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In vitro plant regeneration from cotyledon and hypocotyl segments in two bell pepper cultivars

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Abstract. In vitro plant regeneration has been obtained from *Capsicum annuum* cvs. Pico and Piquillo. Shoot-buds were induced from hypocotyl and cotyledon segments after 15-20 days of culture on MS basal medium supplemented with IAA and BAP or Zeatin. Shoot-buds grew into rosettes that rooted in MS plus NAA (0.1 mg/l) and IBA (0.05 mg/l) after 15 days. The small plantlets were successfully transferred to pots with a mixture of peat and perlite and maintained under greenhouse conditions. Elongation took place when the plantlets were growing in the greenhouse.

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kn, Kinetin; MS, Murashige and Skoog (1962); NAA, alpha-naphthalenacetic acid; Z, Zeatin.

Introduction

Peppers, Capsicum spp., are used around the world in various edible forms. The great variation in size, shape, flavor and color of the fruit, as well as in plant habit, makes it difficult to devise a practical system of classification in *C. annuum*. Smith et al. (1987) reviewed the genus and proposed a horticultural classification of peppers (bell, chile, etc.).

Most of the reports on pepper regeneration from tissue culture deal with different cultivars, mainly the bell and chile types (Gunay and Rao, 1978; Phillips and Hubstenberger, 1985; Sripichitt et al., 1987; Agrawal et al., 1989; Ochoa-Alejo and Moreno, 1990). It has been shown that cultivars of a given species of Capsicum differ markedly in their regeneration requirements (Ochoa-Alejo and Moreno 1990). In addition, the developmental stage (Morrison et al. 1986) and location (Fari and Czako 1981) of the plant tissue are critical to achieve pepper shoot regeneration by tissue culture.

Cultivars Pico and Piquillo, two Spanish bell peppers of *C. annuum*, are the result of many years of careful selection and adaptation to a specific environment. They are of great economic importance in Spain in the Central Valley of the Ebro river because of their high quality. A procedure to regenerate plants from hypocotyl and cotyledon segments of these cultivars is reported in this paper.

Materials and methods

Seeds obtained from the Agriculture Research Station of Logroño (Spain) were surface sterilized by immersion in a 7.5% "Domestos" (commercial bleach) solution for 20 min, followed by three 5 minute washes in sterile distilled water. Seeds were sown on the surface of hormone-free MS medium pH 5.8, solidified with 0.7% (w/v) of Rokoagar AB-50889 (La Coruña, Spain), and germinated under 16h photoperiod at 25°C. Cotyledon and hypocotyl explants of 4-weeks-old seedlings were excised in approximately 1 cm length segments. Proximal and distal parts of the cotyledon, with the upper surface in contact with the medium, and middle to the most acropetal sections of the hypocotyl (basal part was removed), in horizontal position, were cultured in 50 ml flasks containing solidified MS medium supplemented with auxins (1 mg/l of NAA, IAA or 2,4-D) or with combinations of IAA (0.1 and 1.0 mg/l) and cytokinins (0.5, 1.0, 2.0 mg/l of BAP or Z). Cultures were maintained at 25°C under 16h photoperiod (daylight fluorescent tubes, 25 μmol/m2/s). Each treatment consisted of a minimum of 25 replicates of each type of explant. Shoot-buds produced from the isolated seedling segments were rooted in 25 ml tubes with MS medium plus NAA (0.1 mg/l) and IBA (0.05 mg/l). Rooted plants were carefully taken out of the tubes; agar rests were removed from the roots by washing them with tap water, and complete plants were transferred to pots with a mixture of peat: perlite (2:1) and grown in a greenhouse. The first fifteen days the plantlets were covered with a plastic bag.

Results and Discussion

Morphogenic responses of pepper cotyledon and hypocotyl segments, cultured in MS medium supplemented with different growth regulators have been studied for cultivars Pico and Piquillo. In both types of explants from each cultivar, the addition of NAA (1 mg/l) promoted calli and root formation. However, the two cultivars showed a differential response to the application

