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IN VITRO MORPHOGENETIC RESPONSES AND CYTOKININ-AUXIN INTERACTION FOR CALLUS PRODUCTION IN PEPPER

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RESUMEN

Se aislaron y cultivaron *in vitro* meristemos de dos especies de *Capsicum* en medio MS adicionado con citocininas y auxinas en varias combinaciones. Aun cuando se produjeron yemas y plántulas en ambas especies, la eficiencia fue diferente. La mejor respuesta para *C. chinense* fue en la combinación BA (2.0 mg/l) y AIA (1.0 mg/l), en tanto que para *C. annuum* se logró e presencia de K (2.0 mg/l) y AIA (1.0 mg/l). Se cultivaron secciones de hipocótilo de *C. annuum*, observándose una pobre respuesta morfogenética, pero una abundante producción de callo, del cual se evaluaran su peso fresco y seco, los que mostraron un comportamiento exponencial con una tasa máxima de multiplicación del callo a los dos meses de cultivo. La combinación K (2.0 mg/l) con 2,4-D (1.0 mg/l) presentó el mejor rendimiento de callo.

Palabras clave: *Capsicum*, regeneración *in vitro*, producción de callo, hipocótilo, meristemos apicales.

ABSTRACT

Meristems of two *Capsicum* species were cultured in MS medium supplemented with several cytokinin and auxin combinations. Efficiencies in bud and shoot production were different in both species. The best response in *C. chinense* was attained with a BA (2.0 mg/l) IAA (1.0 mg/l) combination, while in *C. annuum* it occurred with K (2.0 mg/l) IAA (1.0 mg/l). Hypocotyl sections of *C. annuum* were also cultured. The morphogenetic response of this explant was poor, even though abundant callus was achieved. Fresh and dry weight were evaluated. An exponential response was observed with the maximum multiplication rate of callus after two months under culture; K (2.0 mg/l), 2,4-D (1.0 mg/l) combinations rendered the best callus production.

Key words: *Capsicum*, *in vitro* regeneration, callus production, hypocotyl, apical shoot meristems.

INTRODUCTION

Plant tissue culture can potentially revolutionize the knowledge and application in several fields of the plant kingdom (Cooking, 1986). Considerable advance has been reached, especially in certain plant families as Solanaceae which is one of the best known (Flick *et al.*, 1983); even though some of the family members, such

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as *Capsicum* remain without an adequate *in vitro* exploration despite their great economic value (Morrison, *et al.*, 1986). *In vitro* studies of *Capsicum* species have been emphasized in the effects of cytokinin and auxins, some of which proved to be ineffective for the induction of morphogenetic responses but efficient for callus production (George and Narayanaswamy, 1973; Gunay and Rao, 1978). Several *Capsicum* explants and their *in vitro* morphogenetic potentiality have been explored with variable degrees of success: cotyledon sections (Gunay and Rao, 1978; Phillips and Hubstenberger, 1985), shoot tip meristems (Phillips and Hubstenberger, 1985) embryos (Agrawal and Chandra, 1983), anther culture (Dumas *et al.*, 1981) and a wide range of explants (Agrawal *et al.*, 1989).

Standardized methods for callus production in *Capsicum* are an important goal in view of the industrial interest in some of its secoundary metabolites (Williams *et al.*, 1988) and particulary capsaicine (Ochoa and Peralta, 1990; Sukrasno and Yeoma, 1990). On the other hand, a friable and abundant callus is desirable in order to trigger somatic embryogenesis (Evans *et al.*, 1981).

As morphogenetic studies in *Capsicum* remain poorly developed, the primary objective of this study was to evaluate the potential responses of shoot tip meristems in two economically important *Capsicum* species: *C. chinense* and *C. annuum* (Pozo *et al.*, 1991) under the exposure to some growth regulators. Callus production with respect to the auxin-cytokinin interaction and the *in vitro* culture of *Capsicum annuum* hypocotyls were also analyzed.

