would potentailly be better able to withstand heavy wind and rain, and perhaps frost, than the seed plants.

Since the parent plant (F-1 seed) was a hybrid plant, second generation comparisons were somewhat difficult. Several differences between F-1 and F-2 seed plants were observed. This is understandable since we began with a hybrid plant (thus highly heterozygous). Thus from one generation to the next we would expect that some independent recombination would occur.

Alternatively, one would logically expect that F-1 regenerated plants would be identical to F-1 seed plants, since these plants arose from cells of the F-1 seed plant, and would thus contain the same genome. However, results showed that very significant differences existed betwen these two plant types. Since it is known that cells of the cotyledonary axil must de-differentiate into meristematic cells, and then

re-differentiate into shoot and/or root cells, it is possible, that some genetic information became lost, un-useable, partitioned, or otherwise unavailable during this totipotent process. Alternatively, it is also known that IAA rapidly increases the population of messenger RNA when administered _in-vitro_ (van der Linde, 1984; Zurfluh and Guilfoyle, 1982). Since these plants were regenerated in the presence of 1.0 mg/l IAA, it is possible that an increase in mRNA, which may have resulted in polyploidy, aneuploidy or chromosome structure changes (Reisch, 1983), caused the observed differences in these plantlets.

When the seed from F-1 regenerated plants produced F-2 regenerated plants, some differences were observed between these two plant types, for example F-2 regenerated plants were taller in field trials, had a later fruit set and harvest, and the fruit had a lower weight. Several similarities were seen between F-2 regenerated and F-2 seed plants, for example both plant types had a similar age of fruit set and harvest in the field and a similar fruit weight in both field and greenhouse trials).

It appears that, in all cases, a field environment produced a more productive plant than the greenhouse

environment. All plants produced many more leaves in the field, and produced more fruit. And although fruit produced in the field had not grown to maturity when they were harvested, they were, on average, heavier than fruit harvested at maturity in the greenhouse. It was also noted that new leaf growth was not arrested when fruit existed on plants in the field, and that fruit were continuously being set. In the greenhouse, when 2 or 3 fruit were set, leaf accumulation would stop, and no more fruit would set until existing fruit were harvested.

Thus, it is concluded that the first generation regenerated plant is superior in quality to all other plant types tested, but that this superior quality is not necessarily retained in subsequent generations.

SUMMARY

As a result of these experiments, I have confirmed reports by previous investigators, that IAA and BAP appear to be the best growth regulators for use in *Capsicum* tissue culture, and established that 28°c, coupled with a 16-hour photoperiod regime, enhances the effects of these regulators. To date, there have been no reports evaluating both media formulations and growth conditions at the same time.

Results of the statistical analysis used in these experiments showed differences in the effects of growth regulators upon different explants cultured under the different incubation environments. No report has been reviewed where results such as these have been reported. Further experimentation in this area may provide important physiological evidence regarding the effects of light and temperature upon endogenous or exogenously applied growth regulators, or upon changes in the physiological state of the explant in culture.

In future experimentation, methods of reporting morphological response should be changed in order that multiple linear regression analysis may be used to its

fullest potential. Instead of the subjective indices used in the present experiments, objective indices, such as number, size, and/or weight of shoots and roots should be used so that data would consist of continuous, not categorical variables. Fewer media formulations, where concentrations of growth regulators are varied systematically, would also result in more accurate interpretation.

In this study, cotyledonary axils were found to be the best explant for plantlet regeneration, and survival rate of plantlets transferred to greenhouse conditions was found to be very good (87%). Evaluation of these regenerated plants in greenhouse and field conditions showed that the first generation regenerated plant was superior in quality to two generations of parent stock in terms of age of fruit set and harvest, and weight of fruit produced. These plants set fruit approximately 20 days earlier than other plant types in comparable trials, and on average, the fruit produced was approximately 10 grams heavier than that produced by other plant types in comparable trials. However, the superior quality of the regenerated plants was not completely retained in the second generation. To be of economic value to the industry, it would be preferable

that this superior quality be retained through several generations. It is felt that, at the present time, costs of maintaining cultures _in-vitro_, would out weight economic benefits of utilizing this regenerated plant for agricultural purposes.

