Morphogenetic Responses *in Vitro* of Hypocotyl Tissues of Chili Pepper (*Capsicum annuum* L.) to Growth Regulators[1]

N. Ochoa-Alejo*, M.A.R. García-Bautista**

ABSTRACT

Cultured hypoctyl sections of chili pepper (*Capsicum annuum* L., ancho type, var. Esmeralda) derived from aseptically germinated seedlings were evaluated on the basis of their morphogenetic responses to combinations of IAA with BA, Kin or 2iP, and NAA or 2, 4-D with BA. Shoot and root differentiation and callus formation were observed on the cut ends of the explants after eight weeks of incubation under continuous light at 25 ± 2°C. Regeneration of adventitious shoots was more prolific in explants cultured on Murashige and Skoog basal medium (MS) containing 5-50 µM IAA plus either 25-50 µM BA or 2iP, whereas rhizogenesis predominated in hypocotyl segments incubated on media supplemented with IAA or NAA alone (1-50 µM). IAA induced compact callus at all the concentrations tested (1-50 µM). NAA produced compact or friable callus depending on the quantity added to the medium, whereas 2, 4-D elicited friable callus development.

COMPENDIO

Secciones de hipocotilo de chile (*Capsicum annuum* L.; tipo ancho var. Esmeralda) derivadas de plántulas obtenidas en condiciones asépticas fueron evaluadas con base en sus respuestas morfogenéticas en combinaciones de ácido indolacético (IAA) con benciladenina (BA), cinetina (Kin) o 2-isopenteniladenina (2iP); y ácido naftalenacético (NAA) o ácido 2, 4-diclorofenoxiacético (2, 4-D) con BA. La diferenciación de brotes adventicios y raíces, y la formación de callos fue observada en los extremos de los explantes después de ocho semanas de incubación en luz continua y a 25 ± 2°C. La regeneración de brotes adventicios fue más abundante en explantes cultivados en el medio básico de Murashige y Skoog (MS) conteniendo 5-50 µM de IAA más 25-50 µM de BA o 2iP, mientras que la rizogénesis prodominó en segmentos de hipocotilo incubados en medios que contenían IAA o NAA solos (1-50 µM). El IAA indujo callos compactos en todas las concentraciones probadas (1-50 µM). El NAA produjo callos compactos o friables dependiendo de la cantidad adicionada al medio de cultivo, mientras que el 2, 4-D indujo el desarrollo de callos friables.

INTRODUCTION

Because of their rich aroma, flavor, pungency and color, chili peppers are important items in the human diet around the world, Latin Americans being the main consumers. Chili peppers help to preserve food, have medicinal value, are an excellent source of vitamins A and C, and are low in caloric value (9). The most important cultivated chili pepper species of the northern hemisphere is *Capsicum annuum*, having its center of domestication in Mexico (19).

Plant cell and tissue cultures have been used as alternative systems for plant improvement (1, 5, 15). Because of their regeneration capability, cells and tissues of Solanaceae have been extensively used for *in vitro* studies. For example, totipotency was first demonstrated in single cells of *Nicotiana tabacum* (20), and the first haploid plants regenerated *in vitro* were derived from anther cultures of *Datura innoxia* (7).

Morphogenetic studies with cells and tissues of *Capsicum* are limited. There are only two reports on plant regeneration from cultured hypocotyl and cotyledon segments (8, 15). Fari and Czako (3) studied the relationship between the position of the explant taken from the hypocotyl and their morphogenetic responses *in vitro*. The isolation and culture of protoplast of *Capsicum annuum* and their regeneration into plants has been described by Saxena *et al.* (17). The obtention of haploid plants through *in vitro* techniques has also been achieved (6, 18). Since the knowledge of the tissue culture responses of chili pepper is still limited, and considering that the optimum concentration of growth regulators for organ induction varies with cultivar (12), it would be convenient to extend the studies of morphogenesis to other *Capsicum* materials. These kind of investigations may be useful for accelerated vegetative multiplication of superior individuals or for the improvement of chili pepper via genetic transformation techniques (10, 13).

