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Tissue Culture and Plant Regeneration of Okra (*Abelmoschus esculentus* L.)

Mihir K. Roy

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Abstract

Tissue Culture and Plant Regeneration of Okra (Abelmoschus esculentus L.)

Mihir K. Roy

Explants (hypocotyl, cotyledon, cotyledonary axil, cotyledonary petiole, internode, leaf node, leaf segment, leaf petiole, anther and immature fruit segments) were excised from okra (Abelmoschus esculentus) seedlings and cultured aseptically on Murashige and Skoog's (MS) basal nutrient medium supplimented with auxins (0.1-2.0 mg/l), cytokinins (0.1-5.0 mg/l) and auxin-cytokinin combinations. Callus formation and root differentiation occured in a medium containing naphthaleneacetic acid (NAA) (1.0 mg/l) or indoleacetic acid (IAA) (1.0 mg/l). There was a greater proliferation of roots on medium supplimented with NAA (1.0 mg/l). The addition of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2 mg/l) to the growth medium suppressed root formation. No shoot bud or shoot development was observed at any of the auxin levels tested. Kinetin (KIN) and zeatin (ZTN) also proved ineffective in inducing shoot buds or shoots. Single shoots were produced at 24^{o} C on cotyledonary

axil, leaf node and cotyledon explants cultured on medium containing 6-benzylaminopurine (BAP) (1.0 mg/l). Multiple shoots were formed on cotyledonary axil and leaf node explants at 28^{o} C. Complete plants were produced on medium supplimented with both BAP (1.0 mg/l) and NAA (1.0 mg/l).

Calli from hypocotyl and cotyledonary axil derived explants of aseptically grown okra were cultured on MS basal medium containing auxin, cytokinin and auxin-cytokinin combinations to stimulate morphogenesis. The hypocotyl-derived callus remained nonmorphogenic when subcultured on fresh BAP (1.0 mg/l) supplimented MS medium whereas, cotyledonary axil-derived callus produced a number of buds. The transfer of the organogenic calli to BAP enriched (1.0 mg/l) MS medium containing various concentrations of silver nitrate (0.0-40.0 mg/l) stimulated multiple shoot development. The presence of 10.0 and 20.0 mg/l of silver nitrate considerably increased the number of calli producing multiple shoots when myo-inositol (0.1 g/l) and casein hydrolysate (1.0 g/l) were omitted from the growth medium. The rooted shoots obtained from all culturing conditions, when transferred to soil, grew normally to their maturity in the greenhouse.

Protoplasts were isolated from the cotyledon and leaf tissue of aseptically grown okra seedlings. The viability of protoplasts was quite high (81%) when they were isolated. Attempts to regenerate plants were not successful.

The project represents the first report on plant regeneration of okra via tissue culture techniques. The in-vitro plant regeneration and viable protoplasts isolation of okra may offer the opportunity to study and possibility to improve this economically important plant.

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To my beloved parents (1901-1988)

Totipotency

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

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

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

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

Each cell contains the secret within
Each one knows where the other has been
Separation will make the study complete
which will you be- root, stem, or leaf?

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

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

Sharon Maraffa

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C. INTRODUCTION

C1. Cell and Tissue Totipotency

Cell and tissue totipotency refers to the ability of a whole organism to regenerate from its constituent parts. Many plant cells exhibit totipotency (RAGHAVAN,1977). One of the expressions of totipotency is that plant cells and tissues are capable of regenerating a whole plant under certain conditions. In 1902, Haberlandt was the first investigator who attempted to regenerate plants from a single cell. His pioneering work was unsuccessful and for the next 30 years very little progress was made to generate plants from in-vitro systems. Nobecourt and Gotheret in the late 1930s independently reported the first in-vitro culture of unorganized carrot tissue and this was followed by White's work with tobacco tissue (In STREET,1977). After these initial reports the techniques of tissue culture developed quickly.

C2. Plant Cell and Tissue Culture

Prior to the development of plant cell and tissue culture techniques, plants were usually propagated by conventional methods of root and stem cuttings and grafting.

Unfortunately, these methods can only be applied to a limited number of plant species. These procedures are time consuming and the results are often poorly reproducible.

Plant cell and tissue culture techniques have, in recent years, developed into very powerful tools for the propagation of economically important species. This in-vitro culturing method has several advantages over the traditional methods of plant propagation. They provide the means of obtaining unlimited number of plants. The increased yield can be achieved through the formation of multiple shoots or plantlets from a single explant or plants can be regenerated from single cells. The technique also allows for the production of disease-free plantlets through meristem culture, somatic hybridization and genetic transformation techniques (DAUB,1986). The tissue culture methods allow for the production of haploid plants (CHIANG et al,1985). Haploid plants are important to geneticists since mutants can be easily detected and homozygous plants of a desired quality may be obtained directly in a single generation. The techniques of tissue culture can also be used to obtain new hybrids by intraspecific and interspecific hybridization or by chromosome aberration (CHIANG et al,1985; OHGAWARA et al,1985; OHMASA,1986; SHINTAKU et al,1986). The use of

isolated protoplasts to accept foreign genetic materials and isolated DNA molecules can further lead to regenerants which are stress tolerant and high yielding (TOYODA et al,1985; MOREHART et al,1985; REICH et al,1986; SILVIERA and AZEVIDO,1987). The tissue culture techniques have also made rapid vegetative propagation possible for many plant species which, under normal condition, grow very slowly or are difficult to grow (RAO,1977).

Apart from producing plants in-vitro, plant tissue culture techniques are also being increasingly applied by industry for synthesising many natural products. These include for example, oils, latex, pigments and compounds with medicinal properties (CRUEGER and CRUEGER,1984; WALTON and EARLE,1985).

C3. Requirements for In-vitro Plant Culture

Plant cell and tissue culture essentially involve isolating cells or tissues from a plant and placing it into a container containing a defined nutrient medium and allowing growth under controlled environmental conditions.

Successful in-vitro culturing of plant cells and tissue depend upon a number of factors. These include the composition of the medium, the plant tissue (explant) and the physical environment of temperature and light. Several

media formulations have been developed (BHOJWANI and RAZDAN,1983); however, Murashige and Skoog's (MS), Gamborg's (B5) and White's media (Appendix I) are the most frequently used by researchers. All culture media consist of mineral salts, a reduced carbon source, plant growth regulators and, in certain cases, organic compounds such as amino acids, vitamins, adenine bases, myo-inositol and casein hydrolysate.

The MS basal medium is one of the most popular media that is nomally used because of its high nitrate, potassium and ammonia contents. When low levels of potassium and nitrogen are required White's basal medium is usually employed.

Under in-vitro conditions, most tissues preferably use readily available carbon source. Therefore, a metabolisable carbon source is essential. The preferred carbon source for most culture media is sucrose. Glucose and fructose are occasionally substituted for sucrose, however, the tissues response is generally poor (WRIGHT et al,1986).

A number of studies have shown that another important additive to the culture medium is the plant growth regulators, particularly auxins and the cytokinins. Some of these growth regulators such as indole acetic acid (auxin),

and kinetin (cytokinin), are produced by the plant itself. Other frequently applied growth substances are synthetically produced. How growth regulators control plant growth and metebolism remains to be discovered. The commonly used auxins are 3-indoleacetic acid (IAA), 3-indolebuteric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4-chlorophenoxyacetic acid (CPA), and 4-amino-3,5,6-trichloropicolinic acid (Picloram or TCP). Often 2,4-D, 2,4,5-T, CPA or TCP are used to induce rapid callus proliferation. In some cases low levels of 2,4-D have been shown to promote morphogenesis (RAO et al,1973). Generally however, IAA, which is less active than 2,4-D, 2,4,5-T or CPA is the auxin of choice to induce morphogenesis (THIMANN, 1951).

Apart from the addition of auxins to the growth media, cytokinins also are very important additives. The routinely used cytokinins are 6-benzyladenine (or 6-benzylaminopurine, BA or BAP), 6-y-y-dimethylaminopurine (isopentenyladenine, 2iP or IPA), 6-furfurylaminopurine (Kinetin) and 6-(4-hydroxy 3-methyl-trans-2-butenylamino-purine (Zeatin). Recent studies have shown that other compounds such as cyclic nucleotides may also mimic cytokinins (MANGAT and JANJUA,1987).

The reason why auxins and cytokinins are important is the fact that studies have shown that interaction between auxin and cytokinin influences morphogenesis. Skoog and Miller (1957) showed that auxin-cytokinin ratios determined the type and extent of organogenesis. They showed that a high auxin to cytokinin ratio initiated root formation. However, this rational does not apply to all plant species and tissues. There is no universal ratio for root and shoot induction. Appropriate auxin and cytokinin levels, therefore, must be determined for each species or variety under study. While both auxin and cytokinin autotrophy have been demonstrated (SKOOG and MILLER, 1957) it is rare that the plant tissue will grow and proliferate in the absence of both.

For the successful in-vitro culturing of plant tissue, the addition of other organic compounds may also be necessary. Addition of vitamins in most cases is not necessary. However, when the nutritional requirements of a tissue are not known, vitamins, casein hydrolysate and myo-inositol are routinely added as a precautionary measure until the nutritional requirements can be determined. The vitamins that are commonly added include pyridoxin, nicotinic acid and biotin. Casein hydrolysate is source of

amino acids and myo-inositol is used for the syntheses of phospholipids, cell wall pectins and cytoplasmic membrane systems (ANDERSON and WOLTER,1966; WOLTER and MURMANIS,1977; HARRAN and DICKINSON,1978). L-glutamine and L-asparagine are added when an additional nitrogen source is required. Adenine sulfate may be incorporated in the medium to enhance growth and shoot formation (SKOOG and TSUI,1948). Coconut milk (or liquid endosperm of coconut) is often substituted for myo-inositol. The liquid endosperm of coconut contains a number of other cell division factors, for example, diphenylurea, 9-B-D-ribofuranosylzeatin and free amino acids that contribute to growth and differentiation (van STADEN and DREWES,1974).

Under certain conditions, in order to enhance growth and morphogenesis, activated charcoal (AC) has been used in a number of studies (FRIDBORG and ERIKSSON,1975, CONSTANTIN et al,1977). In some cases, the explant cultured on medium containing AC may bypass the callus formation and initiates shoot morphogeneses directly (PECK and CUMMING, 1986; ZIV and GADASI,1986). Although AC has been shown to absorb both inhibitory and stimulatory compounds (WANG and HUANG,1976, CONSTANTIN et al,1977), its incorporation to anther culture is an essential component (BANKS et al,1978).

The success of in-vitro cultures is also influenced by the type of explant (a piece of tissue or organ removed from the parent plant). The size, source, physiological age and the genotype of the plant are all important factors. In general, very small explants (less than 1 mm) have low survival rates in culture. When the explants are large (more than 1 cm) shoot production is reduced (GUKASYAN et al,1977).

The success of in-vitro cultures is also influenced by the source of the explants. Because of the nutritional requirements and environmental growth conditions of the parent, explant tissue within any plant differ in their ability to undergo morphogeneses (TAKAYAMA and MISAWA,1979). Further, certain tissues that exhibit morphogenic competence in one species may not be equally competent in another species (PETRU and LANDA,1974; HUSSEY,1975). Explants taken from regenerated plants normally show better morphogenic responses than those taken from parent stocks. Also, explant taken from a plant grown under aseptic condition may show better in-vitro response than that taken from the plants grown under normal conditions of growth (HUSSEY,1975). The physiological age can also influence the type and extent of morphogenic responses. Studies show that young tissues

usually demonstrate a higher degree of morphogenic competence than older tissues (ZIV et al,1970; WEILER and EMERSHAD,1977). Calli obtained from younger tissue are more likely to undergo organogeneses than those obtained from older ones (PIERIK and STEEGMANS,1976). The physiological age of the explant is important in woody species where embryo and seedling tissues have been shown to have higher capacity for regeneration (HU and SUSSEX,1971; CHENG,1975). The genotype of the parent plant may also influence the in-vitro response of an explant (TOMES and SMITH,1985; HODGES et al,1986; CHEN et al,1987).

Since the culture medium normally contains sucrose or other sugars as a carbon source, light in most cases is not essential for the energy source. But as light is an essential environmental factor for the normal development of a plant (MURASHIGE,1974), the quality (wavelength) of light, its intensity and photoperiod have been shown to contribute to the morphogenic responses of in-vitro plant tissues (GAUTHERET,1969; PILLAI and HILDEBRANDT,1969; SEIBERT,1973; BAPAT and RAO,1977; TRAN THANN VAN,1977; KATO,1978; NAEF and SIMON,1978; ECONOMOU and READ,1986; COOKE,1988). The light intensity for tissues in culture is normally lower than that required by regenerated plantlets. However, in some cases,

increased light intensity may effect organogenesis (WELANDER,1978). Occasionally, the culturing of explants in continuous darkness is required to initiate morphogenesis (PIERIK and STEEGMANS,1986). Frequently, a combination of dark and light treatment helps in the successful regeneration of shootlets or plantlets (BAJAJ and PEIRIK,1974; KATO,1978). It has been suggested that photoperiod may influence changes in the endogenous level of auxins and cytokinins and these, in turn could affect the type and extent of morphogenesis in a cultured tissue (HEIDE,1965; HEIDE and SKOOG,1967).

Further, tissues exposed to light generally tend to store more starch than tissues left in the dark (KATO,1978a). Starch has been shown to be important in organogenesis (THORPE and MEIR,1972; MANGAT et al,1989). In some studies, accumulation of starch has been shown to influence the endogenous levels of plant growth regulators of the cultured tissue (KATO,1978).

Temperature also plays a role in the successful regeneration of cultured tissue (PIERIK,1975; SEABROOK and CUMMING,1976; MURASHIGE,1974; COOKE,1988). Some experiments have shown that treating a particular tissue to cold treatment results in specific morphogenic events

(FONNESBECH,1974; BONGA,1977; HENRY,1978; NAKOSTEEN and HUGHES,1978). In other cases increasing temperature or a heat pretreatment severely inhibit shoot formation (FONNESBECH,1974a). Morphogenesis may also be affected with altering day/night temperature cycle (GAUTHERET,1969; SLACK,1986).

Another factor that may influence successful in-vitro cultures is the gaseous environment of the culture flask. A growing cell culture produces ethylene and this production is stimulated by auxins, particularly 2,4-D and IAA (LaRUE and GAMBORG,1971). Although in some cases ethylene has been shown to have a benificial effect (BEASLEY and EAKS,1979), in most cases ethylene tends to inhibit organogenesis (THOMAS and MURASHIGE,1979). Both auxins and cytokinins stimulate ethylene production. Auxins which are normally added to cause callus induction, strongly promotes endogenous production of ethelene (YANG and HOFFMAN,1984). The so called 'IAA effects' may in reality be ethylene effects which include the reduction of greening in cultures with the addition of auxin (DALTON and STREET,1976; SLACK and HAND,1986). In addition to ethylene, accumulation of other volatile compounds such as carbon dioxide, ethanol and acetaldehyde in the culture environment have been reported

(THOMAS and MURASHIGE,1979; 1979a; SLACK and HAND,1986). Therefore, it is a good practice to use a diffusable closure for in-vitro cultures.

The orientation of the explant and repeated subculture has, in some cases, shown to affect the morphogenic potentials of the tissues (ZIV and HELEVY,1970; PIERIK,1975; SEABROOK,1978; PIERIK and STEEGMANS,1986). In many cases the orientation of the explant on the medium may influence its morphogenic capacity. The polarity effect may be due to several factors which include nutrient and/or growth regulator compositions of the medium and anatomical make up of the tissue (RAJU and MANN,1970; GERTSSON,1986). The polarity response of an explant can also be influenced by its natural electric fields (RATHORE,1985). The loss of morphogenic ability through repeated subculturing could occur in several ways. These includes the loss of organizing nature of the tissue, reduced endogenous levels of growth regulators and gradual accumulation of chromosome abnormality (CHEN and GALSTON,1967; MURASHIGE,1967; NISHI and YAMADA,1968; WOCHOK and WETHEREL,1972; SKIRVIN and JANICK,1976; ORTON and NELSON,1979; SINGH,1985; GUPTA and AHMED,1986). Although many tissues loose their morphogenic ability through repeated subculturing, there are some

tissues, however, that retain their morphogenic capabilities over extended periods of subculturing (EARLE,1974; SHERIDAN,1974).

Finally, seasonal variation, a factor that affects morphogenesis, is considered less important than those described so far. Studies, however, show that the morphogenic responses of explant may be limited to the period of the vegetative growth of the parent plant (ROBB,1957; NITSCH,1978; SAUER et al,1985).

Successful in-vitro regeneration and development of plants therefore, require four important steps (MURASHIGE,1977; GEORGE and SHERRINGTON,1984). In step one, a suitable explant from the supraterrestrial-contaminant-free mother plant is excised and cultured on a defined medium. This is followed in step II by the production of propagules (clonal multiplication) or calli. In step III, plantlets are allowed to develop. Plantlets may arise directly via somatic embryogenesis or obtained by transforming shoots to rooting medium for root initiation. In some instances regenerated shoots when transferred directly into soil produce roots. When complete plantlets are obtained, they are transferred to soil. Plantlets are then treated under controlled environment to develop their

anatomical compatibility suitable for the survival condition in nature (step IV). The treated plant is then transferred to the greenhouse to grow to maturity (MURASHIGE,1977; GEORGE and SHERRINGTON,1984).

C4. Importance of Okra

Okra (Abelmoschus esculentus or Lady's finger[1]; 2n= 14) is a member of the Malvaceae. The origin of okra is rather obscure. Wild varieties exist in Ethiopia and in the upper Nile in Sudan, and perenial varieties in West Africa. Its presence in the 'New World' has been attributed to the slaves from Africa (YAMAGUCHI,1983). Okra is grown in all parts of the tropics and is now widely cultivated in Japan and southern states of United States. Its popularity as a vegetable is on the increase in North America. The immature fruits, both fresh and dried or oil-roasted, are used in salads or soup vegetables and are a rich source of protein. Fresh pods are pickled in miso (fermented soybean paste) and both fresh and sun-dried leaves are used in native food (protherb) in Japan. Young shoots as well as fruits are eaten with rice as lalab in Java. Seeds are roasted into

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1. It also is known as Gumbo, Gombo, Ochro, America neri, Tindisha, Bamia, Quiabo, Bhendi, Bendi (TANAKA,1976).

substitute for coffee (TANAKA,1976). When used as grain, okra seeds are similar to other oleaginous crops, for example, cotton (Malvaceae), soybean (Leguminosae) and sunflower (Composatae). Protein and oil content in dry okra seeds are about 33% and 32% respectively (SAVELO et al,1985). The okra meal is used as a substitute for wheat flour and cooking oil to prepare a variety of baked products. A vegetable curd can be made from the seed and organoleptically as acceptable as soybean curd or tofu (MARTIN,1979). Mature plants are often used as a source of fibre and pulp. The okra fibre is gradually replacing that of jute (SCHERY,1972). The flowering stage of okra with its beautiful bright yellow-and-black flowers and colorful pods ("an edible ornamental") of some cultivars contributes to the horticultural beauty of the garden (CORLEY,1985).

The world-wide cultivation and use of okra is thus becoming important and its economic exploitation is increasing. However, relatively little information is available on its ability to be propagated through tissue culture techniques. Plant regeneration has been obtained from tissue cultures of a wide variety of plant species, however, many members of the Malvaceae are recalcitrant to this technique. The presence of mucilage in leaf and stem, a

characteristic feature of the family, is probably one of the reasons why the tissues respond poorly in an in-vitro environment. As well, the sticky mucilage released from the tissue during manipulation prior to culturing creates the condition that makes cultures more susceptible to contamination. Such disadvantages could easily discourage many plant tissue culturists from studying this family.