REFERENCES

Agrawal, S. and Chandra, N. (1983) Differentiation of multiple shoot buds and plantlets in cultured embryos of Capsicum annuum L. Var. Mathania. Current Science. 52(13): 645-646.

Arditti, J. and Nyman, L.P. (1986) In-vitro propagation of the Elephant Yam, Amorphophallus campanulatus var. hortensis Backer (Araceae). Annals of Botany. 57: 11-17.

Bhojwani, S.S., and Razdan, M.K. (1983) Plant Tissue Culture: theory and practice. Elsevier Science Publishing Company Inc.: New York, N.Y.

Campiotti, C.A., Ancora, G., e Saccardo, F. (1986) Results of an attempt of in-vitro culture of pepper shoot apices. Genetica Agraria 35(1): 49-50.

Diamantoglou, S. and Mitrakos, K. (1979) In-vitro culture of olive embryos. Cr. Ac. Sci. D. 288(20): 1537-1540.

Donnelly, D.J., Vidaver, W.E., Lee, K.Y. (1985) The anatomy of tissue cultured red raspberry prior to and after transfer to soil. Plant Cell, Tissue and Organ Culture. 4: 43-50.

Fari, M. and Czako, M. (1981) Relationship between position and morphogenic response of pepper hypocotyl explants cultured in-vitro. Scientia Horticulturae. 15: 207-213.

Favre, J.M. (1977) First results of in-vitro production of bud neoformations in grapevine. Ann. A. Plant. 27(2): 151-169.

Gautheret, R.J. (1942) Hetero-auxines et cultures de tissus vegetaux. Bull. Soc. Chim. Biol. 24: 13-41.

George, E.F. and Sherrington, P.D. (1984) Plant Propagation by Tissue Culture - Handbook and Directory of Commercial Laboratories. Execetics Ltd.: Hants, England.

George, L. and Narayanaswamy, S. (1973) Haploid Capsicum through experimental androgenesis. Protoplasma. 78: 467-470.

Gunay, A.L. and Rao, P.S. (1978) In-vitro plant regeneration from hypocotyl and cotyledon explants of red pepper (Capsicum). Plant Science Letters. 11: 365-372.

Haberlandt, G. (1902) Sber. Akad. Wiss. Wein. 111: 69.

Kurtz, S.M. and Lineberger, R.D. (1983) Genotypic differences in morphogenic capacity of cultured leaf explants of tomato. J. Amer. Soc. Hort. Sci. 108(5): 710-714.

Linsmaier, E.M. and Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum. 18: 100-127.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue culture. Physiol. Plant. 15: 473-479.

____________ (1976) Clonal crops through tissue culture. In Plant tissue culture and its bio-technological application. Barz, W., Reinhard, E., Zenk, M.H. (Eds.) Springer-Verlag: New York, N.Y.

Nie, N.H., Hull, C.H., Jenkins, J.G., Steinbrenner, K., Bront, D. (1975) SPSS Statistical package for the social sciences - Second Edition. McGraw - Hill, Inc.: New York, N.Y.

Norusis, M.J. (1985) SPSS-X - Advanced statistics guide. McGraw - Hill Book Company: New York, N.Y. pp 295-325.

Phillips, G.C. and Hubstenberger, J.F. (1985) Organogenesis in pepper tissue cultures. Plant Cell, Tissue and Organ Culture. 4: 261-269.

Reisch, B. (1983) Genetic variability in regenerated plants. In Handbook of plant cell culture, Volume 1 - techniques for propagation and breeding. Evans, D.A., Sharp, W.R., Ammirato, P.V., Yamada, Y. (Eds.) Macmillan Publishing Co.; New York, N.Y.

Saxena, P.K., Gill, R., Rashid, A., Maheshwari, S.C. (1981) Isolation and culture of protoplasts of Capsicum annuum L. and their regeneration into plants flowering in-vitro. Protoplasma. 108: 357-360.

Seabrook, J.E.A. and Cumming, B.G. (1982) In-vitro morphogenesis and growth of Narcissus in response to temperature. Sec. Hortic. 16: 185-190.