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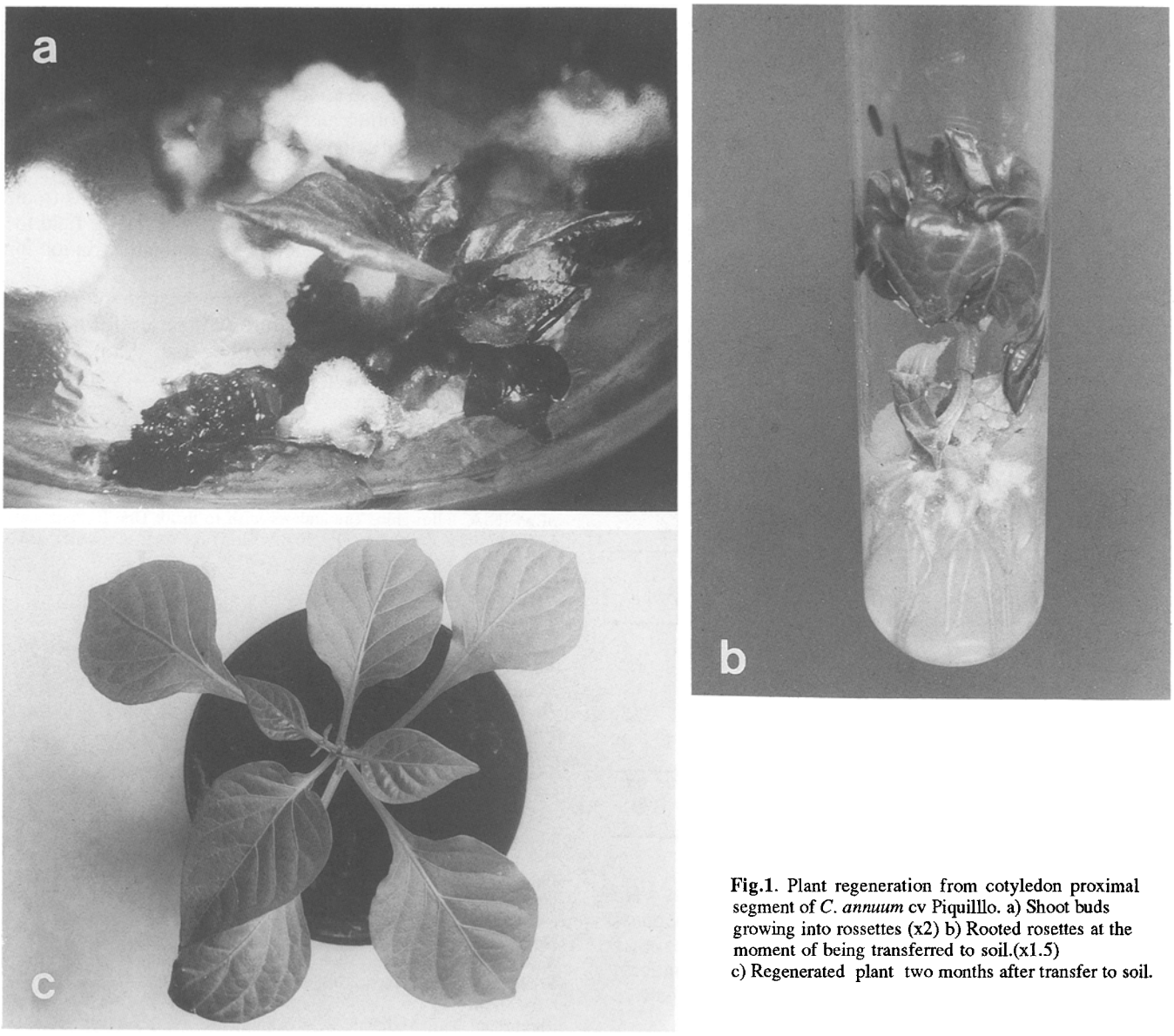

Fig.1. Plant regeneration from cotyledon proximal segment of *C. annuum* cv Piquilllo. a) Shoot buds growing into rossettes (x2) b) Rooted rosettes at the moment of being transferred to soil.(x1.5)
c) Regenerated plant two months after transfer to soil.

of IAA. This auxin induced callus formation in cultivar Pico but root formation in cultivar Piquillo. Both NAA and IAA promoted the growth of a compact calli, whereas 2,4-D developed a friable and vigorously growing callus all over the surface of the explants. From this friable callus, cell suspensions were established and grown in liquid medium MS supplemented with 2,4-D (2mg/l) and Kn (0.25 mg/l). No organogenic response has so far been obtained from these calli or cell suspension. The same result was previously reported by Phillips and Hubstenberger (1985) also with calli and cells of different type of bell and chile pepper plant.

Although regeneration of pepper plants from protoplast-derived-callus has been reported (Díaz et al. 1988), it is known for some plant species, for instance rice (Thompson et al. 1986), that plant regeneration systems established for protoplasts are not always suitable when suspension cells are used as the initial tissue.