Recently several reports on cotton tissue culture have been published (ABDUKARIMOV et al,1987; GILL and BAJAJ,1987; KUZNETSOVA et al,1987; TOLINDER and GOODWIN,1987). However, except for a preliminary report on the production of somatic embryos from callused okra hypocotyl (REYNOLDS and BLACKMON,1981), succsessful in-vitro plant regeneration using tissue culture techniques, has not been reported in this plant

This study was undertaken to investigate the possibilities of in-vitro plant regeneration of okra using tissue culture techniques. The present study included, (a) the establishment of media/explant combination for plant regeneration. (b) micropropagation from a single explant and from explant derived callus tissue, and (c) the isolation and the culture of protoplasts.

D. MATERIALS AND METHODS

D1. Preparation of Media

The MS basal salt medium (MURASHIGE and SKOOG,1962) was used in all experiments (Table 1). The experimentally required medium was prepared from the stock solutions as indicated in table 1. The stocks from I to IV were prepared by dissolving nutrients in distilled deionized water and kept frozen (-22^{o} C) until required. The stocks of growth regulators V to X were prepared by dissolving them in absolute ethanol and kept in the cold (8^{o} C). To prepare 1 litre of MS basal medium, 10 ml each of stock solutions I to IV were taken and added to distilled deionized water. Myo-inositol (0.1 g/l), casein hydrolysate (1.0 g/l), and sucrose (30.0 g/l) were added fresh each time the growth medium was prepared. The pH was adjusted to 5.7$\pm$0.1 with 0.1N NaOH and/or 0.1N HCl and distilled deionised water added to bring the solution to 1 litre. After the addition of 6.0 g/l agar (Agar Bacteriological, Oxoid agar No.1), medium was heated gently on a magnetic stirrer to dissolve the agar followed by the addition of appropriate amount(s) of required growth regulator solution(s). Media containing

Table 1

Stock solutions for the preparation of growth media. Stock from I to IV were prepared by dissolving the chemicals in distilled deionized water. Stock V to X were prepared by dissolving the compounds in absolute ethanol. Appropriate aliquotes of the stock solutions were taken to prepare the desired medium (modified from BHOJWANI, 1983).

Stock-Solutions	Macro-Elements	mg/100ml
Stock I	$CaCl_2.2H_2O$	4400.0
Stock II	KH_2PO_4	1700.0
	KNO_3	19000.0
	$MgSO_4.7H_2O$	3700.0
	NH_4NO_3	16000.0
	Micro-Elements	
Stock III	KI	8.3
	$FeSO_4.7H_2O$	278.0
	Na-EDTA	373.0
	$CoCl_2.6H_2O$	25.0
	$CuSO_4.5H_2O$	25.0
	H_3BO_3	62.0
	$MnSO_4.4H_2O$	223.0
	$NaMoO_4.2H_2O$	2.5
	$ZnSO_4.7H_2O$	86.0
	Vitamins	
Stock IV	glycine	20.0
	nicotinic acid	5.0
	pyridoxine-HCl	5.0
	thiamine-HCl	10.0
	Growth-Regulators	
Stock V	IAA	100.0
Stock VI	NAA	100.0
Stock VII	2,4-D	100.0
Stock VIII	BAP	100.0
Stock IX	KIN	100.0
Stock X	ZTN	100.0

IAA, NAA, 2,4-D, BAP, KIN, ZTN and GA_3 were generally autoclaved (YOMON and MACLEOD,1977). Approximately 25 ml of medium was transferred into 125 ml wide-mouthed culture jar (8.5 cm X 4.5 cm). The jars containing the medium were then capped loosely and autoclaved for 20 minutes at 15 psi and 121^{o} C.

D2. Explant Source

Seeds of okra (Abelmoschus esculentus L. cv. Parkins Mammoth Longpod) were obtained from Dominion Seed House (Georgetown, Ontario, Canada, L7G 4A2). Seeds were surface sterilized first with 80% (v/v) ethanol for 1 minute followed by 50% (v/v) javel [commercial bleach with 6% (w/v) available sodium hypochlorite] for 20 minutes. The sterilized seeds were thoroughly rinsed in 3 changes (5 minutes/rinse) of sterile distilled deionized water. Three sterile seeds were then placed in plastic capped culture jars containing hormone-free MS-basal nutrient medium. The medium was supplemented with 30.0 g/l sucrose. The seeds were allowed to germinate in a 'Conviron' growth chamber at 24^{o} C and 16 h photoperiod.

Seeds were also sown in pots (15 cm X 25 cm) in the greenhouse. No supplimentary light was provided and the

Table 2

Growth regulator formulations used for okra tissue culture. All media except C17 and C19 were made up with full-strength MS basal medium. C17 and C19 were prepared using half-strength MS basal medium. The letter C was used arbitrarily to designate this group of culture media.

Medium #	Growth Regulator mg/l						
	Auxins			Cytokinins			Gibberellin
	IAA	NAA	2,4-D	BAP	KIN	ZTN	GA_3
C O	-	-	-	-	-	-	-
C MS	0.35	-	0.22	-	0.02	-	-
C 1	-	0.1	-	-	-	-	-
C 2	-	1.0	-	-	-	-	-
C 3	0.1	-	-	-	-	-	-
C 4	1.0	-	-	-	-	-	-
C 5	-	-	-	0.1	-	-	-
C 6	-	-	-	1.0	-	-	-
C 7	-	-	-	-	0.1	-	-
C 8	-	-	-	-	1.0	-	-
C 9	-	-	-	-	-	0.1	-
C 10	-	-	-	-	-	1.0	-
C 11	-	-	-	2.0	-	-	-
C 12	-	0.1	-	1.0	-	-	-
C 13	-	1.0	-	1.0	-	-	-
C 14	0.1	-	-	1.0	-	-	-
C 15	0.1	-	-	-	1.0	-	-
C 16	-	0.1	-	-	-	1.0	-
C 17	0.1	-	-	-	-	-	0.1
C 18	-	0.01	-	0.1	-	-	-
C 19	0.1	-	-	-	0.01	-	-
C 20	0.01	-	-	0.1	0.1	-	-
C 21	-	-	-	0.02	-	-	-
C 22	-	-	-	0.05	-	-	-
C 23	-	-	-	0.2	-	-	-
C 24	-	2.0	-	1.0	-	-	-
C 25	-	0.2	-	1.0	-	-	-
C 26	-	0.02	-	1.0	-	-	-
C 27	0.2	-	0.2	-	-	-	-
C 28	-	0.2	-	1.0	-	-	-
C 29	0.05	-	-	10.0	-	-	-
C 30	0.04	-	-	-	0.2	-	-
C 31	0.1	-	-	-	-	0.1	-
C 32	0.1	-	-	-	-	0.5	-
C 33	0.1	-	-	-	-	1.0	-
C 34	0.1	-	-	-	-	2.0	-
C 35	-	0.1	-	-	-	0.5	-
C 36	-	0.1	-	-	-	1.0	-
C 37	-	0.1	-	-	-	2.0	-

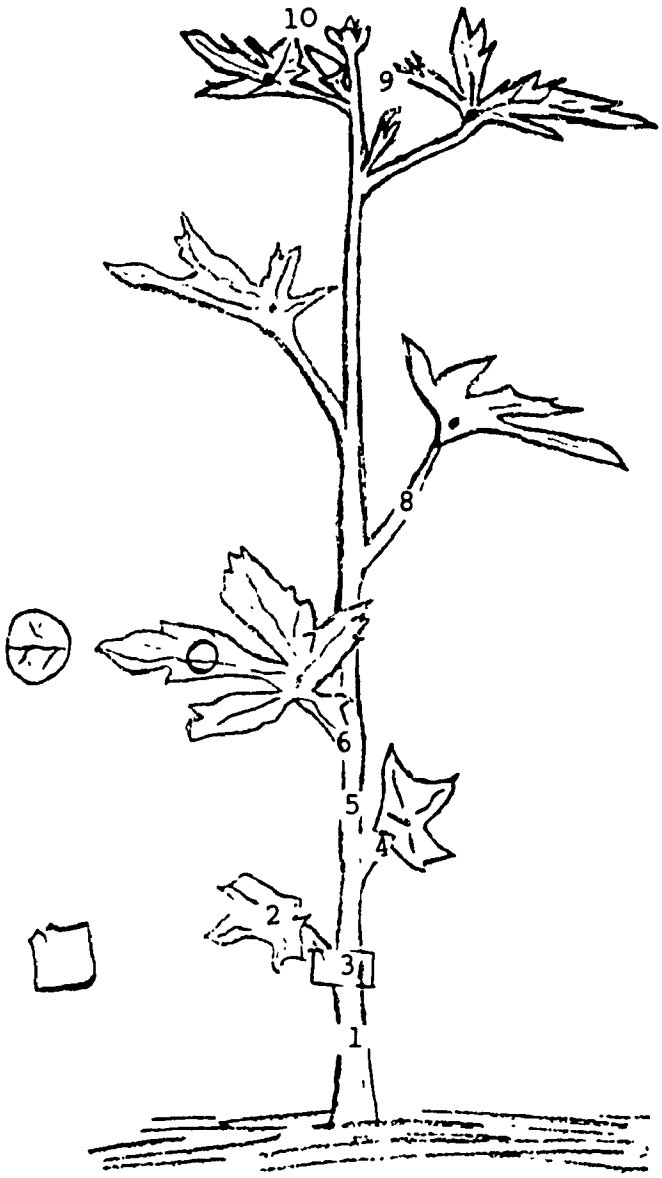
temperature range was 25 to 28^{o} C. Each pot contained 5 seeds in potting soil.

Depending upon the experiment, explants (Figure 1) were taken from either aseptically or greenhouse grown seedlings. Anther and immature fruit explants were taken from greenhouse grown seedlings only.

D3. Culture Procedures

Explants from aseptically grown seedlings were transferred directly under aseptic condition. Explants from greenhouse stocks were first surface sterilized by immersing them into 80% ethanol for 30 seconds and then following the procedures outlined for sterilization of seeds.

The sterile cotyledon and leaf disks were cut from the mid-rib region with a 1 cm diameter sterile cork borer. A longitudinal slit was made along their major vascular bundle regions with a sterile needle. Hypocotyl, internode and petiole explants were slit longitudinally and the cut ends placed on the medium. A few small perforations were made on the exterior surfaces of all explants with a sterile needle. The longitudinal slits and the small perforations allowed increased nutrient absorbing efficiency. The nodal and cotyledonary axil explants were excised and cultured



10. Immature fruit

9. Flower bud (Anther)

8. Petiole

7. Leaf

6. Leaf node

5. Internode

4. Cotyledonary petiole

3. Cotyledonary axil

2. Cotyledon

1. Hypocotyl

Figure 1

Diagramatic representation of an okra plant showing various plant parts used as explants. Excised cotyledonary axil and leaf segment are shown separately (bottom left). Immature fruit and anther explants were cultured from greenhouse grown seedlings only.

without any special treatment. Each culture jar generally contained 3 explants and in most cases five or more replicates of each were cultured. The culture jars were placed in 'Conviron' growth chambers.

D4. Regeneration

(a) Propagation: MS basal nutrient salts and essential organic compounds with different combinations of growth regulators were used to initiate callus root and shoot development from various explants (Figure 1). Thirty nine different media formulations were initially tested (Table 2). All cultures were subjected to 24^{o} C and 16h photoperiod with fluorescent lamps (Westinghouse Cool White, 4000 lux). Cultures were observed regularly and the contaminated jars were replaced by fresh cultures. After a period of 30 days the media and explants were screened and the responses scored.

When a morphogenic response from an explant was observed with a particular media formulation, the experiments were repeated to confirm these observations. When only shoots were produced, these shoots were transferred to a medium containing 1.0 mg/l NAA to form roots. Explants cultured in the medium contining IAA (1.0

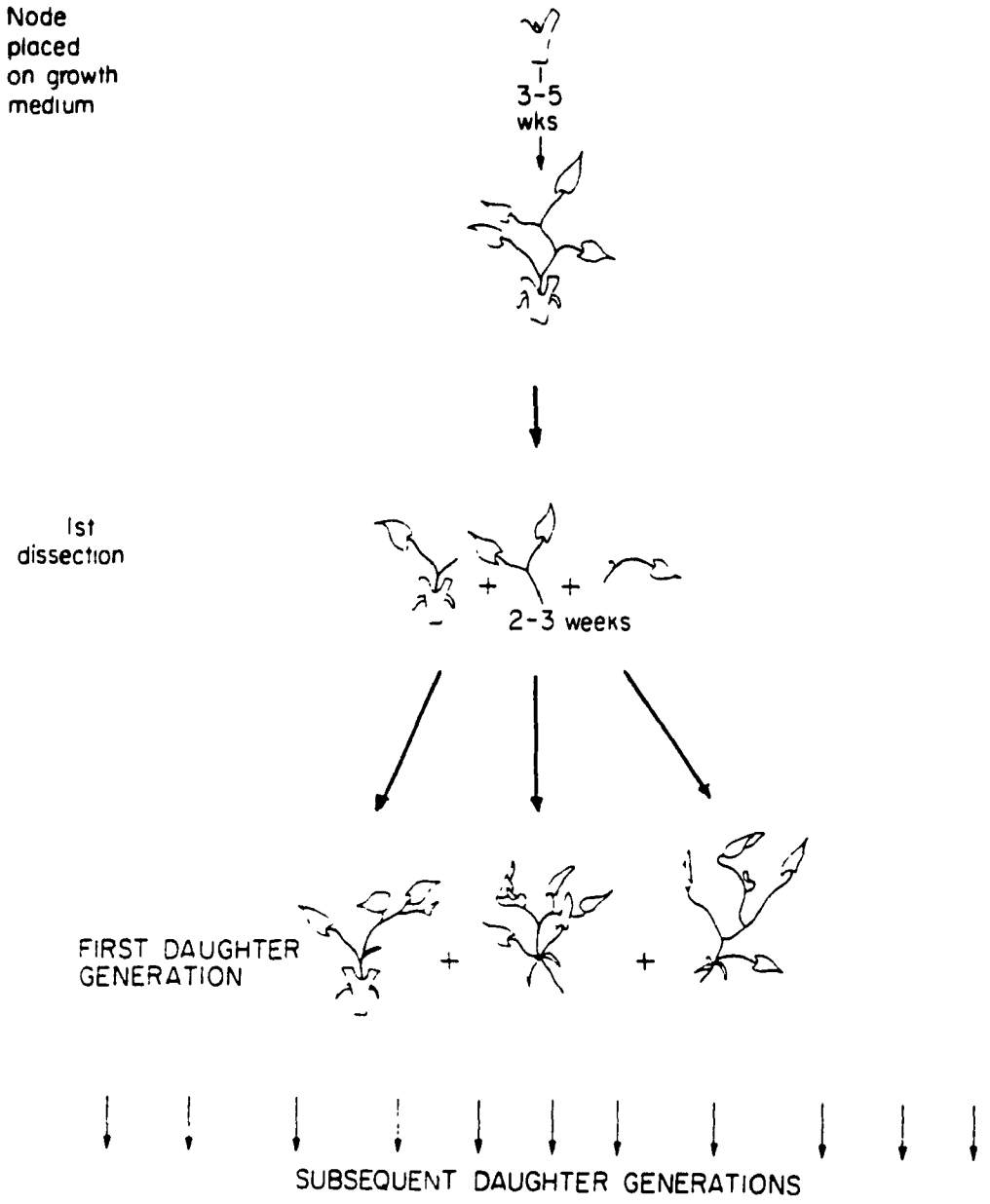


Figure 2

Diagramatic representation of multiple shoot formation from nodal explants. Nodal segment of shoots regenerated from parent explants (seedlings) were excised and cultured again for shoot multiplication. This procedure of micropropagation by nodal cuttings can be continued indefinitely to obtain subsequent daughter generations (modified from MANTELL,1985).

mg/l) and BAP (1.0 mg/l) produced rooted shoots (plantlets). All plantlets were then acclimatized and transferred to the greenhouse and grown to maturity (page 36).

With these early experiments, it was observed that only cotyledons, cotyledonary axils, leaf nodes or nodal tissue from cultured shoots (Figure 2) responded in producing shoots. However, a single shoot was obtained per explant. In order to generate multiple shoots, from an explant or callus tissue a number of other experiments were undertaken.

(b) Micropropagation: (i) Explants. Okra seeds were aseptically placed on MS nutrient medium without growth hormone. Cotyledons, hypocotyls, cotyledonary axils and leaf nodes were removed from 2-week old seedlings. The excised explants were cultured on regeneration media CRS7 to CRS21 (Table 3) at 28^{o}. Multiple shootlets that developed after 4 weeks on cotyledonary axil and leaf nodes were excised and part of them were transferred to rooting medium (NAA 1.0 mg/l) for two weeks.

The remaining shoots were excised into their nodal segments. The nodal cuttings were cultured again on regeneration media CRS7 to CRS11 at 28^{o} C for 4 weeks. Multiple shoots that developed on these nodal cuttings were excised and transferred to rooting medium for another two weeks.

Table 3

Auxin/Cytokinin composition of MS basal nutrient medium used to obtain callus, root shoot and plantlet from various okra explants. In media contain auxin and cytokinin, the auxin concentration was kept constant while that of cytokinin was varied. The abbreviation CRS was arbitrarily used to identify this group of media.

Medium #	Growth Regulator mg/l				
	Auxins		Cytokinins		
	IA	NAA	BAP	KIN	ZTN
CRS 1	0.1	-	-	-	-
CRS 2	1.0	-	-	-	-
CRS 3	2.0	-	-	-	-
CRS 4	-	0.1	-	-	-
CRS 5	-	1.0	-	-	-
CRS 6	-	2.0	-	-	-
CRS 7	-	-	0.5	-	-
CRS 8	-	-	1.0	-	-
CRS 9	-	-	2.0	-	-
CRS 10	-	-	3.0	-	-
CRS 11	-	-	5.0	-	-
CRS 12	-	-	-	0.5	-
CRS 13	-	-	-	1.0	-
CRS 14	-	-	-	2.0	-
CRS 15	-	-	-	3.0	-
CRS 16	-	-	-	5.0	-
CPS 17	-	-	-	-	0.5
CRS 18	-	-	-	-	1.0
CRS 19	-	-	-	-	2.0
CRS 20	-	-	-	-	3.0
CRS 21	-	-	-	-	5.0

continued.....

Table 3 continued....

Medium #	Growth Regulator mg/l				
	Auxins		Cytokinins		
	IAA	NAA	BAP	KIN	ZTN
CRS 22	0.1	-	0.5	-	-
CRS 23	0.1	-	1.0	-	-
CRS 24	0.1	-	2.0	-	-
CRS 25	0.1	-	3.0	-	-
CRS 26	0.1	-	5.0	-	-
CRS 27	0.1	-	-	0.5	-
CRS 28	0.1	-	-	1.0	-
CRS 29	0.1	-	-	2.0	-
CRS 30	0.1	-	-	3.0	-
CRS 31	0.1	-	-	5.0	-
CRS 32	0.1	-	-	-	0.5
CRS 33	0.1	-	-	-	1.0
CRS 34	0.1	-	-	-	2.0
CRS 35	0.1	-	-	-	3.0
CRS 36	0.1	-	-	-	5.0

continued.....

Table 3 continued.....

Medium #	Growth Regulator mg/l				
	Auxins		Cytokinins		
	IAA	NAA	BAP	KIN	ZTN
CRS 37	-	0.1	0.5	-	-
CRS 38	-	0.1	1.0	-	-
CRS 39	-	0.1	2.0	-	-
CRS 40	-	0.1	3.0	-	-
CRS 41	-	0.1	5.0	-	-
CRS 42	-	0.1	-	0.5	-
CRS 43	-	0.1	-	1.0	-
CRS 44	-	0.1	-	2.0	-
CRS 45	-	0.1	-	3.0	-
CRS 46	-	0.1	-	5.0	-
CRS 47	-	0.1	-	-	0.5
CRS 48	-	0.1	-	-	1.0
CRS 49	-	0.1	-	-	2.0
CRS 50	-	0.1	-	-	3.0
CRS 51	-	0.1	-	-	5.0

continued.....