Skoog, F. and Miller, C.O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured in-vitro. Symp. Soc. Exp. Biol. 11: 118-131.

Start, N.D. and Cumming, B.G. (1976) In-vitro propagation of Saintpaulia ionantha Wendl. HortScience 11(3): 204-206.

Van, K. Tran Thanh. (1976) Regulation of morphogenesis. In Plant tissue culture and its bio-technological application. Barz, W., Reinhard, E., Zenk, M.H. (Eds.) Springer-Verlag: New York, N.Y.

van der Linde, P.C.G., Bouman, H., Mennes, A.M., Libbenga, K.R. (1984) A soluble auxin-binding protein from cultured tobacco tissues stimulates RNA synthesis in-vitro. Planta, 160: 102-108.

Walkey, D.G.A. and Woolfitt, J.M.G. (1970) Rapid clonal multiplication of cauliflower by shake culture. J. Hortic. Sci. 45: 205-206.

White, P.R. (1934) Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiology. 9: 585-600.

Williams, D.J. and McHughen, A. (1986) Plant regeneration of the legume Lens culinaris Medik. (lentil) in-vitro. Plant Cell, Tissue and Organ Culture. 7: 149-153.

Wright, M.S., Koehler, S.M., Hinchee, M.A., Carnes, M.G. (1986) Plant regeneration by organogenesis in Glycine max. Plant Cell Reports. 5: 150-154.

Zurfluh, L.L. and Guilfoyle, T.J. (1982) Auxin-induced changes in the population of translatable messenger RNA in elongating maize coleoptile sections. Planta, 156: 525-527.

APPENDIX I

Table 15 presents a partial list of basal nutrient media utilized in studies of plant tissue culture. (Taken from Bhojwani and Razdan, 1983. pp 26-27)

TABLE 15

Composition of some plant tissue culture media[a]

Constituents	Media (amounts in mg l^{-1})[b]						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B_5[g]	Nitsch's[h]	NT[i]
Inorganic							
NH_4NO_3	—	—	1650	1200	—	720	825
KNO_3	80	—	1900	1900	2527.5	950	950
$CaCl_2.2H_2O$	—	75	440	440	150	—	220
$CaCl_2$	—	—	—	—	—	166	—
$MgSO_4.7H_2O$	750	250	370	370	246.5	185	1233
KH_2PO_4	—	—	170	340	—	68	680
$(NH_4)_2SO_4$	—	—	—	—	134	—	—
$Ca(NO_3)_2.4H_2O$	300	—	—	—	—	—	—
$NaNO_3$	—	600	—	—	—	—	—
Na_2SO_4	200	—	—	—	—	—	—
$NaH_2PO_4.H_2O$	19	125	—	—	150	—	—
KCl	65	750	—	—	—	—	—
KI	0.75	0.01	0.83	—	0.75	—	0.83
H_3BO_3	1.5	1	6.2	0.63	3	10	6.2
$MnSO_4.4H_2O$	5	0.1	22.3	2.23	—	25	22.3
$MnSO_4.H_2O$	—	—	—	—	10	—	—
$ZnSO_4.7H_2O$	3	1	8.6	—	2	10	—
$ZnSO_4.4H_2O$	—	—	—	—	—	—	8.6
$Zn.Na_2.EDTA$	—	—	—	15	—	—	—
$Na_2MoO_4.2H_2O$	—	—	0.25	0.025	0.25	0.25	0.25
MoO_3	0.001	—	—	—	—	—	—
$CuSO_4.5H_2O$	0.01	0.03	0.025	0.0025	0.025	0.025	0.025
$CoCl_2.6H_2O$	—	—	0.025	0.0025	0.025	—	—
$CoSO_4.7H_2O$	—	—	—	—	—	—	0.03
$AlCl_3$	—	0.03	—	—	—	—	—
$NiCl_2.6H_2O$	—	0.03	—	—	—	—	—
$FeCl_3.6H_2O$	—	1	—	—	—	—	—
$Fe_2(SO_4)_3$	2.5	—	—	—	—	—	—
$FeSO_4.7H_2O$	—	—	27.8	27.8	—	27.8	27.8
$Na_2.EDTA.2H_2O$	—	—	37.3	37.3	—	37.3	37.3
Sequestrene 330Fe	—	—	—	—	28	—	—
Organic							
Inositol	—	—	100	—	100	100	100
Nicotinic acid	0.05	—	0.5	0.5	1	5	—
Pyridoxine HCl	0.01	—	0.5	0.5	1	0.5	—
Thiamine HCl	0.01	—	0.1	0.5	10	0.5	1
Glycine	3	—	2	2	—	2	—
Folic acid	—	—	—	—	—	0.5	—
Biotin	—	—	—	—	—	0.05	—
Sucrose	2%	—	3%	4%	2%	2%	1%
D-Mannitol	—	—	—	—	—	—	12.7%