MATERIALS AND METHODS

Capsicum chinense L. cv. habanero and *C. annuum* L. cv. serrano seeds were obtained from Productora Nacional de Semillas (PRONASE). These were sterilized by passage through 70% ethanol for two min and disinfected in 0.6% sodium hypochlorite for 15 min on a gyratory shaker at 150 rpm. The seeds were then thoroughly rinsed four times with sterile distilled water and germinated on moistened cotton in sterile 100 ml glass jars at 30°C for 10 days in darkness. Afterwards, they were placed at 27°C with a 16 h light (1000 lux), 8 h darkness photoperiod. Apical meristems 0.4 mm in length were isolated from both species after 8-10 days. Hypocotyl sections of *Capsicum annuum* (10 mm long) were also isolated. Apical meristems and hypocotyl sections were disinfected as described above and rinsed three times in sterile distilled water.

Explants were inoculated in 100 ml glass jars (5 explants/jar) on 30 ml. of MS medium (Murashige and Skoog, 1962) with several growth regulator combinations (see Tables for details). Whenever indolacetic acid (IAA) was used it was added filter-sterile to the medium. The medium's pH was adjusted to 5.8 prior to adding the agar and autoclaving. Explants were incubated at constant 27 ± 2°C with a 16 h light (1000 lux) 8 h darkness photoperiod. Culture procedures were conducted under aseptic conditions. Each treatment comprised from 5 to 6 replicates with 5 explants each. Another set of experiments was done with *C. annuum* hypocotyls which were cultured as stated above and were additionally exposed to 6-benzylandenine (BA) or kinetin (K) (2.0 mg/l) combined with 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0

mg/l) in order to obtain callus; in this case, 35 jars per combination of growth regulators were prepared. Fresh and dry weight were used as growth parameters. After measuring fresh weight, callus tissue was dried on a piece of aluminum foil in an oven at 60°C for 24 h, when dry weight was noted down. Measures of callus production were taken from the third to the ninth week.

RESULTS AND DISCUSSION

Morphogenetic response in Capsicum shoot apical meristems

Table 1 shows the morphogenetic response of *Capsicum chinense* and *C. annuum* shoot apical meristems under the combined influence of several growth regulators at 2.0 and 1.0 mg/l. Both species showed callus formation under all the experimental conditions tested, although at different rates (33-96% *C. chinense;* 19-97% *C. annuum*). Our results partially agree with those from Phillips and Hubstenberger (1985) with respect to the high frequency of callus production in *Capsicum* meristems. Nevertheless, our data show that K + IAA combinations render the best callus response, while the previous authors reported this effect for several auxins + BA combinations. Moreover, in our tests, when 2,4-D was used as the exogenous auxin, a relatively poor callusing was observed, irrespective of the cytokinin tested (Table 1). These results differ from what has been reported by Phillips and Hubstenberger (1985) who found that, 2,4-D ranks as the best callus inductor in *Capsicum,* a fact that has been generally accepted by many authors for many plant species (Georges and Sherrington, 1984). These differences may be due to a genotype influence in the promotion of callus responses in pepper; this genotype effect was clearer when analyzing *Capsicum chinense* and *C. annuum* bud and shoot formation (Table 1). The influence of exogenously supplied growth regulators was different for both species. In *C. chinense* the best performance was promoted with BA with naphtalenacetic acid (NAA) and IAA combinations, particularly with the latter. This observation supports previous conclusions (Gunay and Rao, 1978; Fari and Czako, 1981; Phillips and Hubstenberger, 1985). These authors found that IAA and BA are the best growth regulators for pepper tissue culture. However, their conclusion does not agree with what we observed in *C. annuum* (Table 1), where the best bud and shoot organogenesis was achieved by the interaction of K and IAA, while BA + IAA combinations proved to be cosiderably ineffective. Gunay and Rao concluded (1978) that K was inefficient to induce pepper differentiation and reported only callus production, while Agrawal *et al.* (1989) reported poor morphogenetic responses with K compared with BA in *C. annuum.* These disagreements can be explained based upon the various explants used and their genotypic differences (Rubluo and Kartha, 1985; Chen and Marowitch, 1987). On the other hand, Pillips and Hubstenberger (1985) reported that 2,4-D combined with K can elicit shoot organogenesis, although at low frequencies. We obtained similar results for bud induction for *C. chinense* (Table 1) even though no morphogenetic response in *C. annuum* was observed with 2,4-D irrespective of the cytokinin present.