Table 3 continued.....

Medium #	Growth Regulator mg/l				
	Auxins		Cytokinins		
	IAA	NAA	BAP	KIN	ZTN
CRS 52	1.0	-	0.5	-	-
CRS 53	1.0	-	1.0	-	-
CRS 54	1.0	-	2.0	-	-
CRS 55	1.0	-	3.0	-	-
CRS 56	1.0	-	5.0	-	-
CRS 57	1.0	-	-	0.5	-
CRS 58	1.0	-	-	1.0	-
CRS 59	1.0	-	-	2.0	-
CRS 60	1.0	-	-	3.0	-
CRS 61	1.0	-	-	5.0	-
CRS 62	1.0	-	-	-	0.5
CRS 63	1.0	-	-	-	1.0
CRS 64	1.0	-	-	-	2.0
CRS 65	1.0	-	-	-	3.0
CRS 66	1.0	-	-	-	5.0

continued.....

Table 3 continued.....

Medium #	Growth Regulator				
	Auxins		Cytokinins		
	IAA	NAA	BAP	KIN	ZTN
CRS 67	-	1.0	0.5	-	-
CRS 68	-	1.0	1.0	-	-
CRS 69	-	1.0	2.0	-	-
CRS 70	-	1.0	3.0	-	-
CRS 71	-	1.0	5.0	-	-
CRS 72	-	1.0	-	0.5	-
CRS 73	-	1.0	-	1.0	-
CRS 74	-	1.0	-	2.0	-
CRS 75	-	1.0	-	3.0	-
CRS 76	-	1.0	-	5.0	-
CRS 77	-	1.0	-	-	0.5
CRS 78	-	1.0	-	-	1.0
CRS 79	-	1.0	-	-	2.0
CRS 80	-	1.0	-	-	3.0
CRS 81	-	1.0	-	-	5.0

Following the development of roots, the rooted shoots were planted in soil/vermiculite mixture, climatized and then placed in the greenhouse.

(ii) Callus. Hypocotyl and cotyledonary axil were excised and cultured on the selected media shown in table 4. Two-week-old callus masses were excised from explant tissue and subcultured (3X) on fresh medium (1.0 mg/l BAP). After two weeks, calli pieces (0.5 cm) were cultured on media shown in table 5. The calli showing organogeneses were further subcultured on silver nitrate enriched medium (Table 6). After four weeks, the shoots were excised and subcultured on rooting medium containing NAA (1.0 mg/l). The rooted shoots after climatisation, were transferred to a soil vermiculite mixture and allowed to grow in the greenhouse. All media contained 3% (w/v) sucrose and 0.6% (w/v) agar. The pH was adjusted to 5.7 before sterilization by autoclaving at 15 psi for 20 minutes. Growth regulators were being added to the medium before it was sterilized. Culture flasks (125 ml) containing 30 ml of sterile medium were used for the culture of explants and the subculture of callus. Sterile petri dishes containing 20 ml of sterile medium were used to generate shoots from calli culture and these shoots were allowed to develop and form roots in

Table 4

The growth regulator compositions of selected media used to culture hypocotyl and cotyledonary explants to initiate calli. Growth regulators were incorporated in MS basal nutrient solution.

	Growth Regulator mg/l				
	Auxins		Cytokinins		
Medium #	IAA	NAA	BAP	KIN	ZTN
CRS 8	-	-	1.0	-	-
CRS 13	-	-	-	1.0	-
CRS 18	-	-	-	-	1.0
CRS 23	0.1	-	1.0	-	-
CRS 28	0.1	-	-	1.0	-
CRS 33	0.1	-	-	-	1.0
CRS 38	-	0.1	1.0	-	-
CRS 43	-	0.1	-	1.0	-
CRS 48	-	0.1	-	-	1.0

Table 5

Compositions of MS basal medium containing various amounts of IAA, NAA, BAP, KIN, abscisic acid (ABA) and one medium containing gibberellic acid (GA_3) alone. These growth regulator formulations were used to initiate morphogeneses in okra callus tissue. The use of letters with media numbers is arbitrary.

Medium #	Growth Regulator mg/l					
	Auxins		Cytokinins		GA_3	ABA
	IAA	NAA	BAP	KIN		
IK 1	2.0	-	-	-	-	-
IK 2	2.0	-	-	0.1	-	-
IK 3	2.0	-	-	0.2	-	-
IK 4	2.0	-	-	0.5	-	-
IK 5	2.0	-	-	1.0	-	-
IK 6	-	-	-	2.0	-	-
IK 7	0.1	-	-	2.0	-	-
NK 8	-	2.0	-	-	-	-
NK 9	-	2.0	-	0.1	-	-
NK 10	-	2.0	-	0.2	-	-
NK 11	-	2.0	-	0.5	-	-
NK 12	-	2.0	-	1.0	-	-
NK 11	-	2.0	-	0.5	-	-
NK 13	-	-	-	2.0	-	-
NK 14	-	0.1	-	2.0	-	-
KG 15	-	-	-	-	1.0	-
IB 16	4.0	-	10.0	-	-	-
IB 17	-	-	10.0	-	-	-
IB 18	-	-	5.0	-	-	-
IK 19	0.02	-	-	2.0	-	-
IK 20	1.0	-	-	-	-	-
NB 21		1.0	1.0	-	-	-
IK 22	0.1	-	-	1.0	-	-
IK 23	1.0	-	-	2.0	-	-
IB 24	-	-	1.0	-	-	-
AB 25	-	-	-	-	-	0.1
AB 26	-	-	-	-	-	0.5
AB 27	-	-	-	-	-	1.0
AB 28	-	-	-	-	-	2.0

Table 6

The chemical composition of MS basal medium used to induce multiple shoots from the cotyledonary axil derived calli. The basal nutrients were supplemented with silver nitrate (AgNO3) and BAP when myo-inositol (MI) and casein hydrolysate (CH) either present or absent in the medium. The use of letters with the media numbers is arbitrary.

Chemical Component mg/l				
Medium #	$AgNO_3$	BAP	MI	CH
AG 1	-	1.0	-	-
AG 2	5.0	1.0	-	-
AG 3	10.0	1.0	-	-
AG 4	20.0	1.0	-	-
AG 5	40.0	1.0	-	-
AG 6	-	1.0	100.0	1000.0
AG 7	5.0	1.0	100.0	1000.0
AG 8	10.0	1.0	100.0	1000.0
AG 9	20.0	1.0	100.0	1000.0
AG 10	40.0	1.0	100.0	1000.0

125 ml flasks containing 30 ml of sterile rooting medium. All cultures were incubated in 'Conviron' growth chambers at 28° C with a daily photoperiod of 16h (4000 lux from cool-white Westinghouse fluoroscent lamps).

D5. Growth of Regenerated Plants to Maturity

Plantlets and shoots with well developed root system that were obtained from explants under all conditions described above were removed carefully from the culture jars. The roots were washed thoroughly with running tap water (28° C) to remove solidified culture media. Care was taken to minimize the breakage of the tender roots and soft tissues. Plantlets were then transplanted individually in small pots (4 cm X 7 cm) containing soil vermiculite mixture (4:1) saturated with water and kept in a high humidity environment within an enclosed transparent plastic chamber. This prevented wilting of the planted plantlets. The plantlets were illuminated uniformly with incandescent lamps (3000 lux). The acclimatization period lasted for 4-5 weeks. The regenerated plants were then exposed to the environment by keeping one side of the chamber open for the next 48 hours. Once the acclimatization was acomplished the plants (8-10 cm in height) were ready to be transferred to the greenhouse.

D6. Comparison of Cultured (Regenerated) and Seed Plants

Two-weeks old greenhouse grown seedlings and climatized cultured plantlets were transplanted into bigger pots (15 cm X 20 cm) containing potting soil. Pots were saturated with tap water and placed in the greenhouse under similar conditions. The pots were watered once a day by automated sprinkler system and once a week by hand to ensure sufficient watering. The growth pattern and the yield of the two sets of plants were compared and recorded.

The flowering time (day) was recorded when flower buds opened and the yellow petals could be partially seen. The height of plants, the number and size of fruits and leaves were monitored until the plants reached maturity. Fruit sizes were recorded by averaging the length of the total number of fruits per plant.

D7. Isolation and Culture of Protoplasts

Cotyledons and leaves from aseptically grown four-weeks old okra seedlings were removed. The lower epidermal layers were peeled with a pair of sterile pointed forceps. The peeled areas were excised and separated from the unpeeled portions and cut approximately into 0.5 cm strips. About 10 g of cotyledon and leaf strips were placed separately into

twenty 125 ml erlynmeyer flasks containing 50 ml of cell wall degrading enzymes solution (sterile). The enzymes solution consisted of 4% (w/v) cellulase, 2% (w/v) pectinase (both enzymes purchased from Sigma) and 0.66 M mannitol (pH 5.8). The solution was filter sterilized using millipore filter paper (0.45 um). The plant tissues in the enzyme mixture were shaken on a 'Therm-O-Shake' rotary shaker (80 rpm) at 25^{o} C in the dark

Following the incubation, the solution was filtered through a fine wire mesh (pore size 100 um) to separate protoplasts from other plant debris. The released protoplasts were then allowed to settle to the bottom of the tapered test tubes. The enzymes solution was decanted out slowly without disturbing the protoplast pellets. The protoplasts fraction was washed three times with sterile 0.66 M mannitol solution to free the protoplasts from any extracellular cell wall degrading enzymes. The resultant protoplasts were diluted serially with the sterile mannitol solution to a concentration of approximately $5X10^{4}$ protoplasts per ml.

The viability of protoplasts was tested with 0.04% Evans Blue dye (Sigma) before placing them in the culture medium. A haemocytometer (Improved Neubauer) with 1/400

sq.mm area and 1/10 mm field depth was used to determine both the percentage of viability and the density of the protoplasts in the mannitol solution.

One ml of each protoplast suspension was poured onto 20 ml of semi-solid sterile culture media in plastic petri dish (90 mm X 15 mm). The culture medium contained MS basal nutrients, 2% sucrose, 0.66 M mannitol, 1.0 mg/l NAA, 0.5 mg/l ZTN, 0.2 mg/l 2,4-D and 0.3% agar (SZABADOS,1985). Total of twenty two petri dishes containing protoplasts (11 of cotyledons and 11 of leaf protoplasts) were cultured. The petri dishes were wraped around with parafilm to minimize evaporation and water loss from the cultures and placed at 25^{o} C in the dark for one week followed by the 16h photoperiod for 32 days.

All aseptic manipulations were performed under a laminar flow hood (Forma Scientific, Model 1839) equipped with blower, uv lamp and bunsen burner. The hood was wipe-cleaned with 80% ethanol and then turned on for half an hour prior to placing the culture materials. Sterilized media and all the pertinent sterile materials (i.e., distilled water, pouch, scalpel, forcep, ceramic tile, needle, cork borer) were kept in the hood under uv light and with blower switched on for another half an hour before starting the transferring operations.

The extent of morphological changes was recorded in terms of percent (%) frequency and intensity (4+ high; 1+ low) of responses. The method of reporting responses can be explained as follows:

Response with callus growth for example,

high (++++) = 3

moderate (+++) = 6

medium (++) = 2

low (+) = 2

no (-) = 2

Total explant = 15

Since, 13 out of 15 explants responded to callus growth

therefore, the frequency = 13/15 X 100 = 86.7%

However,

3 explants responded with high (++++)

6 explants responded with moderate (+++)

2 explants responded with medium (++)

2 explants responded with low (+)

and, 2 explant responded with no (-)

callus growth.

Therefore, the total intensity

$$3X4^{(+)} + 6X3^{(+)} + 2X2^{(+)} + 2X1^{(+)} + 2X0^{(+)}$$

$$= 12 + 18 + 4 + 2 + 0$$

$$= 36$$

and the average intensity = 36/15

= 2.40

Thus, it could be seen that although 86.7% of the explants responded in callus formation, the average intensity was only 2.40.

The summary of some of the pertinent experiments is illustrated in the diagram as shown in appendix (Appendix II).

E. RESULTS

E1. Regeneration

(a) **Propagation:** (i) Growth Regulator Composition- Explants of okra seedlings cultured on MS basal medium supplimented with auxins, cytokinins and auxin-cytokinin combinations showed distinct morphogenic responses. Callus was formed in all explants on media containing auxins and cytokinins except explants derived from anther and immature fruit (Table 7). The promotion of root development was observed when IAA and NAA concentration was increased from 0.1 mg/l to 1.0 mg/l. A characteristic difference, however, between two auxins was that on IAA medium the roots were short and poorly developed (Figure 3.2), while on NAA supplimented MS medium the roots were thin and long with a number of lateral roots and/or root hairs (Figure 3.3). The addition of 2,4-D into IAA-supplemented MS medium resulted in the total suppression of root development. However, 2,4-D stimulated the formation of friable whitish callus (Figure 3.1). Shoot bud or shoots were not observed on any of the explant cultured on growth medium containing only an auxin.

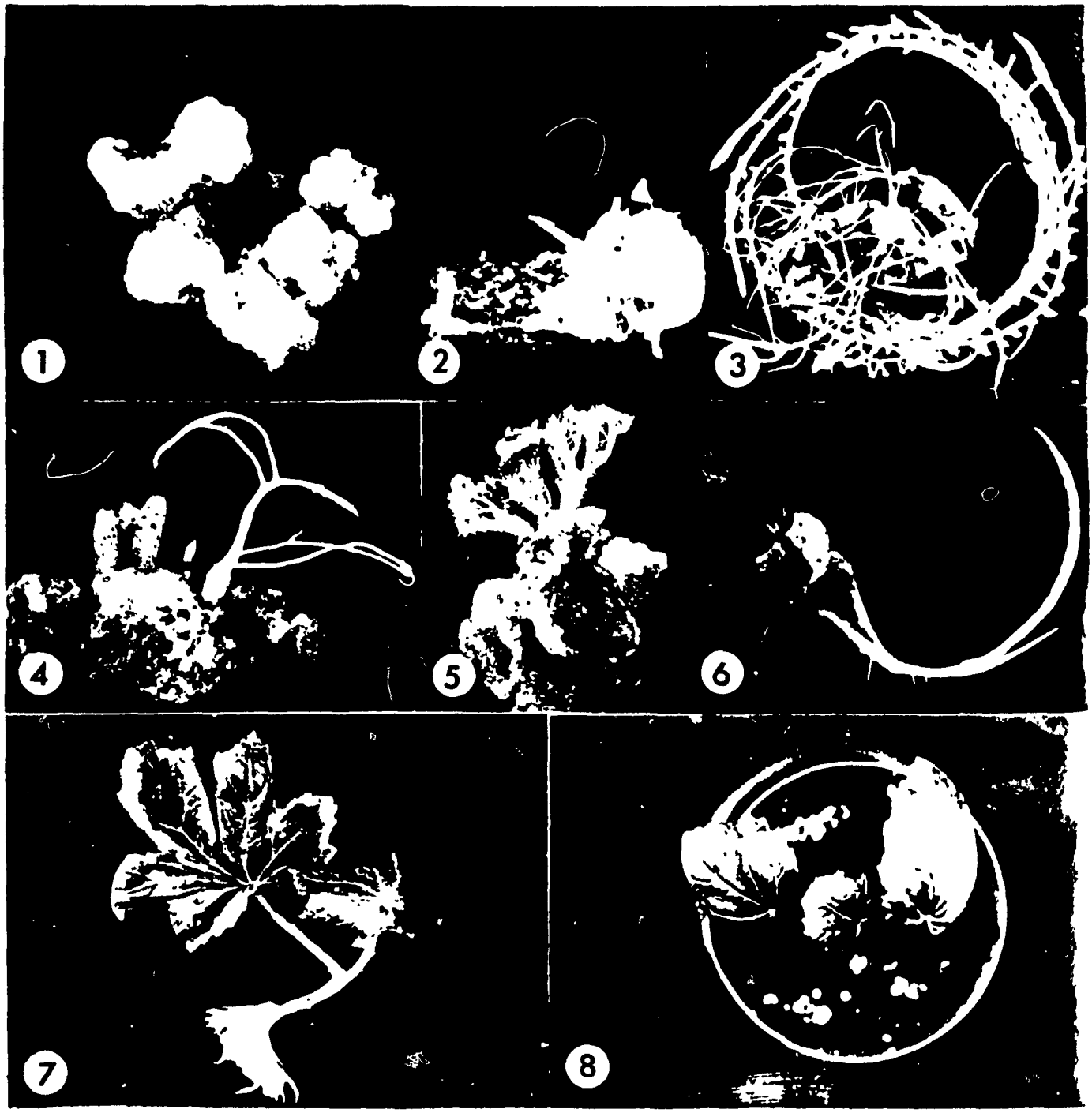
Table 7

Comparative morphogenic responses in okra explants cultured in MS medium supplemented with various hormones. C= callus, R= root, S= shoot. Intensity is represented by 4+ = most, + = least and - = no growth. The explant is represented by H = hypocotyl, c = cotyledon, CA = cotyledonary axil, CP = cotyledonary petiole, I = internode, LN = leaf node, LP = leaf petiole, A = anther, F = immature fruit. Results scored after 4 weeks for a total of 30 replicates per explants from two trials.