[a] Growth regulators and complex nutrient mixtures described by various authors are not included here. The compositions of several media recommended for specific tissue and organ are given in relevant chapters.
[b] Concentrations of mannitol and sucrose are expressed in percentage.
[c] White (1963).
[d] Heller (1953).
[e] Murashige and Skoog (1962).
[f] Eriksson (1965).
[g] Gamborg et al. (1968).
[h] Nitsch (1969).
[i] Nagata and Takebe (1971).

APPENDIX II

Tables 16 to 21 report morphological response of cotyledon, hypocotyl, and cotyledonary axil explants cultured in various media formulations, under three different temperatures and two different photoperiod regimes.

Table 16 : Morphological response of cotyledon, hypocotyl and cotyledonary axil explants cultured for 30 days, under different temperatures and photoperiods, in MS basal medium containing IAA or NAA. (Ten explants were cultured per treatment).

			% FREQUENCY / INTENSITY**					
			TEMPERATURE					
			18°c		22°c		28°c	
			MEDIUM #*					
EXPLANT	PHOTO	RESPONSE	9	1	9	1	9	1
COTY	16hr.	callus	0/0.00	70/0.25	100/1.00	100/1.50	0/0.00	100/2.00
		roots	0/0.00	60/1.60	0/0.00	80/1.25	50/1.30	100/4.50
		shoots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
	24hr.	callus	0/0.00	40/0.40	40/0.20	70/0.90	50/0.70	90/1.70
		roots	0/0.00	10/0.10	0/0.00	80/2.30	0/0.00	80/2.80
		shoots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
HYP	16hr.	callus	0/0.00	100/1.60	90/0.90	80/1.80	50/0.13	90/3.10
		roots	40/1.60	70/1.10	70/2.20	70/0.55	60/1.60	30/0.50
		shoots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
	24hr.	callus	0/0.00	90/0.80	20/0.20	70/1.00	0/0.00	80/0.90
		roots	50/0.70	30/0.30	30/0.90	50/0.80	30/0.60	20/0.10
		shoots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
C. AXIL	16hr.	callus	0/0.00	100/1.00	0/0.00	100/3.00	0/0.00	100/4.00
		roots	70/1.60	40/0.40	90/3.60	70/1.40	90/3.60	90/2.70
		shoots	10/0.05	0/0.00	40/0.80	10/0.05	100/4.00	30/0.30
	24hr.	callus	20/0.20	90/0.58	0/0.00	100/1.00	100/0.25	100/2.00
		roots	50/1.50	30/0.25	30/0.90	60/0.60	70/1.40	50/2.00
		shoots	20/0.20	10/0.10	20/0.30	0/0.00	70/1.40	0/0.00

* 9 = 1.0 mg/l IAA; 1 = 1.0mg/l NAA

** % frequency/intensity = % of explants responding / [sum of the intensity of those responding / total # explants in treatment]

Table 17 : Morphological response of cotyledon, hypocotyl and cotyledonary axil explants cultured for 30 days, under different temperatures and photoperiods, in MS basal medium containing BAP. (Ten explants were cultured per treatment).