The purpose of the present study was to investigate the morphogenetic responses of hypocotyl segments of chili pepper cultured in the presence of different combinations of growth regulators.

1 Received for publication 5 February 1988 [1]
This work was supported by a grant from SEP/SEIT, Mexico (Proyecto estratégico No. 6; Area biología vegetal moderna)

* Centro de Investigación y de Estudios Avanzados del IPN Unidad Irapuato, Apartado Postal 629; 36500-Irapuato, Gto., México

** The preparation of the manuscript was accompanied by the untimely death of M.A.R. García-Bautista.

MATERIALS AND METHODS

Seeds of chili pepper (*Capsicum annuum* L.; ancho type var. Esmeralda) were surface sterilized by immersion in 96% ethanol for 5 min followed by 20 min in 20% (v/v) commercial bleaching solution (Cloralex) containing 0.1% Tween 20. After washing thoroughly in sterile deionized water, the seeds were sown on the basal medium described by Murashinge and Skoog (14) and were incubated at 25 ± 2°C under continuous light (daylight fluorescent tubes; 1 500-2 000 lux) for four weeks. Hypocotyl explants (1 cm in length) were excised from the seedlings and the segments were transferred to glass bottles (100 ml) containing 20 ml of MS basal medium supplemented with auxins and cytokinins at various concentrations. The auxins, indoleacetic acid (IAA), naphtholeneacetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D), and the cytokinins, benzyladenine (BA), 2-isopentenyladenine (2iP) and Kinetin (Kin) were tested at concentrations of 0, 1, 5, 12.5, 25 and 50 μM. Sucrose (3%) was added to the media as carbon source. The pH was adjusted to 5.7 before autoclaving (121°C; 20 min) and the media were gelled with 0.8% agar. Thirty-six treatments (including a control lacking phytoregulators) resulted from each auxin/cytokinin combination. For each treatment ten bottles were inoculated with two explants each. Cultures were maintained for eight weeks under the same conditions described for aseptic seed germination.

All the experiments were carried out at least twice.

RESULTS

Effect of IAA/BA

Figures 1 and 2 show the growth and morphogenetic responses of chili pepper hypocotyl segments to various concentrations of IAA and BA. When the explants were grown on media supplemented with IAA alone there was no shoot induction. Low percentages of shooting (3-5%) were observed in the presence of BA alone (Fig. 2a). However, when BA (12.5-50 μM) was supplemented with IAA (1-50 μM) shoot regeneration occurred on the cut ends of the explants. Adventitious shoot initiation was more pronounced on media containing 5-50 μM IAA plus 25-50 μM BA.

Rhizogenesis occurred predominantly on media supplemented with IAA alone (1-50 μM). The regenerated roots formed on the cut ends of the segments and were long and thin. At all concentrations of IAA tested, root formation decreased as the concentration of BA increased. Higher percentages of root organogenesis were observed on media containing 12.5-50 μM IAA (Fig. 2b).

Most combinations of IAA and BA supported the formation of compact callus, as did IAA or BA alone. As the concentration of IAA was raised, callus growth increased, while increasing the BA concentration had the opposite effect (Fig. 2c).

Effect of IAA/2iP

Cultured hypocotyl tissues demonstrated variable morphogenetic responses to the 36 culture media containing various concentrations of IAA and 2iP (Figs. 1 and 3). Shoot formation in this instance was also limited to the cut surface of the explants. Shooting could occasionally be obtained with 2iP alone (Fig. 3a). Media containing 1-50 μM IAA in conjunction with 5-50 μM 2iP induced adventitious shoot initiation. However, combinations of 5-50 μM IAA plus 25-50 μM 2iP were more effective in bringing about shoot differentiation.