When cotyledon or hypocotyl segments were cultured with auxins and cytokinins, shoot-buds, roots or calli were obtained. Cotyledon explants generally differentiated shoot-buds and only sporadically roots, often with callus formation. Hypocotyl explants produced mainly roots. Only the acropetal part of the hypocotyl showed the ability to differentiate shoot-buds, whereas roots were produced from both acropetal and medium parts. Proximal and distal parts of the cotyledon also showed differential regenerative behavior: 67% of proximal segments differentiated shoot-buds against 33% from distal portions. Similar differential morphogenic responses were observed by Fary and Czako (1981), in the cultivar "T. Hatvani". They suggested that an increased gradient of growth substances from the base of hypocotyl could be responsible for these differences.

The efficiency of BAP and Z in combination with IAA to induce morphogenic responses from hypocotyl and

cotyledon explants of pepper is shown in Table 1 at 65 days of culture. In terms of shoot-bud regeneration percentages, BAP at 2 mg/l plus 1 mg/l IAA was the most effective combination, followed by Z (1 mg/l) plus IAA (0.1 mg/l).

Table1. Effect of different concentrations of growth regulators on the regeneration of shoot-buds from cotyledon and hypocotyl segments of *Capsicum annuum* cvs Pico and Piquillo, after 65 days of culture. Regeneration was measured as the percentage of cotyledon (distal and proximal) and hypocotyl (apical) explants which produced shoot-buds.

Growth regulators (mg/l)	Cotyledon	
	Pico	Piquillo
IAA(0.1)+BAP(0.5)	-	0 c
IAA(0.1)+BAP(1.0)	4 c	4 c
IAA(0.1)+BAP(2.0)	37 b	15 c
IAA(1.0)+BAP(2.0)	50 a	55 a
IAA(0.1)+Z(0.5)	16 c	15 c
IAA(0.1)+Z(1.0)	45 a	40 a
IAA(0.1)+Z(2.0)	32 b	30 b
	Hypocotyl	
	Pico	Piquillo
IAA(0.1)+BAP(0.5)	0 c	0 c
IAA(0.1)+BAP(1.0)	-	10 c
IAA(0.1)+BAP(2.0)	10 c	20 c
IAA(1.0)+BAP(2.0)	60 a	70 a
IAA(0.1)+Z(0.5)	0 c	0 c
IAA(0.1)+Z(1.0)	0 c	0 c
IAA(0.1)+Z(2.0)	0 c	0 c

The significance of the differences between treaments was determinated using the chi-square analysis (Sokal and Rohlf, 1981). a,b values differ ($p<0.05$); a,c ($p<0.01$) and b,c ($p<0.01$).

Similar hormonal combinations and concentrations were found favorable for shoot formation in chile peppers (Ochoa-Alejo and Moreno 1990). However, in other cultivars ("California Wonder" and "Yolo Wonder") Kinetin and 2,4-D combinations were more effective (Phillips and Hubstenberger 1985).

Formation of the first shoot-buds occurred after 15-20 days of culture from hypocotyl explants while they took 40 days to regenerate from cotyledon explants. Hypocotyl explants showed the highest percentage of organogenesis (Table 1) and also were the best in terms of the number of shoot buds produced from each explant; four to six shoot buds were regenerated from each acropetal section that showed organogenic response. However, one to two shoot buds were developed from each cotyledon section.

Shoot-buds grew into rosettes with numerous well developed leaves but did not elongate (Fig. 1a). Several attempts trying to elongate the rosettes, such as culture in hormone free medium, reduction of cytokinin concentration, addition of gibberellic acid or dark and cold treatments were unsuccessful. However, rosettes rooted well after 15 days in MS medium plus NAA and IBA (Fig. 1b). Approximately 15% of the shoot-buds regenerated from apical hypocotyl segments rooted in the same regeneration medium while the rest of the shoot-buds rooted in medium containing NAA and IBA.

Elongation of the plants took place while growing in soil under greenhouse conditions (Fig. 1c). The survival of plants after rooting and acclimation to soil was 70%. No somaclonal variation was observed in the regenerated plants. During the flowering period, plants were covered with nylon bags to avoid cross-pollinization. Seeds from the collected fruits will be sown next year in the field to study the possible appearance of somaclonal variation in the second generation.

The development of an efficient system of pepper regeneration from tissue culture will be useful both to create somaclonal variants (Sripichitt et al. 1988), and for the genetic manipulation of this species. Experiments in this direction are currently in progress.

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