			% FREQUENCY / INTENSITY**		
			TEMPERATURE		
			18°c	22°c	28°c
			MEDIUM #*		
EXPLANT	PHOTO	RESPONSE	12	12	12
COTY	16hr.	callus	100/2.00	100/2.00	100/1.80
		roots	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	10/0.10	10/0.10
	24hr.	callus	70/0.70	100/1.50	100/1.70
		roots	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	0/0.00	0/0.00
HYP	16hr.	callus	100/1.50	100/1.00	100/3.00
		roots	0/0.00	10/0.10	0/0.00
		shoots	0/0.00	0/0.00	0/0.00
	24hr.	callus	70/0.90	100/1.03	100/2.40
		roots	0/0.00	0/0.00	10/0.20
		shoots	0/0.00	0/0.00	0/0.00
C. AXIL	16hr.	callus	100/0.85	100/1.00	100/2.00
		roots	10/0.10	0/0.00	30/0.30
		shoots	30/0.20	90/1.80	100/3.00
	24hr.	callus	100/0.45	100/1.00	100/0.50
		roots	0/0.00	10/0.10	30/0.30
		shoots	70/0.45	60/0.40	100/1.00

* 12 = 2.0 mg/l BAP

** % frequency/intensity = % of explants responding / [sum of the intensity of those responding / total # explants in treatment]

Table 18 : Morphological response of cotyledon, hypocotyl and cotyledonary axil explants cultured for 30 days, under different temperatures and photoperiods, in MS basal medium containing equal concentrations of IAA and BAP. (Ten explants were cultured per treatment).

			% FREQUENCY / INTENSITY**					
			TEMPERATURE					
			18°c		22°c		28°c	
			MEDIUM #*					
EXPLANT	PHOTO	RESPONSE	7	6*	7	6*	7	6*
COTY	16hr.	callus	0/0.00	80/1.60	90/1.30	70/0.60	60/0.60	90/1.40
		roots	0/0.00	10/0.10	0/0.00	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	0/0.00	0/0.00	10/0.20	0/0.00	0/0.00
	24hr.	callus	50/0.10	100/1.50	100/0.28	90/1.80	80/0.50	80/0.80
		roots	0/0.00	0/0.00	0/0.00	20/0.20	0/0.00	0/0.00
		shoots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
HYP	16hr.	callus	10/0.05	90/0.90	90/1.25	90/1.60	70/1.00	100/2.50
		roots	30/0.90	10/0.10	40/1.10	20/0.13	40/0.60	0/0.00
		shoots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
	24hr.	callus	10/0.20	100/1.50	70/0.65	100/1.80	80/0.58	100/1.35
		roots	10/0.10	10/0.10	60/1.20	0/0.00	40/0.60	0/0.00
		shoots	10/0.00	0/0.00	0/0.00	0/0.00	0/0:00	0/0.00
C. AXIL	16hr.	callus	100/0.40	100/0.70	100/0.50	100/1.00	100/1.00	100/0.50
		roots	50/1.30	50/0.50	20/0.20	0/0.00	80/3.20	30/0.30
		shoots	30/0.30	30/0.20	10/0.05	10/0.10	90/3.60	60/1.20
	24hr.	callus	100/0.25	100/1.00	100/0.25	100/0.50	100/0.50	100/2.00
		roots	30/0.50	10/0.10	20/0.20	20/0.05	100/3.00	50/1.00
		shoots	20/0.20	30/0.25	0/0.00	30/0.10	100/2.00	90/2.70

* 7 = 0.05 mg/l each IAA and BAP; 6* = 2.0 mg/l each IAA and BAP

** % frequency/intensity = % of explants responding / [sum of the intensity of those responding / total # explants in treatment]

Table 19 : Morphological response of cotyledon, hypocotyl and cotyledonary axil explants cultured for 30 days, under different temperatures and photoperiods, in MS basal medium containing varying concentrations of IAA and BAP. (Ten explants were cultured per treatment).