Hypocotyl explants rooted in almost all the treatments tested (Fig. 3b). Low percentages of rooting (2-20%) were found to occur in tissues incubated on media supplemented with 2iP only. In general, there was an increase in rhizogenesis in relation to the increase in auxin concentration, whereas the elevation of cytokinin levels progressively inhibited root differentiation. The maximum response occurred in the presence of 5-50 μM IAA with 0-1 μM 2iP.

Callus was induced in most treatments (Fig. 3c). In all cases white-greenish calli were observed. However, callus growth was most prolific on media containing high IAA to 2iP ratios.

Effect of IAA/Kin

Figs. 4 and 5 show the distribution and the degree of responses of explants to different IAA/Kin ratios. Shoot differentiation was erratic in some treatments and was observed in only a few explants (Fig. 5a). Combinations of IAA and Kin resulted in the production of calli or calli with roots. As in the cases of BA and 2iP, Kin alone induced poor root formation (2-15%) (Fig. 5b). Higher root production was obtained on media supplemented with 5-50 μM IAA + 0-1 μM Kin. Compact callus was produced on the cut surfaces of the explants in all treatments. However, callus growth was promoted by high IAA concentrations and inhibited by increasing levels of Kin (Fig. 5c).

Effect of NAA/BA

The morphogenetic responses of hypocotyl tissues to this combination are shown in Figs. 4 and 6. NAA and BA treatments elicited shoot organogenesis, but