Medium	Explant									
mg/l	1H	2C	3CA	4CP	5I	6LN	7L	8LP	9A	10F
BM only	$-_{+}$	$-_{+}$	$-_{+}$	$-_{+}$	$-_{+}$	$-_{+}$	$-_{+}$	$-_{+}$	-	-
MS only	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	-	-
IAA.1	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	-	-
IAA1.O	$C_{+}R^{+}$	$C_{+}R^{2+}$	$C_{+}R^{+}$	$C_{+}R^{+}$	$C_{+}R^{2+}$	$C_{+}R^{2+}$	$C_{+}R^{2+}$	$C_{+}R^{2+}$	-	-
NAA.1	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	C_{+}	-	-
NAA1.O	$C^{+}R^{3+}$	$C^{2+}R^{3+}$	$C^{+}R^{3+}$	$C^{2+}R^{3+}$	$C^{+}R^{3+}$	$C^{+}R^{3+}$	$C^{+}R^{4+}$	$C^{+}R^{2+}$	-	-
IAA.2 +2,4-D.2	C^{4+}	C^{3+}	C^{3+}	C^{4+}	C^{3+}	C^{3+}	C^{4+}	C^{3+}	-	-
BAP.5	C^{2+}	C^{2+}	C^{2+}	C^{2+}	C^{2+}	C^{2+}	C^{2+}	C^{2+}	-	-
BAP1.O	C^{3+}	C^{2+}	$C^{2+}S$	C^{3+}	C^{2+}	C^{2+}	C^{+}	C^{+}	-	-
BAP2.O	C^{2+}	C^{2+}	$C^{2+}S$	C^{2+}	C^{2+}	$C^{+}S$	C^{2+}	C^{2+}	-	-
KIN1.O	C_{+}	C_{+}	-	C_{+}	C_{+}	-	-	C_{+}	-	-
ZTN1.O	C^{2+}	C^{2+}	-	C^{2+}	C^{2+}	-	-	C^{2+}	-	-
IAA.1 +BAP1.O	$C^{2+}R^{+}$	$C^{+}R^{+}$	$C^{+}S$	$C^{2+}R^{+}$	$C^{2+}R^{+}$	$C^{+}S$	C^{3+}	$C^{+}R^{+}$	-	-
IAA.1 +KIN1.O	C^{+}	C^{+}	-	C^{+}	C^{+}	C^{+}	C^{+}	-	-	-
IAA.1 +ZTN1.O	C^{+}	C^{+}	-	C^{+}	C^{+}	C^{+}	-	C^{+}	-	-
IAA1.O +BAP1.O	$C^{2+}R^{+}$	$C^{2+}R^{+}$	$C^{+}R^{+}S$	$C^{+}R^{+}$	$C^{+}R^{+}S$	$C^{2+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	-	-
IAA1.O +KIN1.O	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	-	-
IAA1.O +ZTN1.O	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	-	-
NAA.1 +BAP1.O	$C^{4+}R^{+}$	$C^{3+}R^{+}$	$C^{2+}S$	$C^{2+}R^{+}$	$C^{3+}R^{+}$	$C^{2+}S$	C^{3+}	$C^{+}R^{+}$	-	-
NAA.1 +KIN1.O	C^{+}	C^{+}	C^{+}	C^{+}	C^{+}	C^{+}	C^{+}	-	-	-
NAA.1 +ZTN1.O	C^{+}	C^{+}	C^{+}	C^{+}	C^{+}	C^{+}	C^{+}	C^{+}	-	-
NAA1.O +BAP1.O	$C^{2+}R^{3+}$	$C^{+}R^{3+}S$	$C^{+}R^{2+}S$	$C^{2+}R^{2+}$	$C^{2+}R^{2+}$	$C^{+}R^{2+}S$	$C^{2+}R^{3+}$	$C^{2+}R^{2+}$	-	-
NAA1.O +KIN1.O	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	-	-
NAA1.O +ZTN1.O	$C^{2+}R^{+}$	$C^{2+}R^{+}$	$C^{2+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	$C^{+}R^{+}$	-	-

Figure 3

Responses of various explants of okra to different growth substances. 1, friable whitish callus growth on hypocotyl explant cultured on MS medium containing IAA (0.2 mg/l) and 2,4-D (0.2 mg/l). 2, short root and callus formation on hypocotyl explant cultured on MS medium supplemented with IAA (1.0 mg/l). 3, development of long roots with a number of lateral roots on leaf explant cultured on MS medium containing NAA (1.0 mg/l). 4, formation of roots and shoot buds on cotyledon explant cultured on MS medium supplemented with NAA (1.0 mg/l) and BAP (1.0 mg/l). 5, formation of shoot and callus on cotyledonary node explant cultured on MS medium containing BAP (1.0 mg/l). 6, shoot, root and callus development on cotyledonary node explant cultured on MS medium containing BAP (1.0 mg/l) and NAA (1.0 mg/l). 7, regenerated plantlet exhibiting profuse root growth. 8, regenerated plant growing in the greenhouse.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When explants of okra were cultured on MS medium supplemented with cytokinins, compact green calluses were produced only on hypocotyl, cotyledon, petiole, leaf node, internode and leaf explants (Table 7). When these explants were cultured on KIN and ZTN incorporated MS medium, KIN and ZTN failed to stimulate shoot bud or shoot formation (Table 7). However, the addition of BAP (1.0 mg/l) stimulated shoot formation on cotyledons, cotyledonary axils and leaf nodes followed by callus production (Table 7). Although the presence of cytokinins in the growth medium promoted callus proliferation and generation of shoots, the cytokinins alone failed to produce roots on any of the experimental okra explants (Table 7). The combination of 1.0 mg/l respectively, of NAA and BAP was effective in inducing both shoot buds and root development on cotyledon (Figure 3.4), shoots and roots on cotyledonary axils and leaf nodes (Figure 3.6). When IAA was substituted for NAA, shoots were formed on the cotyledonary axils, but this combination failed to elicit any root development. However, roots developed within 10 days when these shoots were transformed to MS medium containing NAA (1.0 mg/l).

(ii) Temperature Effect- Explants (hypocotyl, cotyledon cotyledonary axil and leaf node) cultured at 24^{o} C in

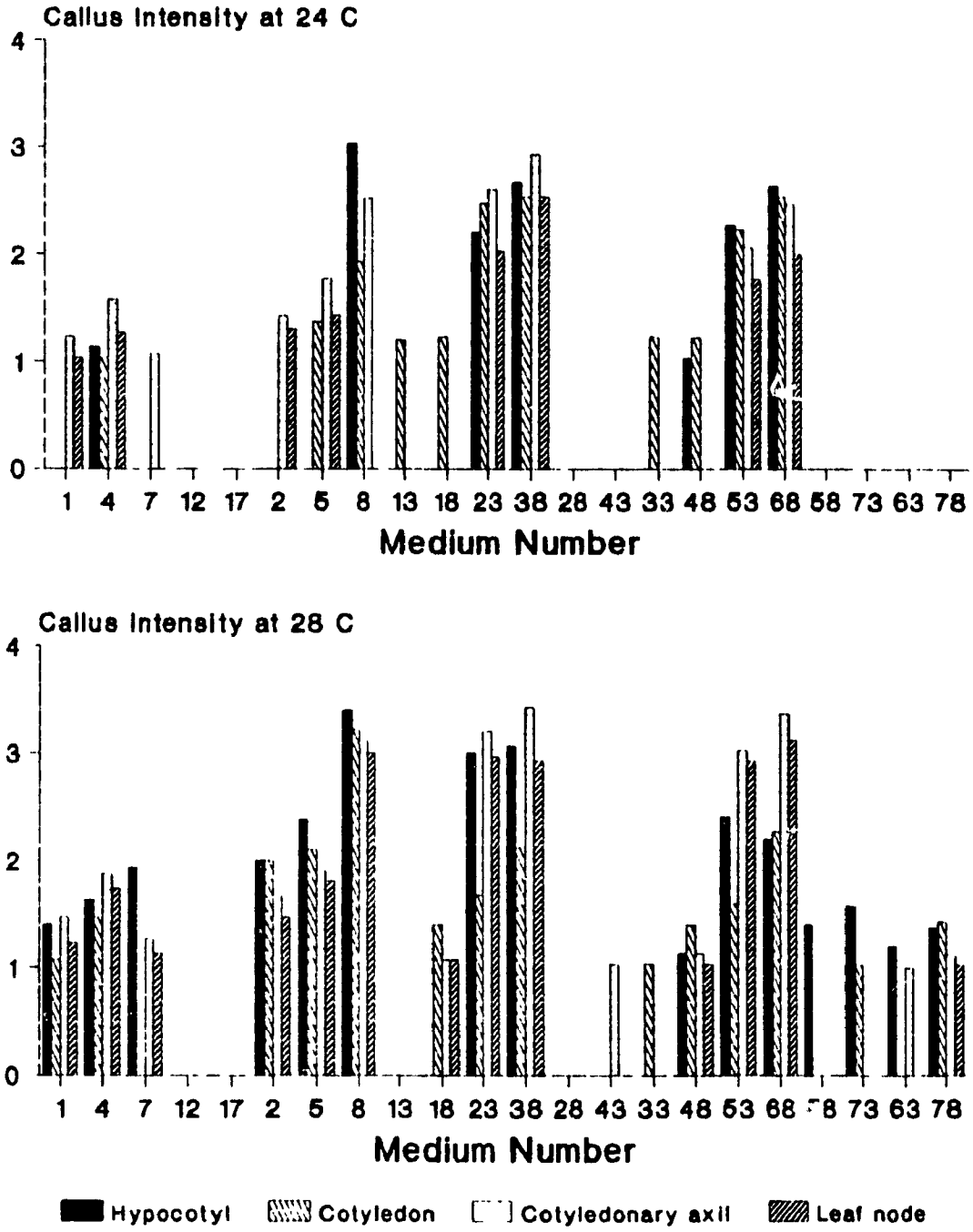


Figure 4

The auxin-cytokinin interaction on calli formation in different okra explants cultured on MS basal medium at 24° C (top) and 28° C (bottom). The result scored after 4 weeks for a total of 30 replicates per explant from two trials. For media number refer to table 3, page 27.

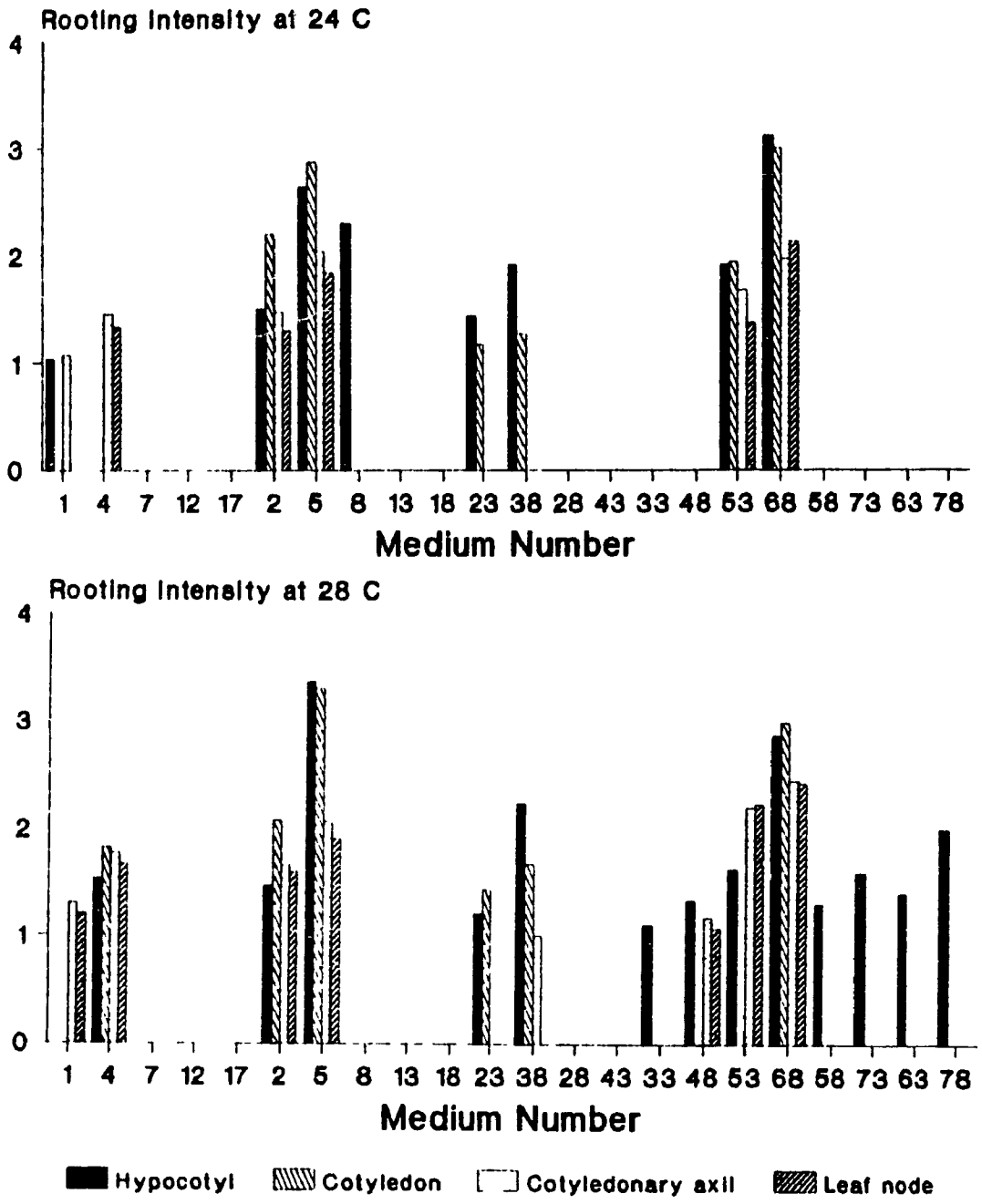


Figure 5

The auxin-cytokinin interaction on root development in different okra explants cultured on MS basal nutrient medium at 24° C (top) and 28° C (bottom). The result is the average of 30 replicates per explant for a total of two trials scored after 4 weeks. For media number see table 3, page 27.

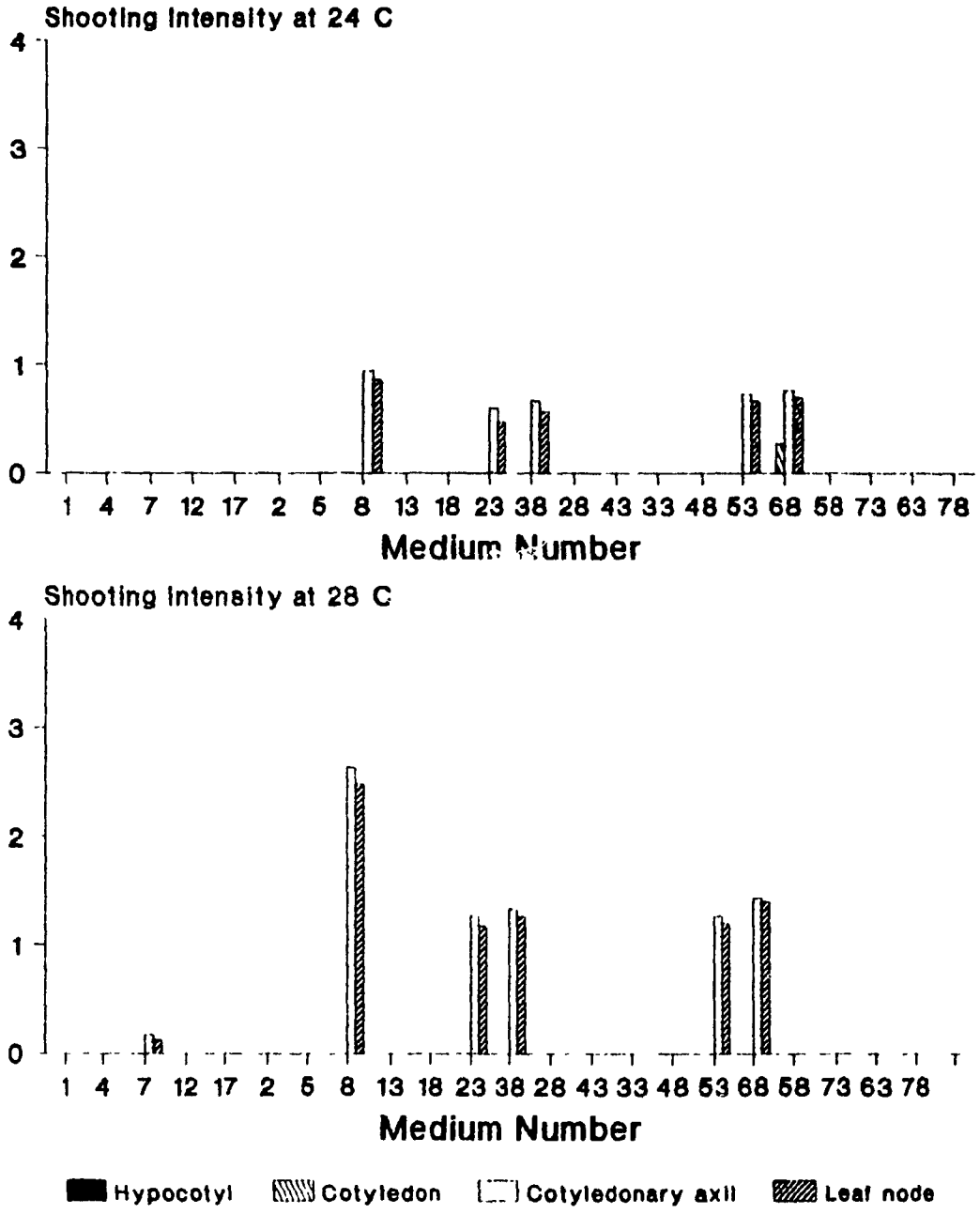


Figure 6

The auxin-cytokinin interaction on shoot initiation in different okra explants cultured on MS basal medium at 24^{o} C (top) and 28^{o} C (bottom). The data scored after 4 weeks from two trials of 15 replicates per explants. For media number refer to table 3, page 27.

media incorporated with various concentrations of auxins and cytokinins produced calli (Figure 4). When these explants were cultured at 28° C in the same media formulations, the intensity of calli formation was enhanced. Also calli formation was stimulated on explants cultured on media number 58, 73, 63 and 78 (Figure 4). Roots developed on hypocotyl, cotyledon, cotyledonary axil and leaf explants at both 24° C and 28° C (Figure 5). The 28° C temperature also stimulated root formation on hypocotyl explants which were cultured on the media 33, 48, 58, 73, 63 and 78 (Figure 5). These explants, when cultured on similar media at 24° C failed to form roots. The rooting intensity however, was increased on explants at 28° C compared to those cultured at 24° C (Figure 5).

Cotyledon, cotyledonary axil and leaf node explants cultured on media 8, 23, 38 53 and 68 produced shoots(Figure 6). When these explants were cultured at 28° C, shoots were only formed on cotyledonary axil and leaf node explants. Shooting intensity on both cotyledonary axil and leaf node explants increased at 28° C. The most shoots were produced when these explants were cultured on medium 8 (Figure 6).

(b) Micropropagation: (i) Explants. Nodal cuttings of regenerated shoots, derived from cotyledonary axil and leaf

Table 8

Shooting frequency of different okra explants cultured on MS basal medium supplimented with various concentration of BAP at 28 C. Explant indicates I= hypocotyl, II= cotyledon, III= cotyledonary axil and IV= Leaf node from seedling; V and VI= leaf nodes of regenerated shoot derived from III and IV. Letter indicates NS= No shoot; S= Shoot and MS= Multiple shoots formed on explants. The numbers indicate total explants produced shoots after a period of 30 days over total cultured.

	Explants									
	I	II	III		V		IV		VI	
Media #			S	MS	S	MS	S	MS	S	MS
CRS7	NS	NS	24/36	6/36	10/14	5/14	21/36	7/36	12/15	4/15
CRS8	NS	NS	36/36	16/36	28/28	18/28	34/36	13/36	30/30	20/30
CRS9	NS	NS	33/36	18/36	24/24	20/24	35/36	17/36	30/30	26/30
CRS10	NS	NS	26/36	12/36	9/18	8/18	28/36	14/36	10/18	9/18
CRS11	NS	NS	15/36	7/36	7/16	4/16	14/36	6/36	9/16	5/16

node explants produced new shoots when cultured at 24° C on regenerating medium (BAP 1.0 mg/l) (Figure 2). Upon transferring these newly produced shoots to rooting medium (NAA 1.0 mg/l) roots were produced.

Cotyledonary axil (III; see figure 8 for definition) and leaf node explants (IV) when cultured on BAP enriched media at 28° C responded by producing shoots. Hypocotyl (I) and cotyledon (II) explants did not form shoot or shoot bud on any of the BAP concentrations in the growth medium (Table 8). The intensity and frequency of shoot formation on other explants (III, V, IV, VI) was highest in the medium with a BAP concentration of 2.0 mg/l and 3.0 mg/l, and with increasing concentration of BAP from 2.0 to 5.0 mg/l, there was a resultant decrease in the intensity and frequency of shoot formation (Figures 7 & 8).