TABLE 1
THE EFFECTS OF SOME GROWTH REGULATORS ON THE MORPHOGENETIC RESPONSE OF *CAPSICUM* MERISTEMS CULTURED *IN VITRO* FOR TWO MONTHS AT 27±2°C, 16 H LIGHT

Growth regulators		*C. chinense* explants forming from calli				*C. annuum* explants forming from calli			
Cytokinins (2.0 mg/l)	Auxins (1.0 mg/l)	*Calli*	*Buds*	*Shoots*	*Roots*	*Calli*	*Buds*	*Shoots*	*Roots*
BA	NAA	12/24(50)*	11/24(46)	0/24(0)	4/24(17)	22/25(88)	0/25(88)	0/25(0)	17/25(68)
BA	IAA	19/25(76)	19/25(76)	10/25(40)	0/25(0)	6/31(19)	3/31(10)	0/31(0)	0/31(0)
BA	2,4-D	10/30(33)	0/30(0)	0/30(0)	0/30(0)	16/30(53)	0/30(0)	0/30(0)	0/30(0)
K	NAA	19/22(86)	0/22(0)	0/22(0)	17/22(77)	16/21(76)	0/21(0)	0/21(0)	12/21(57)
K	IAA	23/24(96)	5/24(21)	0/24(0)	0/24(0)	28/29(97)	12/29(41)	13/29(45)	0/29(0)
K	2,4-D	8/22(36)	1/22(5)	0/22(0)	0/22(0)	10/20(50)	0/20(0)	0/20(0)	0/20(0)

* Explants with response/cultured explants (%).

TABLE 2
THE EFFECT OF GROWTH REGULATORS ON THE MORPHOGENETIC RESPONSE OF *CAPSICUM ANNUUM* HYPOCOTYLS CULTURED *IN VITRO* FOR TWO MONTHS AT 27±2°C, 16 H LIGHT (1000 LUX) 8 H DARKNESS PHOTOPERIOD

Growth regulators			*Explants forming from calli*		
Cytokinins (2.0 mg/l)	*Auxins (1.0 mg/l)*	*Calli*	*Buds*	*Shoots*	*Roots*
BA	NAA	22/25(80)*	0/25(0)	0/25(0)	17/25(68)
BA	IAA	20/23(87)	2/23(9)	1/23(4)	7/23(30)
BA	2,4-D	21/33(64)	0/33(0)	0/33(0)	0/33(0)
K	NAA	22/24(92)	0/24(0)	0/24(0)	12/24(50)
K	IAA	25/28(89)	19/28(68)	0/28(0)	22/28(79)
K	2,4-D	16/20(80)	0/20(0)	0/20(0)	0/20(0)

* Explants with response/cultured explants (%).

Rooting was observed as a direct effect of NAA in both species (Table 1). IAA and 2,4-D were ineffective for the promotion of root growth in *Capsicum*. Once again, our results disagree with those previously reported. Gunay and Rao (1978) found that NAA or IAA induced rooting, while Pillips and Hubstenberger (1985) reported that all auxins tested induced root formation. These observations suggest the influence of a strong genotypic effect.

Hypocotyl segment culture

Callus production in *C. annuum* hypocotyls was the most conspicuous expression and appeared with all the combinations tested, while a poor morphogenetic response was apparent in this explant (Table 2). Phillips and Hubstenberger (1985) also found a low response in *Capsicum* hypocotyl growth in comparison with shoot apical meristems. Nevertheless, our results agree with those from Gunay and Rao (1978) and Fari and Czako (1981) when considering the effects of growth regulators in the induction of a morphogenetic response in this explant. The best performance was achieved by BA in combination with NAA or IAA. Bud induction was very limited and root formation was almost equal with both cytokinins tested combined with NAA or IAA auxins. Once more, 2,4-D seems to suppress any morphogenetic response regardless of the cytokinin present.