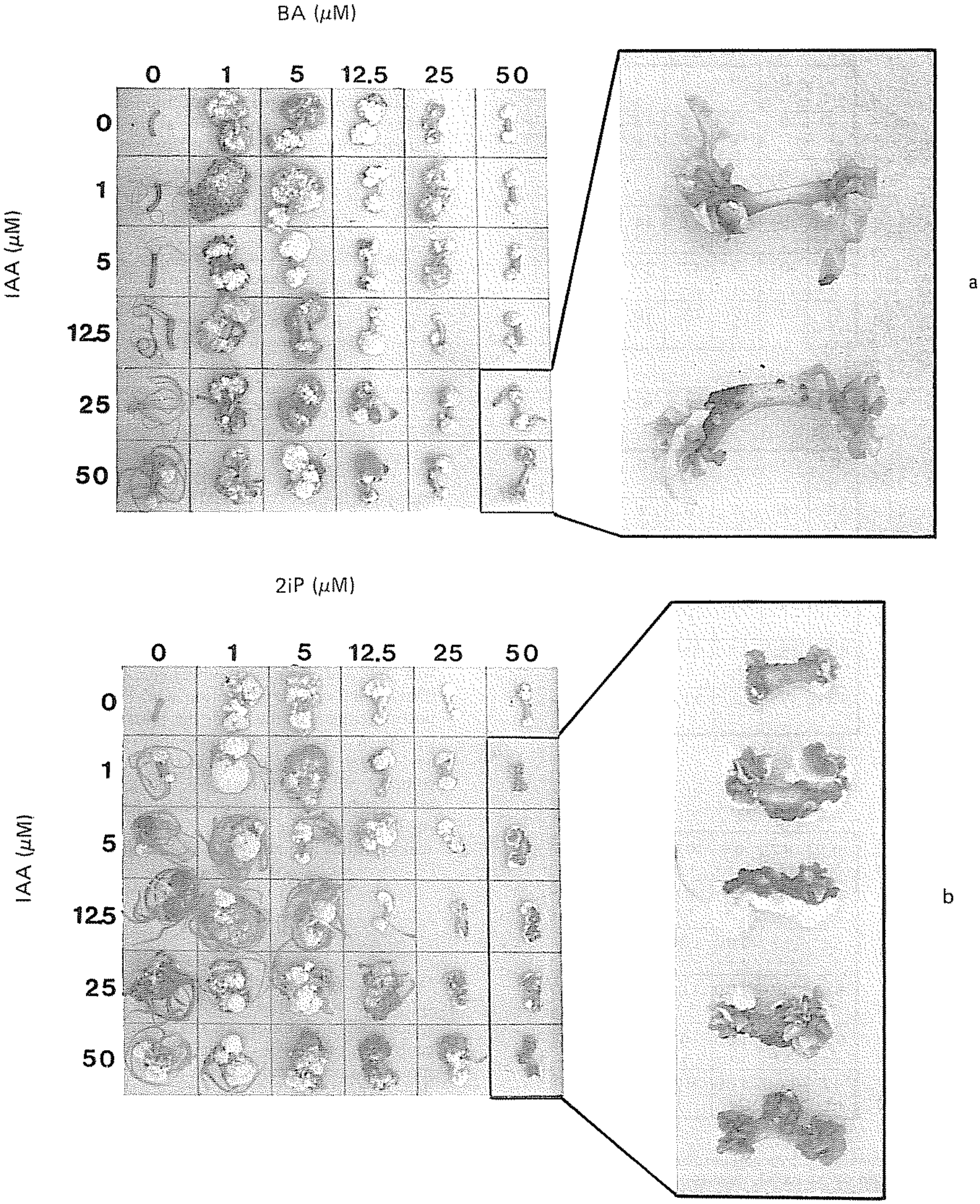

Fig 1 Shoot, root, and callus formation in hypocotyl explants of *Capsicum annuum* var Esmeralda, in the presence of IAA/BA (a), and IAA/2iP (b).

a

IAA (µM) \ BA (µM)	0	1	5	12.5	25	50
0	0	0	0	3 ±	5 ±	5 ±
1	0	0	2 ±	10 ±	14 +	26 +
5	0	0	2 ±	24 +	36 ++	26 ++
12.5	0	0	0	27 +	44 ++	37 ++
25	0	0	7 ±	37 +	39 ++	55 +++
50	0	0	7 ±	23 +	50 ++	47 +++

b

IAA (µM) \ BA (µM)	0	1	5	12.5	25	50
0	0	19 ±	22 ±	6 ±	0	0
1	58 +	20 +	8 ±	10 ±	2 ±	6 ±
5	74 ++	50 ++	7 ±	19 ±	3 ±	0
12.5	92 +++	54 ++	15 +	3 ±	5 ±	0
25	84 +++	78 ++	37 +	22 +	8 ±	5 ±
50	92 +++	37 ++	32 +	21 +	9 ±	6 ±

c

IAA (µM) \ BA (µM)	0	1	5	12.5	25	50
0	9 ±	87 +++	95 +++	95 ++	90 +	95 +
1	46 ±	97 +++	94 +++	91 ++	92 ++	89 +
5	54 +	97 +++	100 +++	100 ++	92 +	92 +
12.5	64 +	100 +++	100 +++	100 ++	92 +	92 +
25	67 ++	97 +++	100 +++	97 ++	82 +	90 +
50	97 ++	100 ++	100 ++	97 ++	90 +	86 +

Fig. 2. Percentages of hypocotyl explants showing differentiation of shoots (a), roots (b) and callus formation (c) in the presence of IAA and BA at various concentrations. 20 replicates per treatment. Extent of responses: ± negligible; +, low; ++, moderate; +++, high

this response was restricted to media containing 1-50 µM NAA plus 12.5-50 µM BA, and the percentages ranged from 2 to 19% (Fig. 6a).

The highest frequencies of root formation were observed in the presence of the auxin alone (Fig. 6b). A trend towards decreased rhizogenesis was found to occur as the concentration of NAA or BA was increased. The roots induced by NAA were short and thick in comparison to those induced by IAA.

High percentages of hypocotyl explants showing callus formation were observed in the presence of NAA, BA or combinations of both growth regulators (Fig. 6c). However, there were differences in the extent of the response and in the texture of the calli. As the NAA concentration was increased from 1 to 50 µM, so more prolific callusing was obtained. Callus produced in explants cultured on media containing 1-25 µM was compact whereas that induced in the presence of 50 µM of this auxin was friable. The extent of callus growth was negatively affected by increasing the BA concentration of either, alone or in conjunction with NAA.