			% FREQUENCY / INTENSITY**					
			TEMPERATURE					
			18°c		22°c		28°c	
			MEDIUM #*					
EXPLANT	PHOTO	RESPONSE	8	11	8	11	8	11
COTY	16hr.	callus	90/1.00	90/1.40	70/1.10	80/0.80	90/1.80	70/0.90
		roots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
		shoots	50/0.38	40/0.18	10/0.20	60/0.60	70/1.10	70/1.30
	24hr.	callus	100/1.50	90/1.40	100/1.00	100/1.25	80/1.00	80/1.58
		roots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
		shoots	10/0.03	0/0.00	10/0.18	20/0.30	40/0.18	0/0.00
HYP	16hr.	callus	100/2.00	90/1.40	100/2.00	100/1.00	100/1.60	70/0.70
		roots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	0/0.00	0/0.00	20/0.05	0/0.00	0/0.00
	24hr.	callus	70/0.70	70/1.20	100/1.70	100/0.63	100/0.38	100/1.25
		roots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00
C. AXIL	16hr.	callus	100/0.90	100/0.80	100/0.50	100/0.50	100/0.50	100/1.00
		roots	0/0.00	0/0.00	0/0.00	0/0.00	0/0.00	20/0.20
		shoots	100/2.00	30/0.30	100/1.00	90/0.45	100/3.00	80/0.80
	24hr.	callus	100/1.00	100/0.25	100/0.25	100/1.00	100/1.00	20/0.05
		roots	0/0.00	0/0.00	0/0.00	0/0.00	20/0.20	0/0.00
		shoots	80/1.60	40/0.20	90/0.80	30/0.30	90/0.90	60/0.30

* 8 = 0.05 mg/l IAA + 10.0 mg/l BAP; 11 = 4.0 mg/l IAA + 10.0 mg/l BAP

** % frequency/intensity = % of explants responding / [sum of the intensity of those responding / total # explants in treatment]

Table 20 : Morphological response of cotyledon, hypocotyl and cotyledonary axil explants cultured for 30 days, under different temperatures and photoperiods, in MS basal medium containing NAA and BAP. (Ten explants were cultured per treatment).

			% FREQUENCY / INTENSITY**		
			TEMPERATURE		
			18°c	22°c	28°c
			MEDIUM #*		
EXPLANT	PHOTO	RESPONSE	23	23	23
COTY	16hr.	callus	100/1.00	100/2.00	100/2.00
		roots	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	80/0.40	40/0.80
	24hr.	callus	100/1.00	100/1.00	100/2.00
		roots	0/0.00	0/0.00	0/0.00
		shoots	60/0.15	60/0.30	10/0.03
HYP	16hr.	callus	100/2.00	100/1.00	100/2.00
		roots	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	10/0.10	10/0.03
	24hr.	callus	100/1.00	100/2.00	100/1.00
		roots	0/0.00	0/0.00	0/0.00
		shoots	10/0.03	10/0.03	0/0.00
C. AXIL	16hr.	callus	100/1.00	100/0.50	100/0.50
		roots	0/0.00	0/0.00	0/0.00
		shoots	100/2.00	100/2.00	100/2.00
	24hr.	callus	100/1.80	100/0.50	100/0.50
		roots	0/0.00	0/0.00	0/0.00
		shoots	90/2.50	90/0.43	60/0.30

* 23 = 0.05 mg/l NAA + 10.0 mg/l BAP

** % frequency/intensity = % of explants responding / [sum of the intensity of those responding / total # explants in treatment]

Table 21 : Morphological response of cotyledon, hypocotyl and cotyledonary axil explants cultured for 30 days, under different temperatures and photoperiods, in MS basal medium containing IAA, BAP and Kin. (Ten explants were cultured per treatment).

			% FREQUENCY / INTENSITY**		
			TEMPERATURE		
			18°c	22°c	28°c
			MEDIUM #*		
EXPLANT	PHOTO	RESPONSE	21	21	21
COTY	16hr.	callus	100/1.00	100/2.00	100/2.00
		roots	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	0/0.00	20/0.05
	24hr.	callus	100/0.25	100/2.00	100/3.00
		roots	0/0.00	0/0.00	0/0.00
		shoots	0/0.00	0/0.00	0/0.00
HYP	16hr.	callus	100/1.00	100/2.00	100/3.00
		roots	50/1.00	20/0.40	20/0.20
		shoots	0/0.00	0/0.00	0/0.00
	24hr.	callus	100/1.00	100/2.00	100/3.00
		roots	0/0.00	20/0.40	0/0.00
		shoots	0/0.00	0/0.00	0/0.00
C. AXIL	16hr.	callus	100/0.25	100/1.00	100/1.00
		roots	0/0.00	0/0.00	50/[illegible].50
		shoots	100/2.00	90/1.80	100/2.00
	24hr.	callus	100/0.30	100/0.50	100/2.00
		roots	10/0.10	10/0.05	30/0.30
		shoots	30/0.30	70/0.45	80/0.80