When cotyledonary axil and leaf node were cultured at 28° C in BAP (0.5 mg/l) enriched MS medium, nearly 64% of the explants produced single shoots (Figure 8) and 17% of those shoot producing explants produced multiple shoots (Figure 9). The frequency of multiple shoot formation increased to nearly 50% when cotyledonary axil and leaf node explants were cultured in medium containing 2.0 mg/l BAP (Figures 8 & 9). Further addition of BAP to the growth

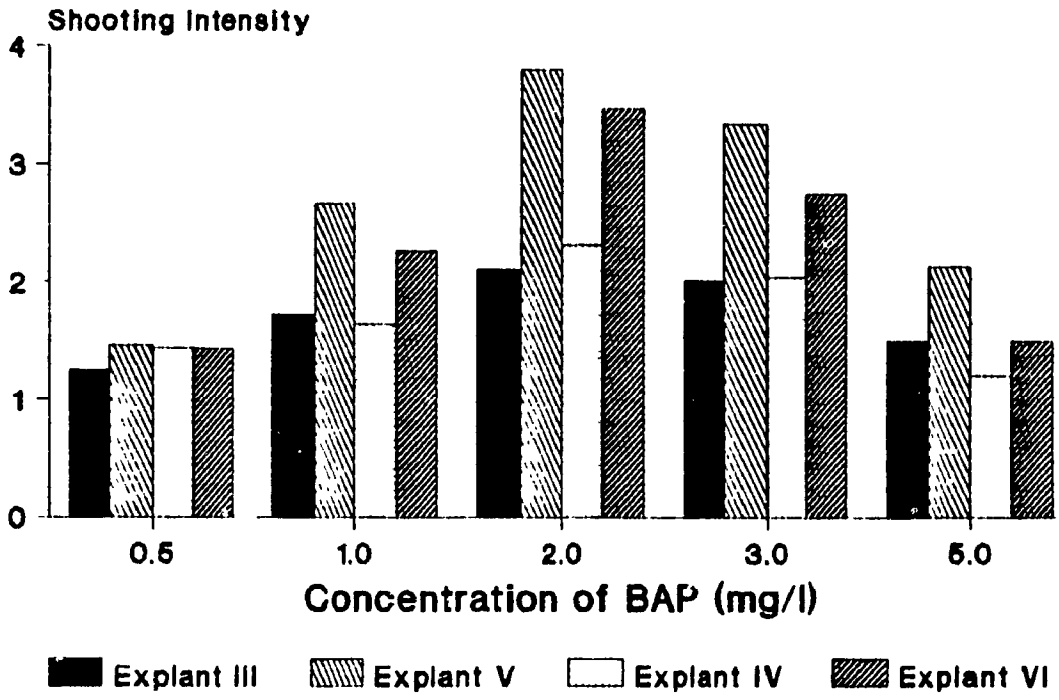


Figure 7

The intensity of shoot formation in different okra explants cultured on MS basal medium containing various concentration of BAP at 28° C. The explant is represented by III, cotyledonary axil and IV, leaf node from aseptically grown seedlings. Explants V and VI are leaf nodes of generated shoots derived from III and IV respectively. The result was scored after 4 weeks.

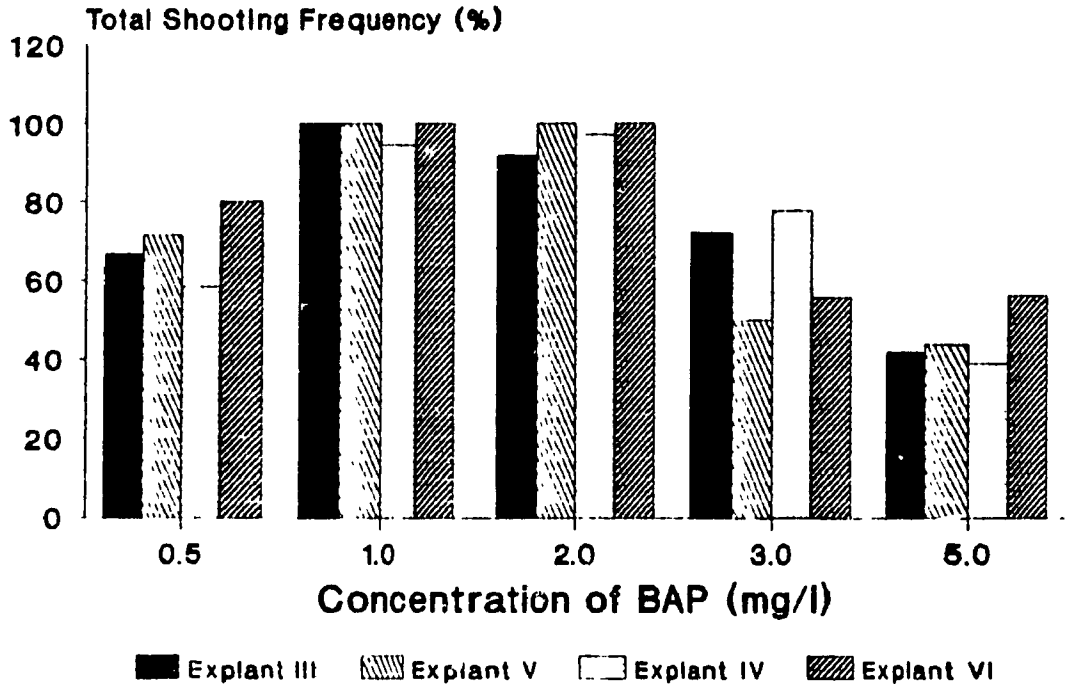


Figure 8

The total shooting frequency in different okra explants cultured on MS basal medium supplemented with various concentrations of BAP at 28° C. Explant is represented by III- cotyledonary axil and IV- leaf nodes from aseptically grown seedlings, V and VI- leaf nodes of generated shoots derived from III and IV respectively. The result was scored after 4 weeks.

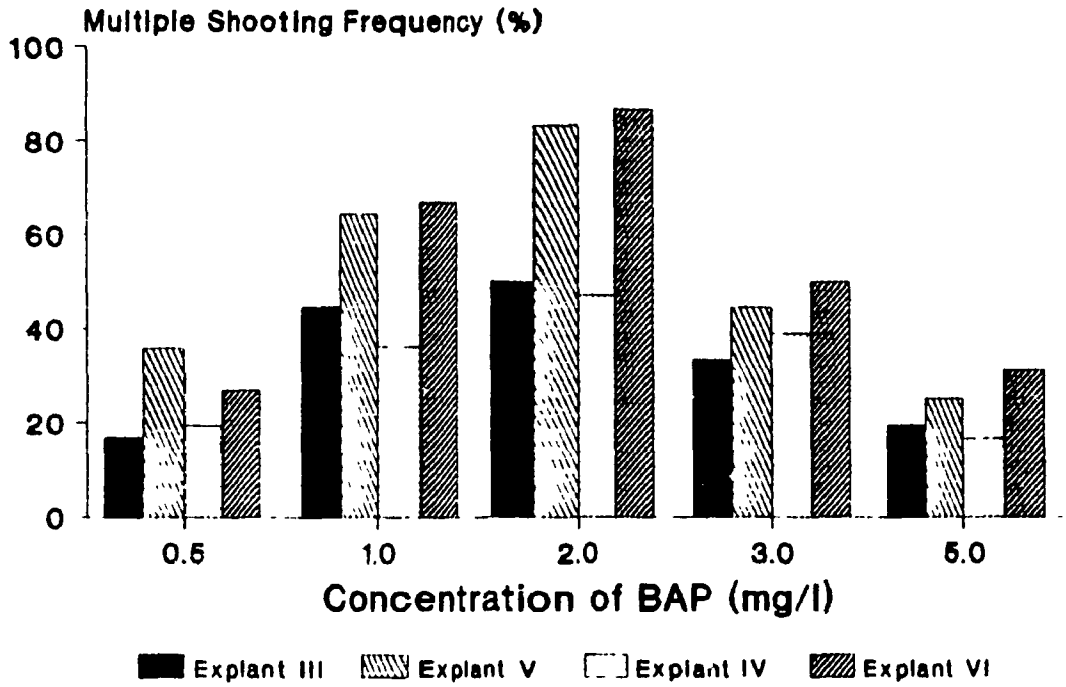


Figure 9

Multiple shooting frequency of various okra explants cultured on MS basal medium supplemented with various concentrations of BAP at 28° C. Explants are represented by III- cotyledonary axil and IV- leaf node from aseptically grown seedlings, V and VI leaf nodes of regenerated shoots derived from III and IV respectively. Results scored after 4 weeks.

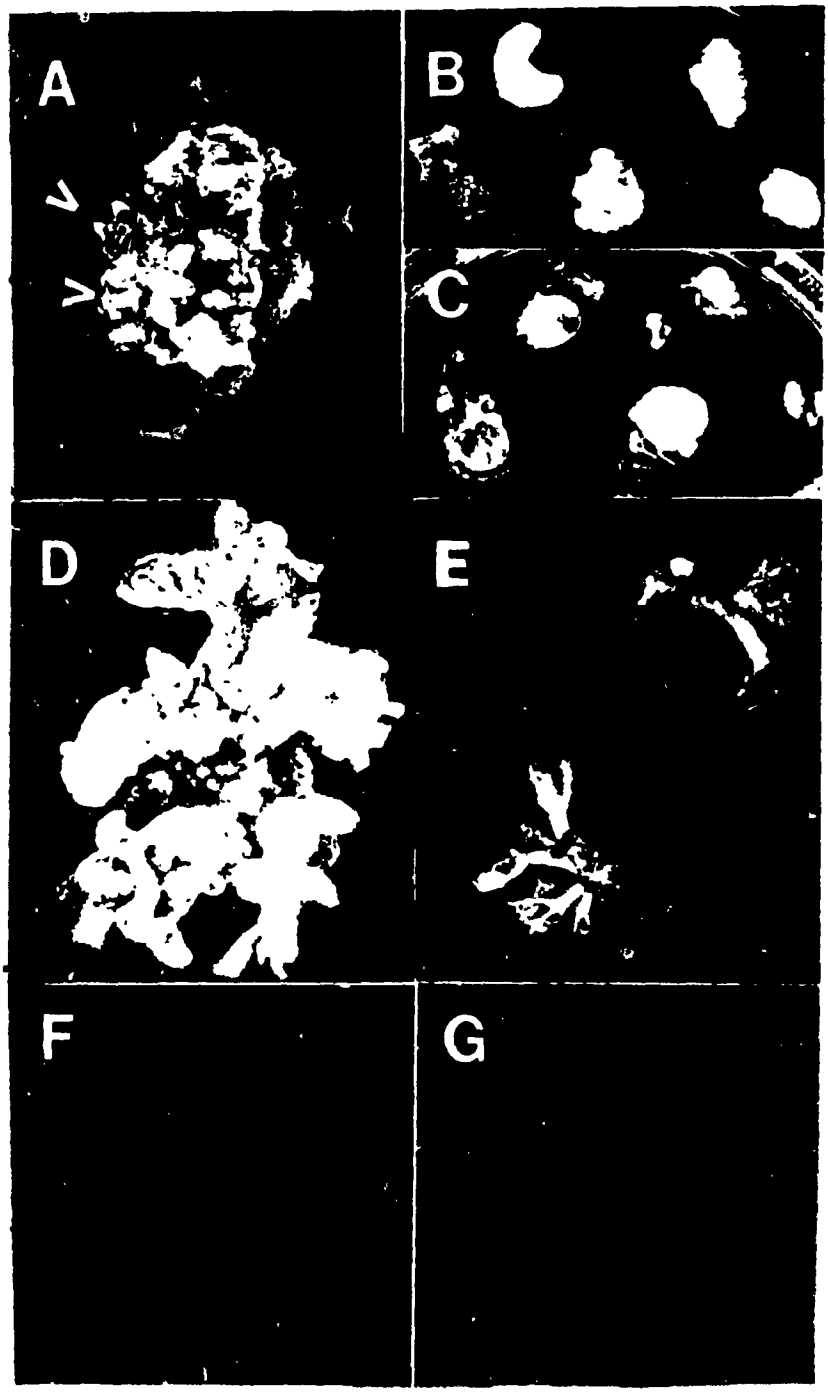
medium resulted in the reduction of multiple shoots.

When shoot nodes (V & VI) from regenerated plants derived from cotyledonary axil and leaf node respectively were cultured at 28° C on 0.5 mg/l BAP supplimented MS basal medium, the total shoot production was increased nearly to 74% (Figure 8) and approximately 38% of those shoot producing explants produced multiple shoots (Figure 9). The frequency of multiple shoot production was increased to nearly 65% when generated shoot nodes were cultured on medium containing 1.0 mg/l BAP and 80% on medium containing 2.0 mg/l BAP. Higher concentration of BAP in the culture medium restrained the multiple shoot formation (Figures 8 & 9).

(ii) Callus. Two-weeks old calli, calli from non-proliferating cotyledonary axil and hypocotyl explants were excised and cultured on BAP (1.0 mg/l) enriched MS medium. Soft white nodular structures appeared after 3-4 weeks on cotyledonary derived calli (Figure 10A). The hypocotyl derived calli did not show any organogenesis and mostly proliferated into an unorganized light colored mass of cells (Figure 10B). When cotyledonary axil calli was serially cultured (X3) on fresh medium, shoot primordia appeared without loss of morphogenic competence of the calli tissue.

Figure 10

Rgeneration of plants from callus tissue of okra. A, a 3-week-old cotyledonary axil derived callus with a number of soft whitish buds, obtained on MS basal medium incorporated with BAP (1.0 mg/l). B, non-oraganogenic calli generated from hypocotyl explant grown on BAP (1.0 mg/l) supplemented MS basal medium. C, muitiple shoot development on 2-week-old cotyledonary axil derived calli cultured on MS basal medium supplemented BAP (1.0 mg/l) and silver nitrate (20.0 mg/l). D, three-week-old multiple shoots on cotyledonary axil derived callus. E, four-week-old excised shoots. F, regenerated shoots growing on rooting medium (MS basal medium and NAA 1.0 mg/l). G, regenerated plant growing in the greenhouse.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There was no further development of the bud primordia when the organogenic calli were left for periods longer than four weeks or when serially cultured on fresh medium. However, the addition of silver nitrate to BAP enriched (1.0 mg/l) medium, with or without myo-inositol and casein hydrolysate, stimulated the buds to organize into multiple shoots within two to three weeks (Figures 10C and 10D). The addition of 5.0 mg/l silver nitrate to myo-inositol (0.1 g/l) and casein hydrolysate (1.0 g/l) enriched medium stimulated nearly 52% of the organogenic calli to regenerate shoots; upon increasing the concentration of silver nitrate, the frequency of shoot regeneration was decreased (Figure 11). On the other hand, the presence of higher amounts of silver nitrate in the medium lacking myo-inositol and casein hydrolysate noticeably increased the number of organogenic calli producing multiple shoots. The addition of 20.0 mg/l silver nitrate stimulated nearly 74% of the calli to form shoots (Table 11). The addition of increased amounts (40.0 mg/l) of silver nitrate in the growth medium with or without myo-inositol and casein hydrolysate resulted in an inhibitory effect on multiple shoot production of the organogenic calli (Figure 11).

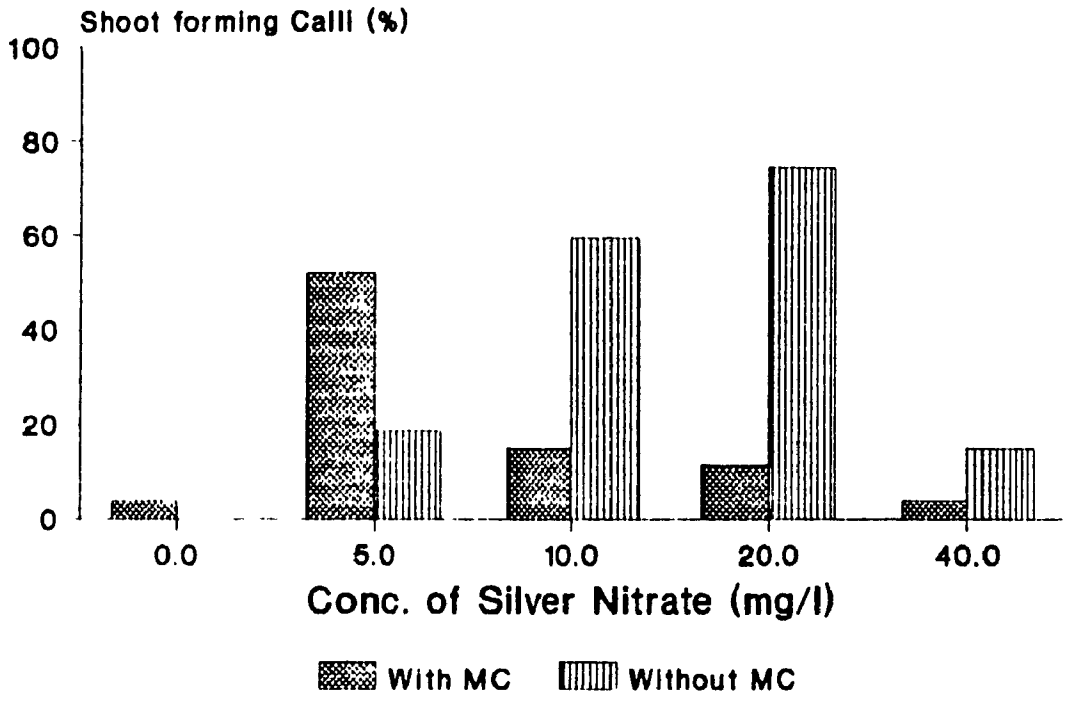

Figure 11

Frequency of shoot generation from cotyledonary axil derived callus of okra cultured on BAP supplemented MS basal medium containing different amounts of silver nitrate ($AgNO_3$) when myo-inositol (M) (0.1 g/l) and casein hydrolysate (C) (1.0 g/l) are present or not in the culture medium. Data scored after 4 weeks and represents a total of two trials.

E2. Comparison of Seed and In-vitro Generated Plants

Okra seeds were sown in the greenhouse three weeks before the cultured plants were ready to be transferred to the greenhouse. These plants and those obtained via in-vitro culturing (TC) were compared for their growth and development (Figure 12). The regenerated plants were short (Figure 13), with fewer number of leaves (Figure 14). However, these leaves were larger than those on plants obtained from the seeds (Figure 14). The regenerated plants flowered approximately 3 weeks earlier (Figure 13) and produced a higher quantity of fruits (Figures 15). The TC2 plant flowered one week earlier than TC1 and approximately a month earlier than the seed plants (control) (Figure 13).

E3. Isolation and Culture of Protoplasts

Protoplasts from both cotyledon and leaf were isolated and cultured on osmotically balanced medium. It had been found that 81% of the protoplasts isolated were viable at the time when they were cultured in callus forming medium. The average frequency of protoplasts to regenerate callus was very poor (0.02%). However, the cotyledon derived protoplasts responded better in forming colonies of micro calli when compared to those obtained from leaf tissue (Table 9).

Figure 12

Okra plants obtained from seeds and through in-vitro propagation shown growing in the greenhouse. The tall plants in the background are from seeds, whereas, the shorter ones propagated from plants are in the foreground.