Effect of 2, 4-D/BA

Low percentages of shoot initiation (2-20%) were obtained only with 1 µM 2, 4-D in combination with 12.5-50 µM BA (data not shown). No root formation was detected on media supplemented with 2, 4-D either alone or in combination with BA (Fig. 4). Hypocotyl explants callused in the presence of 1-50 µM 2, 4-D. As a rule, 2, 4-D alone induced friable callus while combinations of this auxin with BA rendered compact callus. A reduction in callus growth was observed when BA concentration was raised. The medium containing 12.5 µM 2, 4-D proved to be optimal for callus induction and proliferation.

DISCUSSION

The present study has revealed that hypocotyl explants of *Capsicum annuum* var. Esmeralda are capable of forming shoots or roots adventitiously, as well as callus, depending on the combination and concentration of growth regulators present in the culture medium. Combinations of BA and 2iP with IAA were more effective in bringing about adventitious shoot differentiation. In general, the presence of high levels of cytokinin and low or high concentration of auxin were necessary for eliciting shoot formation. IAA (0-5.7 µM) and BA (4.4 to 8.9 µM) have been reported to be the best auxin/cytokinin combination for shoot induction in hypocotyl and cotyledon explants of red peppers (*C. annuum* cvs. California Wonder

2iP(µM)

a

IAA(µM)	0	1	5	12.5	25	50
0	0	0	0	2 ±	0	10 ±
1	0	0	5 ±	8 ±	20 +	40 ++
5	5 ±	0	2 ±	7 ±	47 ++	54 +++
12.5	0	0	0	2 ±	28 +	47 +++
25	2 ±	2 ±	0	18 +	35 ++	54 +++
50	0	0	3 ±	15 +	23 ++	45 +++

b

IAA(µM)	0	1	5	12.5	25	50
0	7 ±	20 ±	5 ±	10 ±	5 ±	2 ±
1	74 +	45 +	26 ±	13 ±	7 ±	0
5	82 ++	87 ++	50 ++	37 +	27 ±	16 ±
12.5	84 +++	75 ++	73 ++	49 +	26 ±	29 ±
25	97 +++	89 ++	77 ++	57 +	36 ±	16 ±
50	84 +++	82 ++	70 +	40 ±	31 ±	22 ±

c

IAA(µM)	0	1	5	12.5	25	50
0	4 ±	100 ++	100 ++	97 ++	100 +	100 +
1	90 ±	100 +++	97 +++	100 ++	100 +	100 +
5	100 +	100 +++	100 ++	97 ++	100 +	100 +
12.5	100 +	100 +++	100 +++	100 ++	100 +	100 +
25	100 ++	100 +++	100 +++	100 ++	100 +	100 +
50	100 ++	97 ++	100 ++	100 ++	100 ++	97 +

Fig. 3. Frequency (%) of hypocotyl segments showing differentiation of shoots (a), roots (b) and bearing callus formation (c), on media containing IAA and 2iP at different concentrations. 20 replicates per treatment. Degreee of responses: see Fig. 2

and Pimiento, and *C. frutescens* cv Bharath) (8). According to Phillips and Hubstenberger (16) MS basal medium containing 0.3-22.8 µM IAA + 44.4-222 µM BA promoted shoot organogenesis in hypocotyl sections, cotyledon pieces and shoot tips of *C. annuum* cvs. California Wonder, Yolo Wonder (sweet bell types), New Mexico No. 6-4, and NuMex R. Naky (long green chili types). The present work showed that the best hormone combination for shoot induction in hypocotyl explants of var. Esmeralda ("ancho" type chili pepper) were 5-50 µM IAA with 25-50 µM BA. These differences in growth regulator requirements for shoot regeneration could be the result of genotypic and developmental differences of the explant source as has been observed for *Lycopersicon esculentum* tissues (4, 12).