Callus culture and growth

It was found that *Capsicum annuum* hypocotyls easily produce callus tissue (Table 2). Within 3 weeks, a mass of callus was produced by a proliferation of the segments. Despite the fact that callus appears in all growth regulators combinations tested, the consistency was different among treatments and those growing with BA or K combined with 2,4-D gave the best callus quality (friable, loose, white and abundant). This callus tissue was maintained in the conditions stated for nine weeks in culture, after this period it turned brown and died.

It order to analyze the interaction of the cytokinins tested with 2,4-D, fresh and dry weights were evaluated. As Figs. 1a, b show, the dynamics of pepper callus shows and exponential curve of growth progress. This kind of response has been reported for callus formations in several materials (Yeoman and Macleod, 1977; Wei-hua *et al.*, 1980). The maximum multiplication rate of the callus in fresh and dry weight occurred within the 8-9 weeks. Consequently, a two month period is considered to be the best for the callus harvest with both cytokinins. A clear effect of interaction of the cytokinins tested in combination with 2,4-D was apparent in callus production and K showed to be the most effective for callus induction in pepper (Figs. 1a, b).

It has been proposed (Evans, *et al.*, 1981) that a good, friable and abundant callus culture should be obtained previous to the somatic embryogenesis.

Hypocotyl has been found as a suitable explant to attain this goal (Arrillaga *et al.*, 1986). On the other hand, K and BA combined with 2,4-D have been used as callus inductors for somatic embryogenesis (Litz, 1984; Chen and Marowitch, 1987). However, the results reported for both cytokinins are conflictive and some

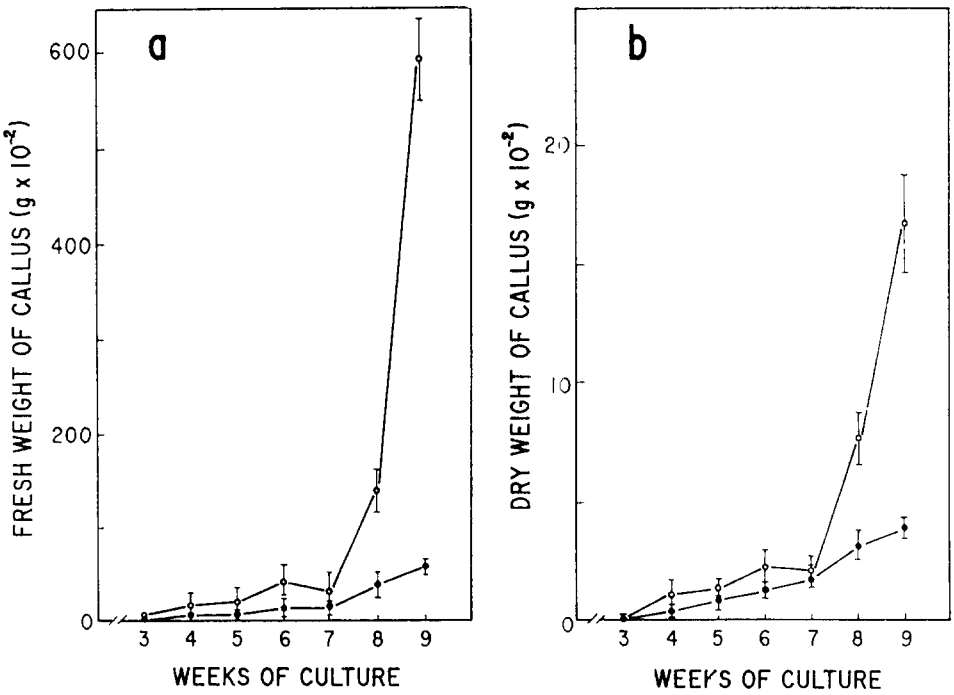

FIGURE 1. Growth curves of *Capsicum annuum* hypocotyl callus. a) fresh weight. b) dry weight. Bars represent standard error. —●— BA (2.0 mg/l) + 2,4-D (1 mg/l). —●— K (2.0 mg/l) + 2,4-D (1 mg/l).

authors obtain a better callus production with K, while others recommend BA (Litz, 1986).

Based on our data we can suggest that K combined with 2,4-D is the best way to obtain an efficient harvest of callus in *C. annuum* hypocotyl after two moths in culture, which could possibly be used as a source to attempt several *in vitro* ways to breed this important crop.

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