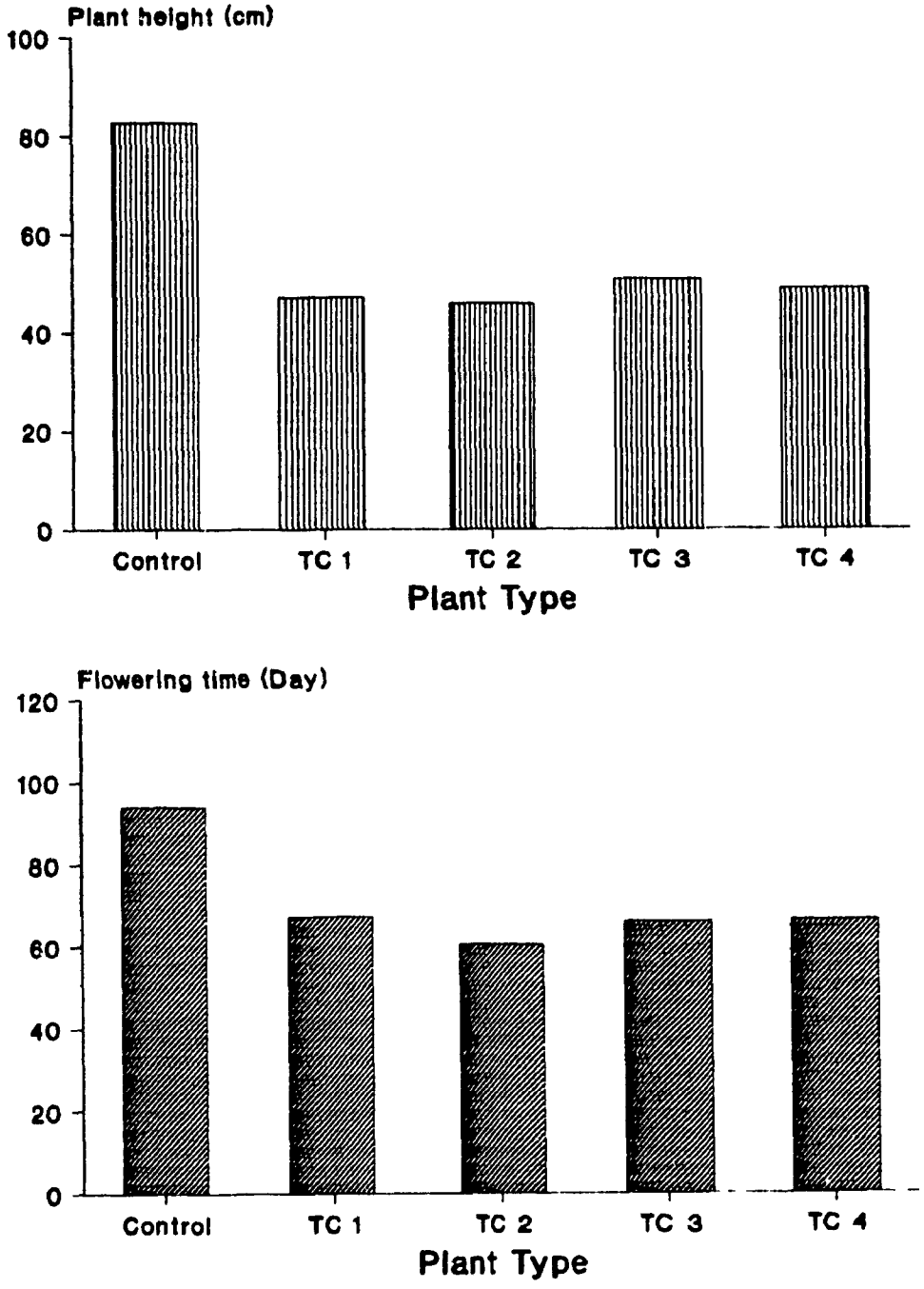

Figure 13

Plant height (top) and flowering time (bottom) of okra plants obtained from seeds and through in-vitro culture. Plants obtained represent control- from seeds, TC1 and TC2- from cotyledonary axil (explant III) and leaf node (explant IV) of aseptically grown seedlings, TC3 and TC4- from the nodes of generated shoots derived from III and IV respectively.

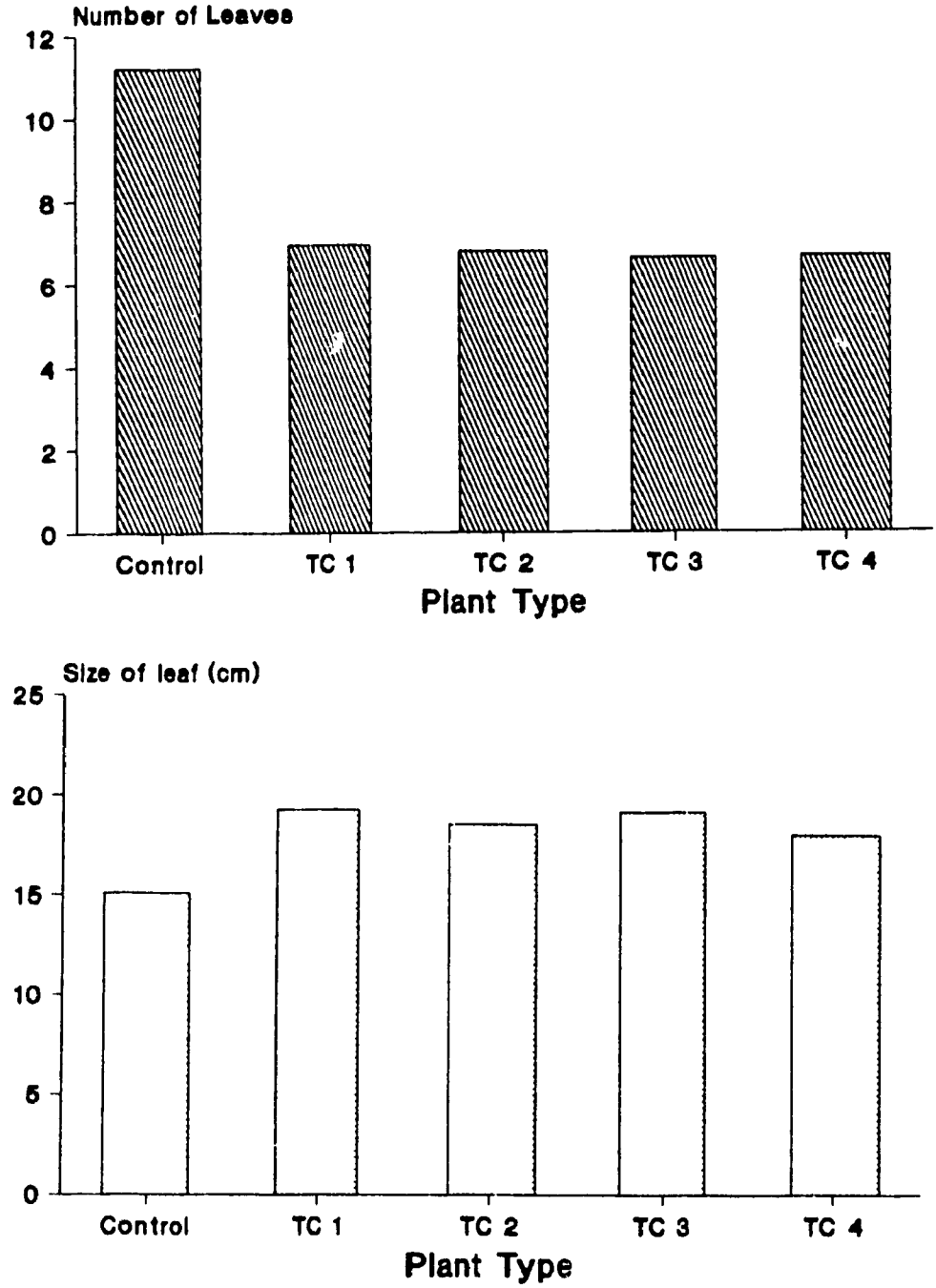

Figure 14

Number of leaves (top) and leaf size (bottom) of okra plants obtained from seeds and via tissue culture. Plants are represented by control- from seeds, TC1 and TC2- from cotyledonary axil (explant III) and leaf node (explant IV) of aseptically grown seedlings, TC3 and TC4- from the nodes of generated shoots derived from III and IV respectively.

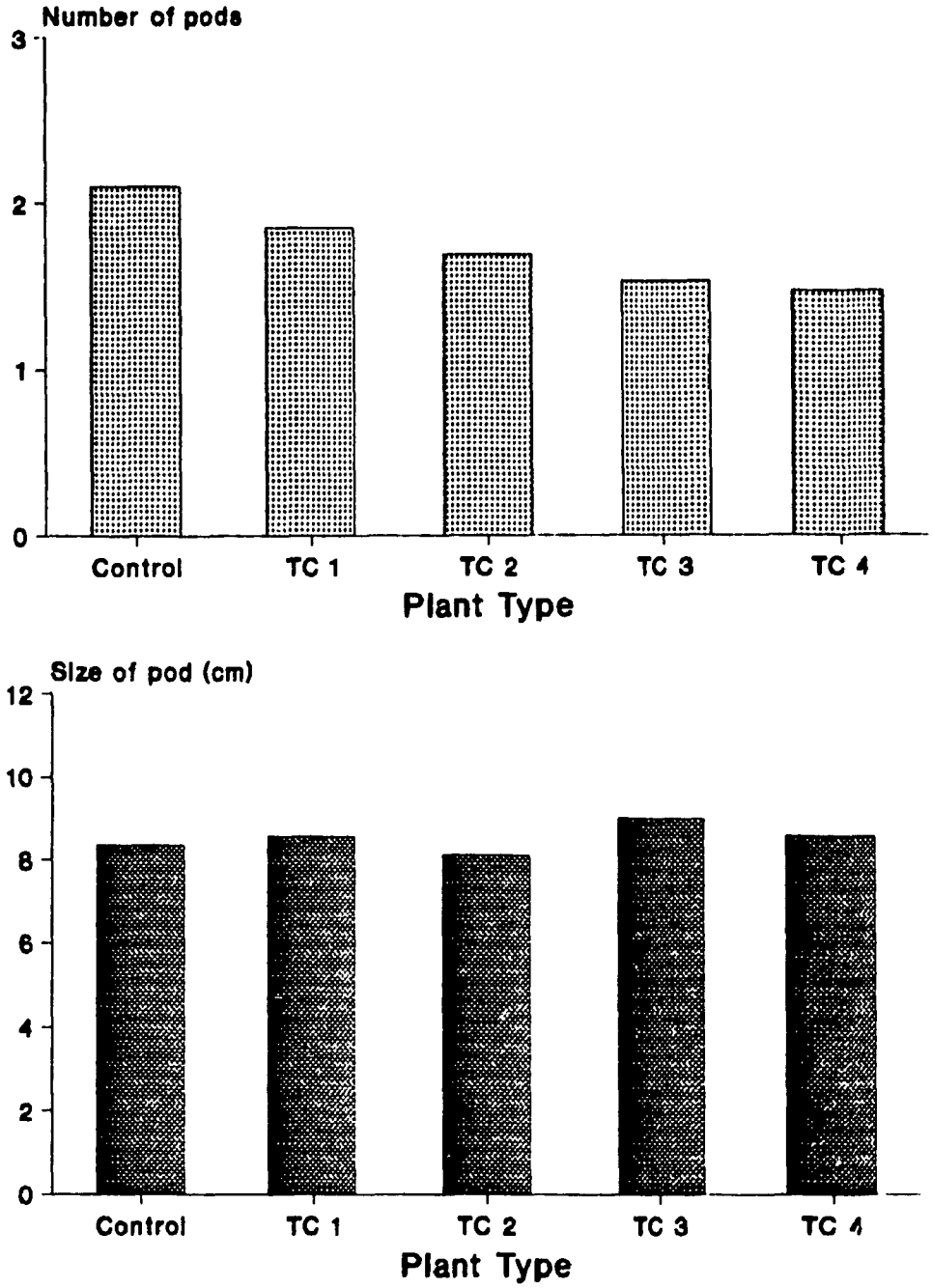

Figure 15

Number of pods per plant (top) and the pod size (bottom) of okra plants obtained from seeds and through tissue culture. Plants are represented by control- from seeds, TC1 and TC2- from cotyledonary axil (explant III) and leaf node (explant IV) of aseptically grown seedlings, TC3 and TC4- from the nodes of generated shoots derived from III and IV respectively.

Table 9

Colonies of micro calli formed from the cotyledon and leaf derived protoplasts of okra. Each petri dish was cultured with 1.0 ml of osmoticum solution (0.66 M) mannitol) containing approximately $5X1^{4}$ protoplasts. Colonies were counted after 32 days following inoculation. The result is the average of 11 petri dishes from each of the protoplasts sources.

Protoplast source	Number of calli colonies per 50000 protoplasts derived from	Frequency of Regeneration (%)
Cotyledon	6.1	0.02
Leaf	4.4	0.01

F. DISCUSSION

The culturing of okra explants on MS medium supplimented with auxins, cytokinins and auxin-cytokinin combinations evoked clear-cut morphogenetic responses. Callus formation was observed on almost all explants that were cultured in media containing either auxins or cytokinins or their combination (Table 7). The presence of auxins in the nutrient medium promoted mostly the formation of roots. The auxin promotion of roots in in-vitro tissue has been observed for a number of tissues including Brassica, (KARTHA et al,1974), Petunia (RAO et al,1973) and Capsicum (GUNAY and RAO,1978). In the present studies IAA and NAA both stimulated root development, however, in IAA supplimented medium, the roots were short and poorly developed (Figure 3.2); while NAA promoted the formation of extensive roots with a number of lateral roots and root hairs (Figure 3.3). This difference in root development was similar to that observed for yam (ARDITTI and NYMAN,1985) and not similar to Capsicum, which gave a better response with IAA (GUNAY and RAO,1974; HUBSTENBURGER and PHILLIPS,1985; COOKE,1988). The synthetic auxin, NAA, in

some plant tissues is more soluble and stable than the natural auxin, IAA (ELIASSON and AREBLAD,1984). Eliasson and Areblad working on melon tissue cuture had shown that a large number of root primordia formed when explants were cultured on NAA rich medium. They found that NAA was not broken down as readily as IAA. This likely may be the case in okra, where IAA is probably degraded quickly thus resulting in poor root development.

The effect of 2,4-D on in-vitro tissue has been studied with various plant species. Development of roots was observed when leaf segments of wheat were cultured in medium containing 2,4-D (WERNICKE et al,1986). A complete inhibition of root development on Petunia (RAO et al,1973) and Antirrhinum (POIRIER-HAMON,1974) was noted when 2,4-D was incorporated in the culture media. The addition of 2,4-D to the culture medium in the present studies did not elicit root development but stimulated the formation of friable callus. As with most tissues, the addition of auxin alone to the growth medium generally does not stimulate shoot bud or shoot development and okra tissue was no exception. None of the okra explants produced shoot buds or shoots when cultured on media containing auxins only.

When okra explants were cultured on media containing KIN and ZTN, no shoot buds or shoots were produced. The presence of these two cytokinins stimulated the production of compact green calluses on most explant except cotyledonary axil, leaf nodes and leaf segments (Table 7). Such observations have been made with Petunia when KIN and ZTN were added to the culture media (RAO et al,1973). However, in some plant species, KIN has been shown to stimulate shoot development (SKOOG and MILLER,1957). Zeatin also appeared to stimulate shoot development (GUNAY and RAO,1978). This seems to be a normal observation, where some tissues do not respond to KIN or ZTN, whereas others responds to the two cytokinins. It appears that BAP is generally a better cytokinin for shoot initiation. When BAP was incorporated in the growth medium in the present studies, some of the okra explants produced shoots (Figure 3.5). This finding is similar to the result reported recently on sugar beet (ECONOMOTO and OHYAMA,1985), mulberry (HO-RAK et al,1985) and Brassica (KARTHA et al,1978) explants.

The combination of auxin and cytokinin was most effective in inducing both shoot and root development in cotyledonary axil and leaf node of okra. This response of

okra to BAP and NAA was similar to that observed for a number of other plants . The combination of NAA and BAP in the growth medium was best in promoting shoots and roots in Brassica (KARTHA et al,1974). The result of other investigation showed the development of numerous plantlets on the explants of Petunia, when MS medium was supplimented with both IAA and BAP (RAO et al,1973). Phillips (1987) found that the sequential exposure to auxin and cytokinin resulted in cytodifferentiation on artichoke tuber.

GA_3-stimulated organogenesis in some tissues such as buckwheat (SREJOVIC and NESCOVIC,1985) and tomato (COLEMAN and GREYSON,1977) has been reported. However, in the majority of studies, GA_3 appears to inhibit organogenesis. This inhibition has been observed in Begonia (HEIDE,1969) and tobacco (MURASHIGE,1964). In the present studies with the okra results also indicate that GA_3 in the culture medium did not stimulate callus proliferation or root/shoot morphogeneses. It has been suggested (MANGAT,1988; THORPE,1974;) that the presence of starch is essential for organ initiation and GA_3 has been shown to inhibit starch accumulation and concomitantly suppress shoot formation (MURASHIGE,1974). This may also be the case with okra.

A number of studies have shown that temperature is an important factor in regeneration (ELIASSON and AREBLAD,1984; HUBSTENBURGER and PHILLIPS,1985; WELENDER,1987 COOKE,1988). In the present studies when okra explants were cultured at 24° C, only single shoots were obtained from cotyledonary axils and leaf nodes. When these explants were cultured at 28° C, multiple shoots were produced on them.

The temperature 25° C is ambient in most tissue culture practice (MURASHIGE,1974). The temperature requirements for culturing plant tissues differ considerably from species to species. Ipomoea for example, can be cultured between 15° and 34° C. However, the temperature requirement for its optimum growth is 25° to 32° (HUGHES,1981). The temperature 24° to 28° C may be adequate for the culturing of many annual and tropical species. The culturing of plant tissue at low temperature, may initiate callus, root and single shoot. When they are cultured at high temperature nomally produce multiple shoots (MURASHIGE,1974). Okra is a warm season crop and can grow in a wide range of temperature (YAMAGUCHI,1983). Therefore, the single shoot formation at 24° C and multiple shoot formation at 28° C on okra explants conform to the facts described by Murashige (1974).

The explants obtained from in-vitro generated plants showed a higher frequency of multiple shoot formation than those taken from seed plants. Increased multiple shoot production in explants from cultured plants has been observed for other plant species (PIERIK and STEEGMANS,1986). There could be several reasons why explants from generated plants should produce more shoots. For example, the generated explants comprise young and growing tissues compared to their parents; they are cultured under controlled environmental conditions; they are grown in the nutrient rich medium and lack of auxin which sometimes antagonizes BAP action.

In-vitro plant regeneration from calli tissue of many plant species has been reported. In the early experiments, the calli obtained from explants at 24^{o} C on media supplemented with various growth regulators failed to organize into shoots or roots. However, at 28^{o} C in medium with added NAA (1.0 mg/l) and/or BAP (1.0 mg/l), the hypocotyl derived calli remained nonorganogenic whereas, cotyledonary axil derived calli produced shoot buds on them (Figure 10A). There was no further development of these bud primordia when the nonorganogenic and organogenic calli were left for a period longer than 4 weeks and serially cultured

(3X) either on similar or other shoot forming medium (Table 5). These growth regulators have been shown to stimulate organogenesis in other tissues (PATTERSON and AVERETT,1985; KATHAL et al,1986; RENGEL,1986). When they were cultured on BAP (1.0 mg/l) containing medium incorporated with silver nitrate with or without the presence of myo-inositol and casein hydrolysate, the shoot bud production was stimulated on the cotyledonary axil derived organogenic calli and resulted in the formation of multiple shoots. However, the hypocotyl derived calli remained nonorganogenic. The stimulation of shoot morphogenesis on callus tissue upon the addition of silver nitrate, an inhibitor of ethylene production, on Nicotiana and Triticum has been reported (PURNHAUSER et al,1987).

In some investigations ethylene has been reported to stimulate bud and shoot development (KRISHNAMOORTHY,1970; BATTEN and MULLINS,1978; VAN AARTRIJK et al,1985; EVERETT,1982). A possible role of ethylene has also been observed in pollen embryogenesis of Solanum carolinens (REYNOLDS,1987). However, in most cases, the gaseous plant growth regulator has an inhibitory effect on morphogenesis in various tissue and cell culture systems. Inhibition of somatic embryogenesis in wild carrot (WOCHOK and

WETHRELL,1971); TISSERAT and MURASHIGE,1977), shoot generation in tobacco (HUXTER et al,1981), root formation on pea explants (NORDSTROM and ALIASSON,1984) and leaf growth and development in carnation explants (MELE et al,1982), are all examples of negative effects which have been attributed to ethylene. Silver nitrate is a strong antagonist of ethylene (BEYER,1976). Both auxin and cytokinin stimulate ethylene production. However, auxins which are normally added to callus induction and maintenance medium to stimulate callus formation (PURNHAUSER et al,1987), strongly promotes endogenous production of ethylene (YANG and HOFFMAN,1984). In the present studies, however, the callus induction and regeneration medium did not necessarily require an auxin. The promotion of shoot generation on the presence of silver ions may suggest that in-vitro, okra tissues likely generates elevated levels of ethylene. The probable production could be the result of higher endogenous levels of auxins, since the present observations show that the addition of an auxin to the growth medium is not necessary to promote callus induction. The premise of evolution of ethylene in-vitro may also explain why okra, a member of the Malvaceae is recalcitrant to tissue culture techniques (MANGAT, and ROY,1986). However this aspect requires further investigation.