Our results show that regeneration of shoots was also possible on media containing IAA combined with 2iP, and the percentages of induction were similar to that of IAA + BA. This observation has not been reported before and therefore this combination could be used as an alternative for shoot induction in *Capsicum* tissues.

The poor shoot differentiation induced by IAA/Kin in the present work confirms the results reported previously (8, 16). This combination has been found to elicit shooting in hypocotyl explants of *Solanum melongena*, and in stem, leaf and cotyledon segments of *Lycopersicon esculentum* (11, 21), indicating that the hormonal requirements for shoot differentiation varies with species.

Rooting of hypocotyl explants occurred mainly on the media supplemented with IAA or NAA (1-50 µM). Nevertheless, morphological differences were observed: IAA induced long, thin roots whereas NAA produced short, thick roots. On the other hand, 2, 4-D, when added alone to the culture medium, prevented root and shoot organogenesis. However, when combined with BA, shoot differentiation took place at low frequencies. Gunay and Rao (8), and Phillips and Hubstenberger (16) have also reported inhibition of root and shoot induction in hypocotyl and cotyledon explants of chili peppers grown in the presence of 2. 4-D. Furthermore, occasional shoot differentiation has been observed in treatments with 2, 4-D and Kin (16).

In general, 2, 4-D has been shown to induce friable callus formation in several species (2) and this response was also observed in the present work. This auxin is being used in our laboratory for establishing callus and cell suspension cultures.