* 21 = 0.1 mg/l IAA + 1.0 mg/l BAP + 0.1 mg/l Kin

** % frequency/intensity = % of explants responding / [sum of the intensity of those responding / total # explants in treatment]

APPENDIX III

Tables 22 to 24 report results of the stepwise multiple regression analyses of seperate treatments temperature and photoperiod manipulation experiments.

Table 22: Results of stepwise multiple regression analysis on Shooting Response of explants in seperate treatment data. Only those regression coefficients (R-values) for individual variables in the equation that were found to be statistically significant ($P<0.05$) are reported. Independent variables are in sequence according to how often they were significant in regression equations. (a) Cotyledons; (b) Hypocotyls; (c) Cotyledonary Axils.*

(a)

TEMP(°c)	PHOTO	BAP	IAA	NAA	KIN	R^2	P
18	16-hr	0.40***	-0.18**			0.16	0.000
	24-hr	0.41***				0.27	0.000
22	16-hr	0.50***	0.40**			0.32	0.000
	24-hr	0.30***				0.09	0.004
28	16-hr	0.68***				0.46	0.000
	24-hr	0.32**	-0.13*			0.17	0.000

(b)

TEMP(°c)	PHOTO	BAP	IAA	NAA	KIN	R^2	P
18	16-hr						
	24-hr						
22	16-hr	0.37***				0.14	0.000
	24-hr						
28	16-hr						
	24-hr						

(c)

TEMP(°c)	PHOTO	BAP	KIN	IAA	NAA	R2	P
18	16-hr	0.51***	0.47***	-0.34***	-0.28*	0.85	0.000
	24-hr	0.59***		-0.27***		0.58	0.000
22	16-hr	0.25***	0.36***	-0.33***	-0.32**	0.46	0.000
	24-hr	0.35**	0.11*			0.16	0.000
28	16-hr	-0.05*	-0.08***	-0.42***	-0.52***	0.68	0.000
	24-hr	-0.36***	-0.09***		-0.41***	0.50	0.000

* Data = R-values for variables in equation significant at $P<0.05$; P = Overall probability of equation
* = $P < 0.05 - 0.01$; ** = $P < 0.01 - 0.001$; *** = $P < 0.001$

Table 23: Results of stepwise multiple regression analysis on Rooting Response of explants in seperate treatment data. Only those regression coefficients (R-values) for individual variables in the equation that were found to be statistically significant (P<0.05) are reported. Independent variables are in sequence according to how often they were significant in regression equations. (a) Cotyledons; (b) Hypocotyls; (c) Cotyledonary Axils.*

(a)

TEMP(°c)	PHOTO	NAA	BAP	IAA	KIN	R^2	P
18	16-hr	0.74***				0.53	0.000
	24-hr	0.30**				0.30	0.004
22	16-hr	0.82***				0.68	0.000
	24-hr	0.85***				0.72	0.000
28	16-hr	0.91***	-0.38**			0.84	0.000
	24-hr	0.87***				0.87	0.000

(b)

TEMP(°c)	PHOTO	BAP	NAA	IAA	KIN	R^2	P
18	16-hr	-0.43***				0.18	0.000
	24-hr	-0.28**				0.08	0.007
22	16-hr	-0.40***				0.16	0.000
	24-hr	-0.36***				0.13	0.000
28	16-hr	-0.35**				0.12	0.001
	24-hr	-0.27*				0.07	0.011

(c)

TEMP(°c)	PHOTO	BAP	KIN	NAA	IAA	R^2	P
18	16-hr	-0.40***	-0.17**	-0.02*		0.30	0.000
	24-hr	-0.30**				0.09	0.004
22	16-hr	-0.41***	-0.16**			0.23	0.000
	24-hr	-0.30**				0.09	0.004
28	16-hr	-0.60***	-0.15***			0.45	0.000
	24-hr	-0.53***	-0.17**			0.37	0.00