Regenerated plantlets obtained via in-vitro tissue culture techniques were successfully grown to maturity in the greenhouse (Figure 12). The regenerated plants showed many characteristics which were different from seed generated plants. These differences included larger leaves and shorter plants. One important characteristic noted was that the regenerated plants flowered and produced more pods much earlier than plants obtained from seeds(Figure 13). The reason for these different characteristics could be due to several factors which include the nutrient content of the growth medium, environmental condition of the growth; somaclonal variation and the influence of growth regulator(s). Growth regulators affect the plant at their molecular levels. The application of IAA for example, increases the production of mRNA (ZURFLUH a. i GUILFOYLE1982; van der LINDE,1984). The increased production of mRNA results in the change in chromosomal sructure (REISCH,1983).

After having achieved propagation from explant tissue and organogenesis from callus, experiments were initiated to regenerate plants from okra protoplasts. To date there is no evidence in the iterature on isolation of viable protoplasts from okra. Because of the dificulties presented by the presence of mucilaginous material in the okra , the

initial attempts to isolate viable protoplasts were difficult. However, with repeated attempts using different enzyme combinations and time of incubation, success was achieved in obtaining viable protoplasts. The viability of protoplasts was quite high when they were isolated. However, attempts to regenerate plants were not successful. The isolation of viable protoplasts and regeneration of plants from protoplasts cultures require further investigation. In future experiments a number of points should be taken under consideration which includes avoiding over-night incubation of the plant tissue in the medium containing cell wall degrading enzymes, addition of cell growth enhancing substances such as polyvinylpolypirolidone in the regeneration medium (SAXENA,1987) and allowing longer culture period for the proliferation of the tissue (WU and KUNIYUKI,1985).

This project represents the first report where okra plants have been regenerated via tissue culture techniques. The morphogenic competence of the calli culture may offer the opportunity to produce a cell suspensions. These suspensions can be further employed to generate protoplasts or to raise plants via somaclonal variation. The gradual success may bring this crop plant at the stage of its

biotechnological manipulations which, in future, may lead to the development of genetically tailored and economically desired plants.

SUMMARY

The following could be summerized from the study:

(1) The establishment of media/explant combination for plant regeneration.

(2) Multiple plant generation from a single explant.

(3) Multiple plant generation from the explant derived callus tissue.

(4) Isolation of viable protoplasts.

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H. Appendix I

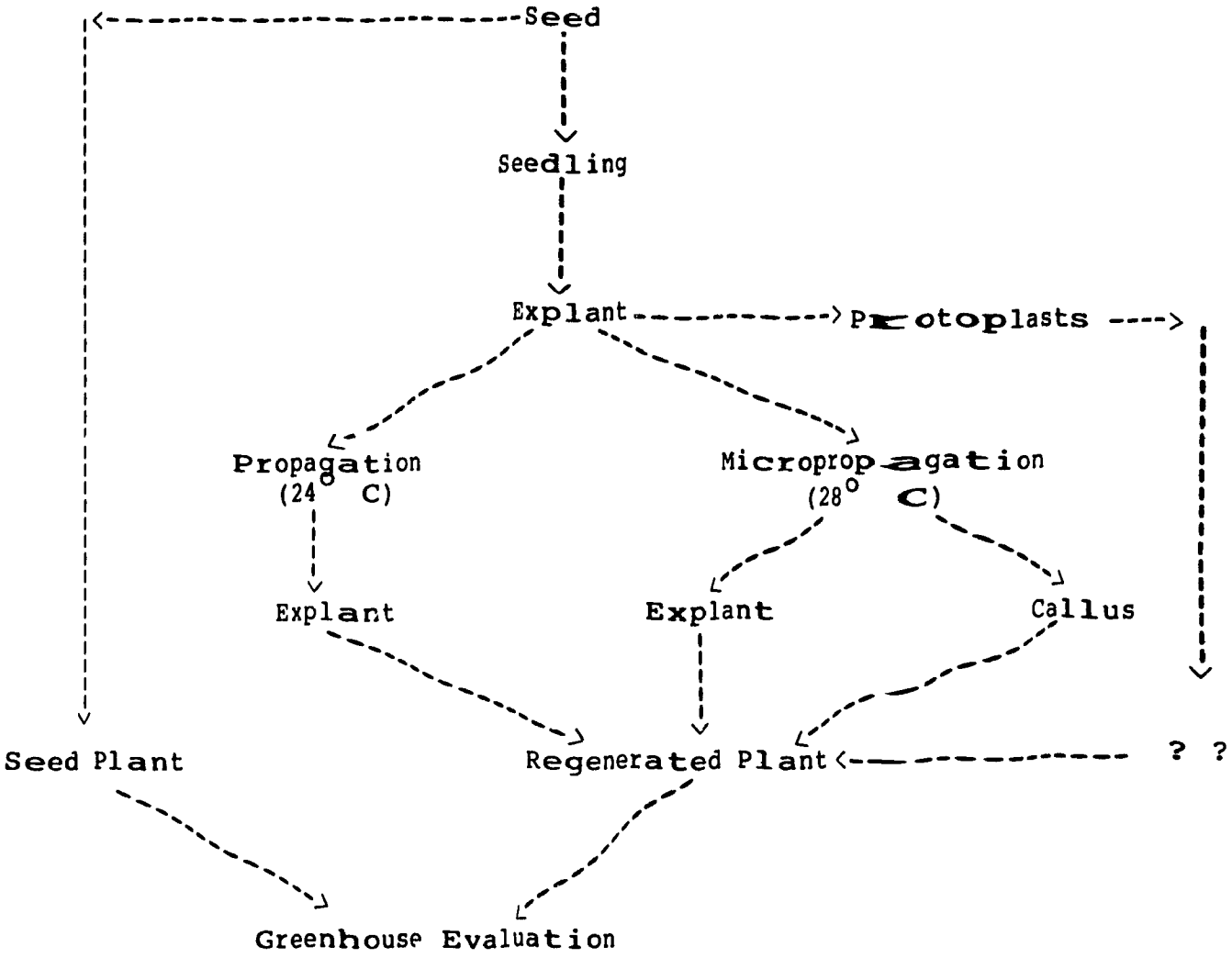
Composition of some basal nutrient media that are commonly used for plant tissue culture (BHOJWANI, 1983).

Constituents	Media (amounts in mg l⁻¹)[b]						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B.[g]	Nitsch's[h]	NT[i]
Inorganic							
NH_4NO_3	–	–	1650	1200	–	720	825
KNO_3	80	–	1900	1900	2527 5	950	950
$CaCl_2 \cdot 2H_2O$	–	75	440	440	150	–	220
$CaCl_2$	–	–	–	–	–	166	–
$MgSO_4 \cdot 7H_2O$	750	250	370	370	246 5	185	1233
KH_2PO_4	–	–	170	340	–	68	680
$(NH_4)_2SO_4$	–	–	–	–	134	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	300	–	–	–	–	–	–
$NaNO_3$	–	600	–	–	–	–	–
Na_2SO_4	200	–	–	–	–	–	–
$NaH_2PO_4 \cdot H_2O$	16	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
$MnSO_4 \cdot 4H_2O$	5	0 1	22 3	2 23	–	25	22 3
$MnSO_4 \cdot H_2O$	–	–	–	–	10	–	–
$ZnSO_4 \cdot 7H_2O$	3	1	8 6	–	2	10	–
$ZnSO_4 \cdot 4H_2O$	–	–	–	–	–	–	8 6
Zn Na EDTA	–	–	–	15	–	–	–
$Na_2MoO_4 \cdot 2H_2O$	–		0 25	0 025	0 25	0.25	0 25
MoO_3	0 001	–	–	–	–	–	–
$CuSO_4 \cdot 5H_2O$	0 01	0 03	0 025	0 0025	0 025	0 025	0 025
$CoCl_2 \cdot 6H_2O$	–	–	0 025	0 0025	0 025	–	–
$CoSO_4 \cdot 7H_2O$	–	–	–	–	–	–	0.03
$AlCl_3$	–	0 03	–	–	–	–	–
$NiCl_2 \cdot 6H_2O$	–	0 03	–	–	–	–	–
$FeCl_3 \cdot 6H_2O$	–	1	–	–	–	–	–
$Fe_2(SO_4)_3$	2 5	–	–	–	–	–	–
$FeSO_4 \cdot 7H_2O$	–	–	27 8	27 8	–	27 8	27 8
Na 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%	7%
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] Camborg et al. (1968).
[h] Nitsch (1969).
[i] Nagata and Takebe (1971).

I. Appendix II

The flow diagram of experiments showing in-vitro propagation/micropropagation of okra.


Seed
Seedling
Explant
Protoplasts
Propagation
(24° C)
Micropropagation
(28° C)
Explant
Explant
Callus
Seed Plant
Regenerated Plant
? ?
Greenhouse Evaluation

J. Appendix III

Copies of two publications which ensued from this study.

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REGENERATION OF PLANTS FROM CALLUS TISSUE OF OKRA (*ABELMOSCHUS ESCULENTUS*)

M.K. ROY and B.S. MANGAT*

Department of Biology, Concordia University, 1455 de Maisonneuve Blvd. West, Montreal, Quebec H3G 1M8 (Canada)

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Hypocotyl and cotyledonary axil explants of aseptically grown okra (*Abelmoschus esculentus*) seedlings were induced to form callus when cultured on Murashige and Skoog's (MS) basal medium supplemented with cytokinins or auxin-cytokinin combinations. The presence of kinetin (KN) or zeatin (Z) in the induction medium stimulated callus formation on hypocotyl explants. The cotyledonary axil explants failed to yield callus when cultured on media containing KN or Z or the combination of either of these two cytokinins with indoleacetic acid (IAA) or naphthaleneacetic acid (NAA). Rapid callus induction resulted from all explants cultured on MS medium supplemented with benzyladenine (BA) ($1.0\ mg \cdot l^{-1}$). The hypocotyl-derived callus remained non organogenic, when subcultured on fresh BA-supplemented MS medium, whereas, cotyledonary-axil-derived callus produced number of buds. The transfer of the organogenic calli to BA-enriched MS media containing various concentrations of silver nitrate ($0–40\ mg \cdot l^{-1}$) stimulated multiple shoot development within 2–3 weeks. The presence of 10 and 20 mg l^{-1} of silver nitrate significantly increased the number of calli producing multiple shoots when myo-inositol and casein hydrolysate were omitted from the growth medium. Roots developed quickly when regenerated shoots were subcultured on MS medium containing BA ($1.0\ mg \cdot l^{-1}$) and NAA ($1.0\ mg \cdot l^{-1}$). The plantlets, on transfer to soil, grew normally.

Key words: Okra, *Abelmoschus esculentus*; tissue culture; callus; organogenesis; plant regeneration; silver nitrate

Introduction

Okra (*Abelmoschus esculentus*) is an important crop which has special agronomical importance in a number of countries because of its multi-product usage in food, fibre and production of oil and proteins [1–3]. Although the world-wide cultivation and use of okra is becoming important, its economic exploitation is still hampered at present by the lack of selected clones and the low yields from present cultivars [4].

Since the early demonstration of cellular totipotency and differentiation in vitro, plant tissue culture techniques have been widely used in the clonal multiplication of plants [5]. Further, the increase in genetic variation that is frequently associated with regenerating mature plants from undifferentiated callus tissue and single cells provides added benefits for crop improvement through selection of useful variants and application of genetic engineering [6]. Therefore, the development of tissue culture methods for the reliable induction and maintenance of callus, with regeneration of plants, is imperative for the successful application of tissue culture techniques to the improvement of the okra crop.

In an earlier paper [7] we reported the development of plantlets obtained from okra explants cultured in vitro into mature plants. During these studies callus tissue was produced on the test explants. However, regeneration of plants from the callus tissue was not possible. This paper outlines methods for consistent production and maintenance of callus and successful regeneration of plants from callus cultures.

*To whom correspondence should be addressed

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Materials and methods

Seeds of Okra (*A. esculentus* (L) Moench, cv. Parkins Mammoth Longpod) were obtained from Dominion Seed House (Georgetown, Ontario, Canada). Seeds were sterilized and 2-week-old seedlings obtained as described previously [7]. Explants (hypocotyl and cotyledonary axils) were excised and cultured on callus initiation and maintenance media. The explants were placed on the medium randomly. All callus initiation and maintenance media contained salts and vitamins according to Murashige and Skoog (MS) [8]. The media were supplemented with different phytohormones (Table I). Two-week-old callus masses were excised from explant tissue, making certain by microscopic examination that no residual auxillary meristem was present, and subcultured on fresh MS 1 medium (Table I). After 2 weeks, calli (0.5 cm) were cut and subcultured on fresh MS 1 medium. Cotyledonary axil derived calli, with multiple buds, were transferred to BA ($1.0\ mg \cdot l^{-1}$) supplemented MS media containing various amounts ($0\text{-}40\ mg \cdot l^{-1}$) of silver nitrate, when myo-inositol ($0.1\ mg \cdot l^{-1}$) and casein hydrolysate ($1.0\ mg \cdot l^{-1}$) were present or not in the culture media. After 4 weeks the shoots were excised and subcultured on rooting medium containing NAA ($1.0\ mg \cdot l^{-1}$). The rooted shoots, after acclimatization [7], were transferred to a soil/vermiculite mixture (4 : 1, w/w) and allowed to grow in the greenhouse.

All media contained 3% (w/v) sucrose and 0.6% (w/v) agar. The pH was adjusted to 5.7 before sterilization by autoclaving at $1\ kg/cm^2$ for 15 min. Growth regulators were filter-sterilized before being added to the sterile medium. Culture flasks (125 ml) containing 30 ml of sterile medium were used for the culture of explants and the subculture of callus. Sterile petri dishes containing 20 ml of sterile medium were used to recover shoots and these shoots were allowed to develop and form roots in 125-ml flasks containing 30 ml of

Table I. Callus induction from cotyledonary axils and hypocotyl explants of Okra (*A. esculentus*) cultured on Murashige and Skoog's (MS) basal medium supplemented with various growth regulators Data scored after 2 weeks, each treatment composed of 15 replicates, repeated twice IAA, indoleacetic acid, NAA, naphthaleneacetic acid, BA, benzyladenine, KN, kinetin, Z, zeatin

Medium	Growth regulator ($mg\ l^{-1}$)					Explant	Callus induction/ no of explants
	IAA	NAA	BA	KN	Z		
MS 1	–	–	1.0	–	–	Hypocotyl	30/30
						Cotyledonary axil	30/30
MS 2	–	0.1	1.0	–	–	Hypocotyl	28/30
						Cotyledonary axil	30/30
MS 3	0.1	–	1.0	–	–	Hypocotyl	28/30
						Cotyledonary axil	29/30
MS 4	–	–	–	1.0	–	Hypocotyl	26/30
						Cotyledonary axil	0/30
MS 5	–	0.1	–	1.0	–	Hypocotyl	24/30
						Cotyledonary axil	0/30
MS 6	0.1	–	–	1.0	–	Hypocotyl	18/30
						Cotyledonary axil	0/30
MS 7	–	–	–	–	1.0	Hypocotyl	15/30
						Cotyledonary axil	0/30
MS 8	–	0.1	–	–	1.0	Hypocotyl	20/30
						Cotyledonary axil	0/30
MS 9	0.1	–	–	–	1.0	Hypocotyl	16/30
						Cotyledonary axil	0/30

sterile rooting medium. All cultures were incubated in 'Conviron' growth chambers at 28 ± 2 C with a daily photoperiod of 16 h (4000 lux from cool-white Westinghouse fluorescent lamps).

Results and discussion

Almost all explants cultured on BA (1.0 mg l^{-1}) enriched medium formed callus at their cut ends within 2 weeks when hypocotyl and cotyledonary explants of *A. esculentus* were incubated on MS basal media containing BA, kinetin (KN) or zeatin (Z) alone or the combination of either of these cytokinins with IAA or NAA (Table I). Callus was induced on 86% of the hypocotyl explants when these were cultured on KN enriched (1.0 mg l^{-1}) MS medium. The callus induction frequency however, dropped when KN was combined with IAA (1.0 mg $\cdot l^{-1}$) or NAA (1.0 mg $\cdot l^{-1}$) (Table I). Similarly, only half the hypocotyl explants formed callus tissue when cultured on Z (1.0 mg $\cdot l^{-1}$) supplemented MS medium. The frequency of callus generation dropped further when zeatin was combined with IAA or NAA. Although nearly all cotyledonary axil explants generated callus on BA enriched growth medium, these explants failed to yield callus when cultured on media containing KN or Z or the combination of these two cytokinins with IAA or NAA (Table I).

Although the presence of KN or Z in the induction medium stimulated callus formation on hypocotyl explants, the earliest visible signs of callus induction were noticeable on most explants cultured on medium containing BA alone or in combination with NAA. It has also been reported that BA or BA in combination with NAA promotes callus induction in a number of other plant tissues [9-11].

The 2-week-old-light green compact calluses formed on both the cotyledonary axil and hypocotyl explants were excised from non-proliferating tissue and the calli (0.5 cm) were subcultured on fresh MS 1 medium. After 3-4 weeks, soft white nodular structures appeared on cotyledonary-axil-derived calli (Fig. 1A).

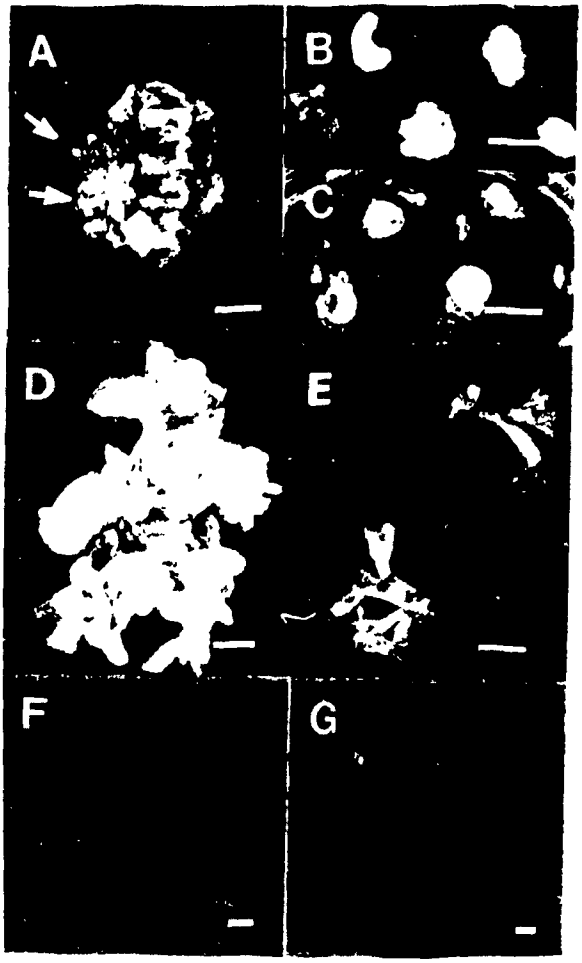

Fig. 1. Regeneration of plants from callus tissue of *A. esculentus*. A. a 3-week old cotyledonary axil derived callus, showing a number of soft whitish buds (arrows), obtained on MS medium supplemented with BA (1.0 mg l^{-1}). B non organogenic calli growing from hypocotyl explant generated callus growing on MS medium supplemented with BA (1.0 mg l^{-1}) (3 weeks). C: multiple shoot development on 2-week-old calli derived from cotyledonary-axil-generated callus cultured on BA (1.0 mg l^{-1}) enriched, myo-inositol and casein hydrolysate free, MS medium containing silver nitrate (20.0 mg l^{-1}) D multiple shoots on cotyledonary axil derived callus (3 weeks) E. 4-week old excised shoots. F: regenerated shoot growing on rooting medium (MS medium supplemented with NAA, 1.0 mg l^{-1}). G: regenerated plant growing in the greenhouse. (Bar = 10 mm).

The hypocotyl derived calli did not show any organogenesis and mostly proliferated into an unorganized light-coloured mass of cells (Fig. 1B). When cotyledonary-axil-derived calli were

serially subcultured 3 times on fresh induction medium, bud primordia appeared without loss of morphogenic competence of the callus tissue.