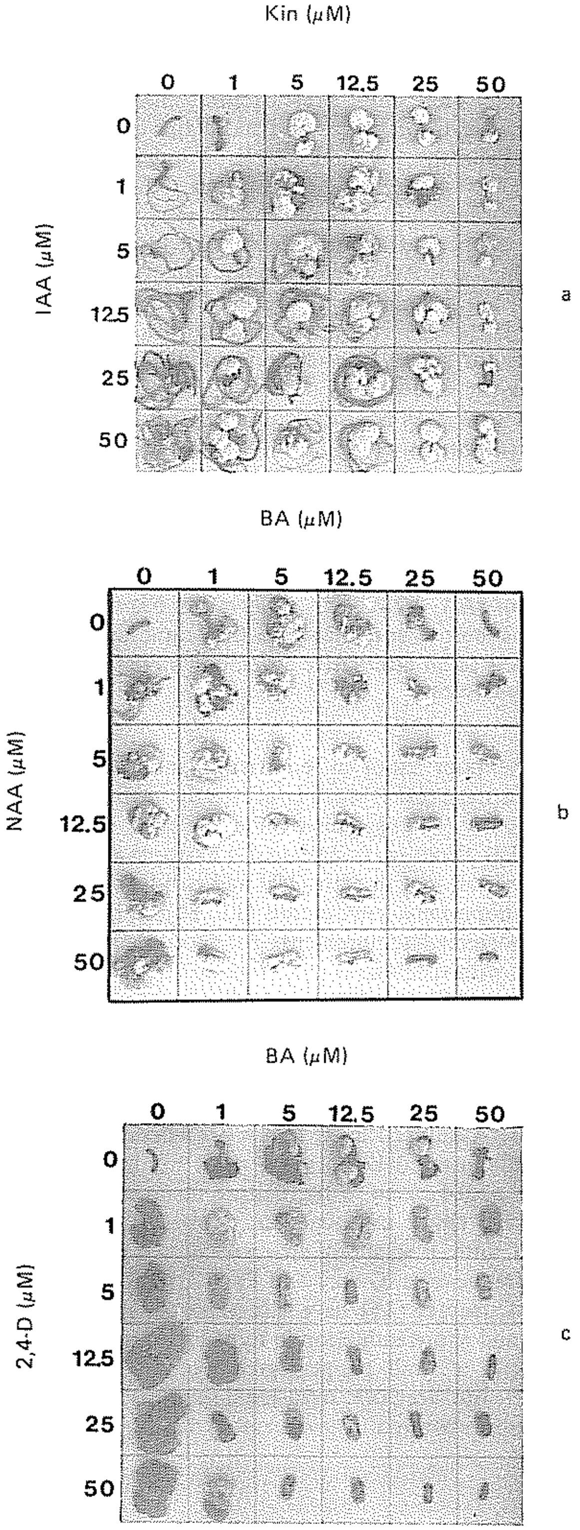

Fig. 4. Growth and morphogenetic responses of hypocotyl tissues of chili pepper cultured on media with IAA/Kin (a), NAA/BA (b), and 2,4-D/BA (c), at various concentrations

a

IAA (μM) \ Kin (μM)	0	1	5	12.5	25	50
0	0	0	0	0	0	0
1	0	0	0	0	3 ±	0
5	3 ±	0	0	0	0	0
12.5	0	3 ±	5 ±	0	0	0
25	0	5 ±	0	0	2 ±	8 ±
50	3 ±	5 ±	0	0	0	2 ±

b

IAA (μM) \ Kin (μM)	0	1	5	12.5	25	50
0	17 ±	10 ±	2 ±	15 ±	2 ±	8 ±
1	59 +	59 +	27 +	27 +	20 ±	3 ±
5	79 ++	75 +	71 +	46 ±	31 ±	14 ±
12.5	75 +++	84 ++	64 ++	46 +	47 ±	30 ±
25	84 +++	92 +++	85 ++	65 ++	34 ±	28 −
50	83 +++	92 +++	71 ++	81 ++	52 +	31 ±

c

IAA (μM) \ Kin (μM)	0	1	5	12.5	25	50
0	7 ±	55 +	72 ++	87 ++	90 +	80 +
1	67 ±	91 +++	90 +++	100 +++	95 ++	85 +
5	92 +	97 ++	97 +++	100 ++	100 ++	100 +
12.5	92 +	100 +++	93 ++	100 +++	92 ++	100 +
25	100 ++	100 ++	97 ++	100 +++	100 ++	100 +
50	95 ++	100 +++	97 ++	100 ++	100 ++	100 ++

Fig. 5. Percentages of hypocotyl segments showing shoot (a), root (b) and callus formation (c), on media supplemented with IAA and Kin at various concentrations 20 replicates per treatment. Extent of responses: see Fig. 2.

a

NAA(μM) \ BA(μM)	0	1	5	12.5	25	50
0	0	0	0	0	7 ±	5 ±
1	0	0	0	15 +	15 +	19 +
5	0	0	0	0	2 ±	8 ±
12.5	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0

b

NAA(μM) \ BA(μM)	0	1	5	12.5	25	50
0	7 ±	15 +	5 ±	7 ±	0	0
1	92 +	65 +	45 +	21 ±	10 ±	0
5	84 +	47 +	21 +	12 ±	7 ±	7 ±
12.5	68 +	16 ±	12 ±	0	0	0
25	44 ±	0	0	5 ±	0	0
50	0	0	0	0	0	0

c

NAA(μM) \ BA(μM)	0	1	5	12.5	25	50
0	27 ±	90 ++	100 +++	100 ++	97 +	100 ±
1	95 +	100 +++	95 ++	100 ++	100 +	100 +
5	97 ++	100 ++	100 ++	100 +	100 +	100 +
12.5	97 ++	100 ++	100 ++	97 +	97 +	100 +
25	100 ++	100 ++	100 +	92 +	100 +	93 +
50	100 +++	100 ++	100 +	87 +	89 +	74 ±

Fig 6 Frequency (%) of hypocotyl segments showing differentiation of shoots (a), roots (b) and callus formation (c), on culture media containing NAA and BA at different concentrations 20 replicates per treatment Degree of responses: see Fig 2