* Data = R-values for variables in equation significant at P<0.05; P = Overall probability of equation
* = P < 0.05 - 0.01; ** = P < 0.01 - 0.001; *** = P < 0.001

Table 24: Results of stepwise multiple regression analysis on Callus Response of explants in seperate treatment data. Only those regression coefficients (R-values) for individual variables in the equation that were found to be statistically significant (P<0.05) are reported. Independent variables are in sequence according to how often they were significant in regression equations. (a) Cotyledons; (b) Hypocotyls; (c) Cotyledonary Axils.*

(a)

TEMP(°c)	PHOTO	KIN	BAP	IAA	NAA	R^2	P
18	16-hr		0.31**		-0.29*	0.14	0.002
	24-hr		0.61***	0.39*		0.42	0.000
22	16-hr	0.33**		-0.52***		0.32	0.000
	24-hr	0.43***		0.13*		0.23	0.000
28	16-hr	0.24**	0.19**	-0.32**	0.25**	0.36	0.000
	24-hr	0.52***	0.08*		0.10**	0.38	0.000

(b)

TEMP(°c)	PHOTO	KIN	IAA	BAP	NAA	R^2	P
18	16-hr	-0.07*	-0.08**	0.62***	0.22***	0.63	0.000
	24-hr		0.26*			0.07	0.013
22	16-hr	0.31**		0.20*		0.16	0.001
	24-hr	0.33**	-0.22**	0.25**		0.30	0.000
28	16-hr	0.32**	-0.37***		0.35**	0.31	0.000
	24-hr	0.59***				0.35	0.000

(c)

TEMP(°c)	PHOTO	NAA	KIN	BAP	IAA	R^2	P
18	16-hr	0.33***	-0.37*	0.48***		0.50	0.000
	24-hr			0.48***	-0.20***	0.37	0.000
22	16-hr	0.92***	0.05***			0.87	0.000
	24-hr	0.45***			0.26***	0.34	0.000
28	16-hr	0.38***				0.77	0.000
	24-hr	0.43***	0.43***			0.43	0.000

* Data = R-values for variables in equation significant at P<0.05; P = Overall probability of equation;

* = P < 0.05 - 0.01; ** = P < 0.01 - 0.001; *** = P < 0.001

APPENDIX IV

Table 25 reports results of ONEWAY analyses indicating differences in trials of individual plant types for parameters measured. (R = regenerated plants; S = seed plants). (G1 = Greenhouse Trial #1; G2 = Greenhouse Trial #2; F = Field Trial). Significant differences are indicated as follows:

* = P < 0.05 - 0.01

** = P < 0.01 - 0.001

*** = P < 0.001

Table 24 : Results of ONEWAY analyses indicating differences in trials of individual plant types for parameters measured.

		PLANT TYPE										
		F-1 R			F-2 R		F-1 S			F-2 S		
PARAMETER	TRIAL	TRIAL G1	G2	F	G1	F	G1	G2	F	G1	G2	F
Max.Plant height	G1											
	G2											
	F						***	***			***	
Max. leaf number	G1											
	G2						***					
	F	***	***		***		***	***		***	***	
Age at Fruit Set (Yield 1)	G1											
	G2	***		***						***		***
	F				***		***	***				
Age at Fruit Harvest (Yield 1)	G1		**	**								
	G2									***		***
	F				***		*	*				
Time between Fruit Set and Harv. (Yield 1)	G1											
	G2											
	F	***	***		***		***	***				
Number of Fruit (Yield 1)	G1											
	G2											
	F	***	***		**		*	*		***	***	

Table 24 Con't:

		PLANT TYPE										
		F-1 R			F-2 R		F-1 S			F-2 S		
PARANETER	TRIAL	TRIAL										
		G1	G2	F	G1	F	G1	G2	F	G1	G2	F
Weight of Fruit (Yield 1)	G1											
	G2									***		***
	F	*	*									
Fruit Data (Yield 2)	G1	No individual differences found.										
	G2											
	F											