There was no further development of the bud primordia when the organogenic calli were left for periods longer than 4 weeks or when serially subcultured on fresh MS 1 medium. However, the addition of silver nitrate to BA-enriched medium (MS 1), with or without myo-inositol and casein hydrolysate, stimulated the buds to organize into multiple shoots within 2–3 weeks (Fig. 1C and D). The addition of 5.0 mg · l^{-1} silver nitrate to myo-inositol (1.0 mg · l^{-1}) and casein hydrolysate (1.0 mg · l^{-1}) enriched medium stimulated nearly 52% of the organogenic calli to regenerate shoots; upon increasing the concentration of silver nitrate, the frequency of shoot regeneration however, was lowered (Table II). On the other hand, the presence of higher amounts of silver nitrate in media lacking myo-inositol and casein hydrolysate noticeably increased the number of organogenic calli producing multiple shoots. The addition of 20.0 mg · l^{-1} silver nitrate stimulated nearly 74% of the calli to form shoots (Table II). The results are similar to those observed for *Triticum aestivum* and *Nicotiana plumbaginifolia* callus tissue. Silver nitrate, over a range of 5–50 mg · l^{-1}, stimulated shoot regeneration and further, the incorporation of myo-inositol and casein hydrolysate into the growth medium, was not necessary for a dramatic promotion of shoot regeneration in wheat callus tissue [12].

Table II. Frequency of shoot regeneration from cotyledonary axil derived callus of *A esculentus* cultured on benzyladenine supplemented MS media containing different amounts of silver nitrate ($AgNO_3$) when myo-inositol (M) (0.1 mg l^{-1}) and casein hydrolysate (C) (1.0 mg l^{-1}) are present or not in the culture media Data scored after 4 weeks and represents a total of 2 experiments The χ^2 test for $AgNO_3$ in promoting shoot regeneration was significant at $P < 0.005$

$AgNO_3$ (mg l^{-1})	No of shoot regenerating calli/ no of calli tested	
	+M,C	−M,C
0	1/27	0/27
5.0	14/27	5/27
10.0	4/27	16/27
20.0	3/27	20/27
40.0	1/27	4/27

In some investigations ethylene has been reported to stimulate bud and shoot development [13,14]. However, in most cases, the gaseous plant growth regulator has an inhibitory effect on morphogenesis in various tissue and cell culture systems. Inhibition of wild carrot somatic embryogenesis [15,16], of shoot regeneration in tobacco [17], of root formation in pea explants [18] and of leaf growth and development in carnation explants [19], are all examples of the negative effects which have been attributed to ethylene. Silver nitrate is a strong antagonist of ethylene [20]. Both auxins and cytokinins stimulate ethylene production. However, auxins, which are normally added to callus induction and maintenance medium to stimulate callus formation [12], strongly promote endogenous production of ethylene [21]. In the present studies, however, the callus induction and regeneration medium did not require an auxin. The promotion of shoot regeneration in the presence of silver ions may suggest that in vitro, *A. esculentus* tissues likely generate elevated levels of ethylene. The probable production of ethylene could be the result of higher endogenous levels of auxins, since the present observations show that the addition of an auxin to the growth medium is not necessary to promote callus induction. The premise of evolution of ethylene in vitro may also explain why okra, a member of the Malvaceae is recalcitrant to tissue culture techniques [7]. However, this aspect requires further investigation.

After 4 weeks, the regenerated shoots, with 3–6 leaves, (Fig. 1E) were excised and aseptically transferred to culture flasks containing rooting medium (Fig. 1F). Roots developed quickly on all shoots and, after 10 days, the rooted shoots were ready to be planted in a soil/vermiculite mixture. After acclimitization

the plantlets developed into mature plants (Fig. 1G).

The demonstration of a rapid callus formation and the regeneration of multiple plants from callus tissue offers a great potential for fast, mass propagation of okra. The fact that there was no loss of morphogenic competence after serially subculturing the calli and that plant regeneration is possible, makes the material described above an alternative source of tissue for the establishment of cell suspensions and the isolation of potentially totipotent protoplasts for biotechnological manipulations [6, 22]. Presently, studies are in progress to isolate viable protoplasts and to optimize conditions to stimulate organogenesis and embryogenesis in nonregenerative calluses.

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TISSUE CULTURE AND PLANT REGENERATION OF OKRA (*ABELMOSCHUS ESCULENTUS*)

B.S. MANGAT and M K. ROY

Department of Biology, Concordia University, 1455 de Maisonneuve Blvd. West, Montreal, Quebec H3G 1M8 (Canada)

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Explants (hypocotyl, cotyledon, cotyledonary node and leaf segment) were excised from aseptically grown okra (*Abelmoschus esculentus*) seedlings The explants were cultured on a Murashige and Skoog basal nutrient medium supplemented with auxins, cytokinins and auxin-cytokinin combinations. Callus formation and root differentiation occurred in a medium containing naphthaleneacetic acid (NAA) or indoleacetic acid. There was a greater proliferation of roots on medium supplemented with NAA. The addition of 2,4-dichlorophenoxyacetic acid (2,4-D) to the growth medium suppressed root formation. No shoot bud or shoot development was observed at any of the auxin levels tested. Both kinetin (KN) and zeatin (Z) also proved ineffective in inducing shoot buds or shoots. Shoots were produced on cotyledon and cotyledonary node explants cultured in a medium supplemented with benzyladenine and NAA. These shoots developed roots on the same medium. The plantlets, on transfer to soil, grew normally.

Key words okra, *Abelmoschus esculentus*, tissue culture; plant regeneration

Introduction

Since the early demonstration of cellular totipotency and differentiation in vitro, plant tissue culture techniques have been widely used for the clonal multiplication of plants [1]. To date, many economically important plants have been successfully propagated through this technique [2]. Okra (*Abelmoschus esculentus*), a member of the Malvaceae, is an important food crop which is grown in a number of countries. Although plant regeneration has been obtained from tissue cultures of a wide variety of plant species, many members of the Malvaceae have been rather recalcitrant. The ability to regenerate okra plants in vitro would permit the rapid asexual propagation of this important agricultural plant.

Except for a preliminary report on the production of somatic embryos from callused okra hypocotyl [3], the micropropagation of *Abelmoschus* has been little studied. It was therefore thought worthwhile to apply the technique of tissue and organ culture to explore the possibility of vegetative propagation of okra. The present investigation outlines the comparative morphogenetic responses of hypocotyl, cotyledon, cotyledonary node and primary leaf explants of aseptically grown okra seedlings cultured in different media combinations. Evidence is also presented for the regeneration of okra plants from plantlets formed in vitro.

Materials and methods

Seeds of okra (*A. esculentus* cv. Parkins Mammoth Longpod) were obtained from Dominion Seed House (Georgetown, Ontario, Canada). Seeds were surface sterilized with 80% (v/v) ethanol for 1 min and then with

Abbreviations: BA, benzyladenine, 2,4 D, 2,4-dichlorophenoxyacetic acid, IAA, indoleacetic acid, KN, kinetin, MS, Murashige and Skoog, NAA, naphthaleneacetic acid, Z, zeatin.

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50% (v/v) commercial bleach (3% sodium hypochlorite) for 20 min. The sterilized seeds were thoroughly rinsed in three changes of sterile deionised water and germinated aseptically on a hormone-free Murashige and Skoog (MS) basal nutrient s[illegible] medium [4]. Two-week-old seedlings were used to obtain explants (hypocotyl sections, cotyledons, cotyledonary nodes and primary leaf segments) and these explants were cultured aseptically on a modified MS basal medium. The medium was supplemented, according to experimental requirements, with auxins such as indoleacetic acid (IAA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinins such as benzyladenine (BA), kinetin (KN) and zeatin (Z). The culture medium also included vitamins [5], 3% (w/v) sucrose and 0.6% (w/v) agar. The pH of the medium was adjusted to 5.7 with 1 N NaOH or 1 N HCl. After autoclaving at 15 lb/sq. in. for 15 min, 20 ml of the warm medium was poured into sterile wide-mouthed culture jars (85 × 45 mm). All cultures were incubated in 'Conviron' growth chambers at 22 ± 2°C with a 16-h photoperiod (300 ft-c from cool-white Westinghouse fluorescent lamps). The comparative morphogenetic responses of the explants to auxins, cytokinins and auxin-cytokinin combinations were scored after 30 days. Shoots without roots were transferred to MS medium supplemented with NAA (1.0 mg/l) and BA (1.0 mg/l) and allowed to form roots for the next 15 days. Plantlets with well-developed roots were removed from culture jars, washed thoroughly with running tap water and planted in a soil-vermiculite mixture saturated with water. The plantlets were kept under high humidity in an enclosed chamber to prevent wilting. Once the acclimatization was accomplished, the plants (8–10 cm in height) were transferred to the greenhouse.

Results and discussion

The culturing of explants of aseptically grown okra seedlings on MS medium supplemented with auxins, cytokinins or auxin-cytokinin combinations evoked clear-cut morphogenetic responses. Callus formation was observed on all the explants that were cultured in media containing auxins (Table I). Apart from callus proliferation, the presence of NAA in the nutrient medium promoted the formaton of roots on hypocotyl, cotyledon and leaf explants. Root differentiation was also observed on hypocotyl and cotyledon explants that were cultured on IAA supplemented nutrient medium (Table I). The promotion of root differentiation in vitro on *Brassica*, *Petunia* and *Capsicum* explants by NAA or IAA has been reported by other investigators [6–8]. A characteristic difference, however, between the two auxins was that on IAA medium, the roots were short and poorly developed (Fig. 2), while on NAA-supplemented MS medium, the roots were thin and long with a number of lateral roots or root hairs (Fig. 3). This difference in root development was found to be opposite to that observed on *Capsicum* explants [8]. When 2,4-D was incorporated into IAA-supplemented MS medium the explants responded differently; 2,4-D totally suppressed root formation but promoted the growth of friable whitish callus (Fig. 1). The ineffectiveness of 2,4-D in inducing root formation has also been noted in the case of *Petunia* [9] and *Antirrhinum* [10].

None of the explants cultured on auxins alone showed any development of shoot buds or shoots.

When explants of okra were cultured on MS medium containing cytokinins, both KN and Z were ineffective in inducing shoot bud or shoot formation, instead compact green calluses were produced on hypocotyl and cotyledonary explants. KN (1.0 mg/l) and Z (1.0 mg/l) failed to elicit any callus formation on cotyledonary nodes and primary leaf segments (Table I). With BA, shoots were formed on cotyledonary nodes followed by callus production (Fig. 5). Some recent reports have also indicated the formation of shoot buds or shoots when explants of sugar beet [11] and mulberry [12] are cultured on BA-supple-

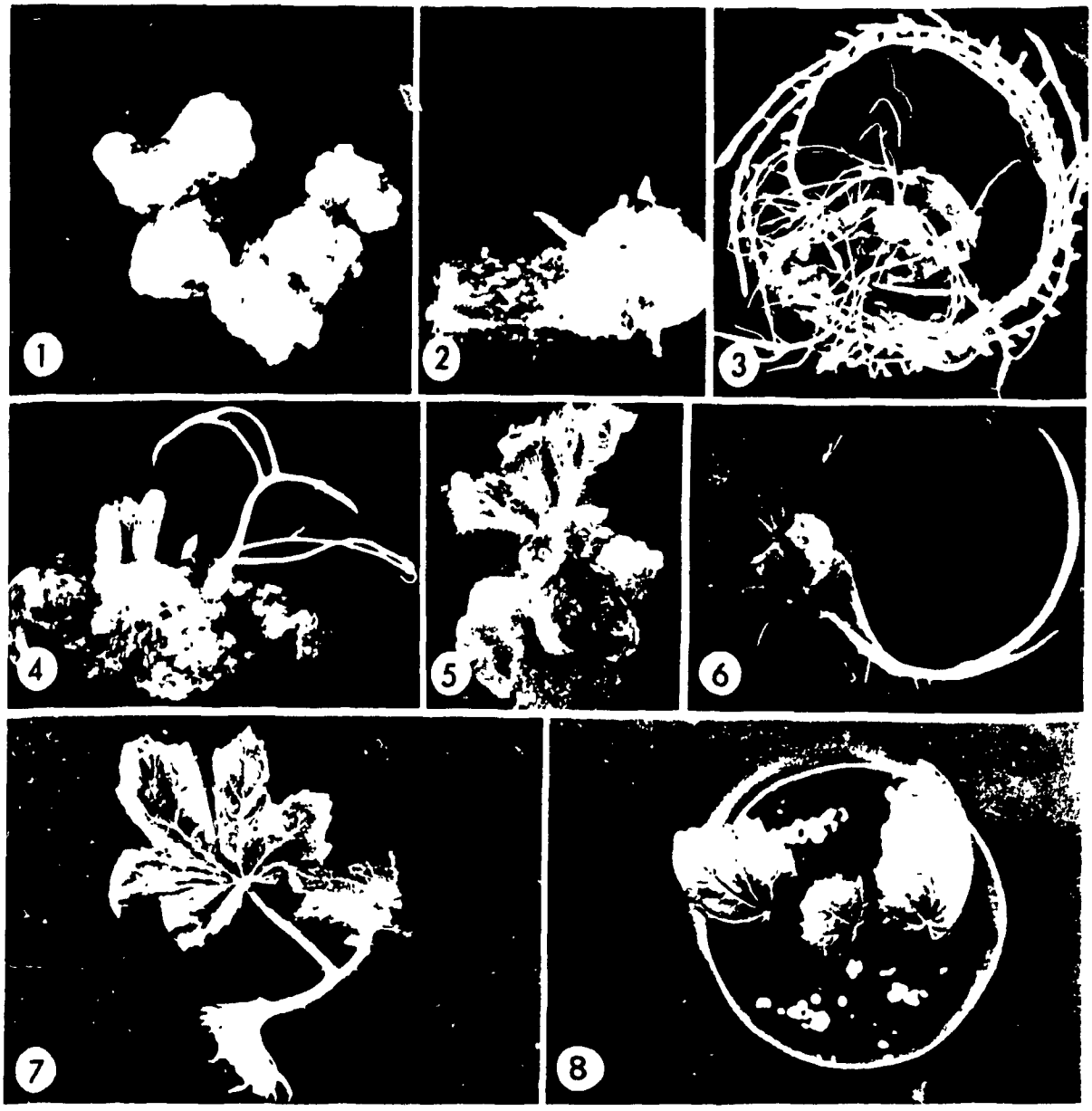
Table I. Comparative morphogenetic responses of hypocotyl, cotyledon, cotyledonary node and primary leaf explants of aseptically grown okra (*A. esculentus*) seedlings cultured in MS medium supplemented with various hormones. Data scored at the end of 30 days, each treatment composed of 10 replicates, repeated twice. The explants used were of similar size and physiological age. NG = no growth; C = callus; R = root, Sh = shoot or shoot bud; C + R = callus and root; C + Sh = callus + shoot or shoot bud; C + Sh + R = callus, shoot or shoot bud and root.

	Hypocotyl	Cotyledon	Cotyledon node	Leaf segment
Basal medium (MS)	NG	NG	NG	NG
MS + NAA (0.1 mg/l)	C	C	C	C
MS + NAA (1.0 mg/l)	C + R	C + R	C	C + R
MS + IAA (0.1 mg/l)	C	C	C	C
MS + IAA (1.0 mg/l)	C + R	C + R	C	C
MS + IAA (0.2 mg/l) + 2,4-D (0.2 mg/l)	C	C	C	C
MS + BA (1.0 mg/l)	C	C	C + Sh	NG
MS + BA (2.0 mg/l)	C	C	C + Sh	C
MS + KN (1.0 mg/l)	C	C	NG	NG
MS + Z (1.0 mg/l)	C	C	NG	NG
MS + NAA (0.1 mg/l) + BA (1.0 mg/l)	C + R	C + R	C	C
MS + NAA (1.0 mg/l) + BA (1.0 mg/l)	C + R	C + Sh + R	C + Sh + R	C + R
MS + IAA (0.1 mg/l) + BA (1.0 mg/l)	C + R	C + R	C + Sh	C
MS + IAA (0.1 mg/l) + KN (1.0 mg/l)	C	C	NG	C
MS + NAA (0.1 mg/l) + Z (1.0 mg/l)	C	C	NG	NG

mented MS medium. Similarly, the presence of BA in the growth medium has been shown to promote the development of shoot buds and shoots on *Brassica* explants cultured in vitro [6]. In the same studies, Gunay and Rao further reported a sporadic formation of shoots with Z and none with KN-supplemented MS medium. Although the presence of cytokinins in the growth medium promoted callus proliferation and generation of some shoots in BA-supplemented medium, the cytokinins failed to produce root differentiation on any of the okra explants (Table I).

A strong interaction of auxin and cytokinin in inducing shoot bud, shoot or callus formation was observed. NAA (1.0 mg/l) in combination with BA (1.0 mg/l) or IAA (0.1 mg/l) was effective in producing shoots or buds (Table I). The combination of 1.0 mg/l, respectively, of NAA and BA was more effective in inducing both shoot buds and root development on cotyledonary explants (Fig. 4) and shoot and roots on cotyledonary nodes (Fig. 6). It has also been observed, in culturing *Brassica*, that a combination of NAA and BA was best in promoting shoots and roots [6]. When IAA was substituted for NAA in the present work, shoots were formed on cotyledonary nodes, but this combination failed to elicit any root development (Table I). However, roots developed within 10 days when these shoots were transferred to MS medium containing BA (1.0 mg/l) and NAA (1.0 mg/l).

After initial climatization, the plantlets from cotyledon or cotyledonary node explants eventually developed into complete plants (Fig. 8) with extensive roots (Fig. 7) when



Figs. 1—8. Responses of various explants of okra (*A. esculentus*) to different growth substances and plant regeneration. Figs. 1—6 taken after 30 days *Fig. 1.* Friable whitish callus growth on hypocotyl explants cultured on MS medium containing IAA (0.2 mg/l) and 2,4-D (0.2 mg/l). *Fig. 2.* Short roots and callus formation on hypocotyl explant cultured on MS medium supplemented with IAA (1.0 mg/l). *Fig 3.* Development of long roots with a number of lateral roots on leaf explant cultured on MS medium containing NAA (1.0 mg/l). *Fig. 4.* Formation of roots and shoot buds on cotyledon explant cultured on MS medium supplemented with NAA (1.0 mg/l) and BA (1.0 mg/l). *Fig 5.* Development of shoot and callus on cotyledonary node explant cultured on MS medium containing BA (1.0 mg/l). *Fig. 6.* Shoot, root and callus development on cotyledonary node explant cultured on MS medium supplemented with NAA (1.0 mg/l) and BA (1.0 mg/l). *Fig. 7.* Regenerated plantlet exhibiting profuse root growth. *Fig. 8.* Regenerated plant growing in the greenhouse.

Table II. Frequency of plant regeneration in cultured explants of okra (*A esculentus*). Data obtained at the end of 30 days; each treatment composed of 10 replicates, repeated twice

Media	Explants			
	Hypocotyl	Cotyledon	Cotyledon node	Leaf segment
MS	–	–	–	–
MS + NAA (1.0 mg/l)	–	–	–	–
MS + IAA (1.0 mg/l)	–	–	–	–
MS + BA (1.0 mg/l)	–	–	+	–
MS + NAA (1.0 mg/l) + BA (1.0 mg/l)	–	++	++++	–
MS + IAA (1.0 mg/l) + BA (1.0 mg/l)	–	–	++	–

– = nil; + = low; ++ = moderate; ++++ = very high.

these plantlets were transferred to the greenhouse.

Thus, our experiments demonstrate the development into complete plants of plantlets obtained from cultured explants of okra cotyledons and cotyledonary nodes. Further, a single medium was sufficient for initiation as well as the subsequent development of the rooted plantlets. This coupled with the reproducibility of the technique (Table II) prompts us to believe that this method offers a great potential for rapid, mass propagation of okra plants.

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