LITERATURE CITED

1. CHALEFF, R S ; PARSON, M F. 1978. Direct selection *in vitro* for herbicide-resistant mutants of *Nicotiana tabacum*. Proceedings of the National Academy of Science 75:5104-5107

2. EVANS, D A ;SHARP, W.R ;FLICK, C.E 1981 Growth and behavior of cell cultures In Plant Tissue Culture Methods and Applications in Agriculture. Ed. by Thorpe, T A. New York, Academic Press. p. 45-113

3. FARI, M.; CZAKO, M 1981. Relationship between position and morphogenetic response of pepper hypocotyl explants cultured *in vitro* Scientia Horticulturae 15:207-213.

4. FRANKENBERGER, E.A.; HASEGAWA, P.M ; TIGCHELAAR, E.C. 1981 Influence of environment and development state on the shoot-forming capacity of tomato genotypes Zeitschrift für Pflanzenphysiologie 102:221-232

5. GENGENBACH, B G.; GREEN, C.E. 1975. Selection of T-cytoplasm maize callus cultures resistant to *Helminthosporium maydis* race T pathotoxin. Crop Science 15:645-649.

6. GEORGE, L.; NARAYANASWAMY, S. 1973. Haploid *Capsicum* through experimental androgenesis Protoplasma 78:467-470

7. GUHA, S.; MAHESHWARI, S C. 1964. *In vitro* production of embryos from anthers of *Datura* Nature 204:497

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

9. HEISER, C.B. JUNIOR; SMITH, P.G 1953 The cultivated *Capsicum* peppers. Economic Botany 7:214-227.

10. HORSCH, R.B.; FRY, J.E.; HOFFMANN, N.; WALLROTH, M.; EICHHOLTZ, D.; ROGERS, S.G.; FRALEY, R.T. 1985. A simple and general method for transferring genes into plants. Science 227: 1229-1231.

11. KAMAT, M.G.; RAO, P.S. 1978. Vegetative multiplication of eggplants (*Solanum melongena*) using tissue culture techniques. Plant Science Letters 13:57-65.

12. KURTZ, S.M.; LINEBERGER, R.D. 1983. Genotypic differences in morphogenic capacity of cultured leaf explants of tomato. Journal of the American Society for Horticultural Science 108:710-714.

13. McCORMICK, S.; NIEDERMEYER, J.; FRY, J.; BARNASON, A.; HORSCH, R.; FRALEY, R. 1986. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Reports 5:81-84.

14. MURASHIGE, T.; SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

15. NABORS, M.; GIBBS, S.E.; BERNSTEIN, C.S.; MEIS, M.E. 1980. NaCl-tolerant tobacco plants from cultured cells. Zeitschrift für Pflanzenphysiologie 97: 13-17.

16. PHILLIPS, G.C.; HUBSTENBERGER, J.F. 1985. Organogenesis in pepper tissue cultures. Plant Cell Tissue Organ Culture 4:261-269.

17. SAXENA, P.K.; GILL, R.; RASHID, A.; MAHESHWARI, S.C. 1981. Isolation and culture of protoplasts of *Capsicum annuum* L. and their regeneration into plants flowering *in vitro*. Protoplasma 108:357-360.

18. SIBI, M.; DUMAS DE VAULX, R.; CHAMBONNET, D. 1979. Obtention de plantes haploides par androgenese *in vitro* chez le piment (*Capsicum annuum* L.). Annales l'Amelioration des Plantes 29:583-606.

19. SMITH, P.G.; HEISER, C.B. 1951. Taxonomic and genetic studies on the cultivated peppers, *Capsicum annuum* L. and *C. frutescens* L. American Journal of Botany 38:362-368.

20. VASIL, V.; HILDEBRANDT, A.C. 1965. Differentiation of tobacco plants from single isolated cells in microculture. Science 150:889-892.

21. VNUCHKOVA, V.A. 1978. Development of a method for obtaining regenerate tomato plants under tissue culture conditions. Soviet Plant Physiology 24:884-889.