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Plant Regeneration *via* Organogenesis from Seed Explants in Red Pepper (*Capsicum annuum* L.)

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Efficient plant regeneration has been achieved *via* organogenesis in the red pepper plant (*Capsicum annuum* L.). Shoots were induced from seed explants of cultivar 'Friendship' on Murashige and Skoog's (MS) basal medium supplemented with; NAA or IAA, and BAP or zeatin. Seed explants on the medium supplemented with 0.1-0.3 mg/L IAA and 2-5 mg/L zeatin for 2 weeks vigorously formed normal shoots in more than 90% of the explants. When these were transferred to MS medium containing 0.5-1.0 mg/L GA, 90-100% of the shoots have elongated within 1-2 weeks. The elongated shoots rooted in media supplemented with 0.3 mg/L NAA. It was revealed that this method is a very rapid and efficient regeneration system for red pepper and regenerated plants can be obtained after only 5-6 weeks of culture.

Keywords: pepper (*Capsicum annuum* L.), organogenesis, plant regeneration, seed explant

Red peppers (*Capsicum annuum* L.) are economically a very important vegetable belonging to the family Solanaceae along with tobacco, tomato potato. They are used as condiments, a source of red pigment and medicine in many countries of the world. The major research fields of the pepper are the secondary metabolism of the pepper fruits, the biochemical aspects of carbohydrate metabolism of the seeds and fruit flesh, and the development of plants resistant against viruses, bacteria and fungi.

As the usual breeding methods for the improvement of disease resistance and fruit quality of the pepper requires much time and effort, molecular biological techniques have been applied to its genetic improvement (Liu *et al.*, 1990; Lee *et al.*, 1993; Oren-Shamir *et al.*, 1993; Romer *et al.*, 1993). For the selection of useful variants and regenerated plants from transformed cells, the establishment of an efficient plant regeneration system is essential. An *in vitro* study of the pepper was first reported in 1978 by Gunay and Rao on regeneration of plantlets from seedling hypocotyls and cotyledons. Since then several reports have followed (Fari and Czako, 1981; Agrawal and Chandra, 1983; Phillips and Hubstenberger, 1985; Agrawal *et al.*, 1989; Arroyo and Revilla, 1991; Ebida and Hu, 1993). And the regeneration systems *via* somatic embryogenesis have been developed subsequently (Harini and Lakshmi Sita, 1993; Jeong *et al.*, 1994; Jo *et al.*, 1996). The greatest problem in pepper regeneration is the difficulty of shoot elongation. The previous works showed that the shoots elongated only when rooting, or after rooting, in greenhouse (Agrawal *et al.*, 1989; Arroyo and Revilla, 1991). To solve this problem, Valera-Montero and Ochoa-Alejo (1992) proposed a new approach using rooted hypocotyls as explants. And later regeneration, independent of exogeneous growth regulators, was reported using precultured seed explants as explants (Ezura *et al.*, 1993). Within 4 weeks, shoots were induced and after 3 more weeks the shoots elongated.

In this study we report a faster and more efficient system for plant regeneration using seed explants to overcome the difficulty of shoot elongation.

MATERIALS AND METHODS

Plant materials

Pepper (*Capsicum annuum* cv. Friendship) seeds were surface-sterilized by soaking for 15 s in 70% ethanol and for 15 min in 1% NaOCl. Then, the seeds were rinsed three times with sterile DDW.

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The surface-sterilized seeds were precultured on filter paper wetted with DDW for 1 day then cut in two (Fig. 1), the portion (part B) consisting of the proximal part of the hypocotyl and the radicle of the embryo was used as the explant.

Shoot induction

Basal MS medium (Murashige and Skoog, 1962) with 100 mg/L myo-inositol at pH 5.7-5.8 gelled with 0.2% phytagel (Sigma) was used in the experiments for shoot induction. The growth regulators tested were NAA and IAA (0.05, 0.1, 0.3, 0.6 mg/L) as auxins, and BAP and zeatin (0.1, 1, 2, 3, 5, 10 mg/L) as cytokinins. The shoots were induced for 2 weeks.

Shoot elongation

The seed explants with adventitious buds were washed to remove the remaining medium and placed on fresh MS medium without any growth regulators, or MS medium supplemented with 0.2, 0.5, or 1 mg/L GA. Shoot elongation frequency (%) was calculated as [(explants with elongated shoots)/(explants with adventitious shoot buds)].

Root induction and acclimatization

The elongated shoots were placed on MS medium with 0.1-1.0 mg/L NAA or IAA or, without any growth regulators. Three to four weeks later, rooted shoots were placed on vermiculite wetted with Hoagland solution then potted in a 3 : 1 mixture of soil and compost.

RESULTS

Shoot induction

More efficient shoot differentiation was induced with growth regulators than with basal MS medium. The NAA/BAP, NAA/zeatin, IAA/BAP, and IAA/zeatin combinations all improved shoot induction with IAA/zeatin proving the best combination, and NAA/zeatin showing the lowest efficiency (Fig. 2).

Seed explants cultured on MS medium supplemented with 0.3 mg/L NAA and 3 mg/L BAP, showed best in a preliminary experiment using cotyledons. After 7 days the shoots began to differentiate (Fig. 3A) and grow (Fig. 3B). After more than 3 weeks of culture, the shoot induction frequency reached 81.2% and calli were also induced. The shoots were thick, multiple and rosette-shaped. The petioles did not differentiate (Fig. 3C).

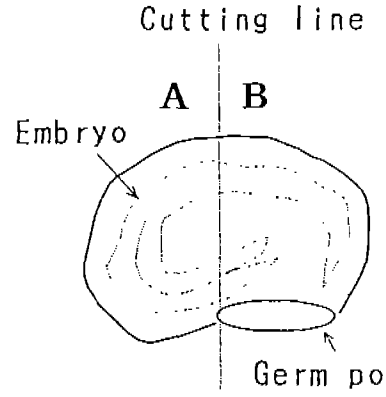

Fig. 1. Preparation of explants from mature seeds of the pepper. Part B was used for culture in this experiment (Ezura *et al.*, 1993).

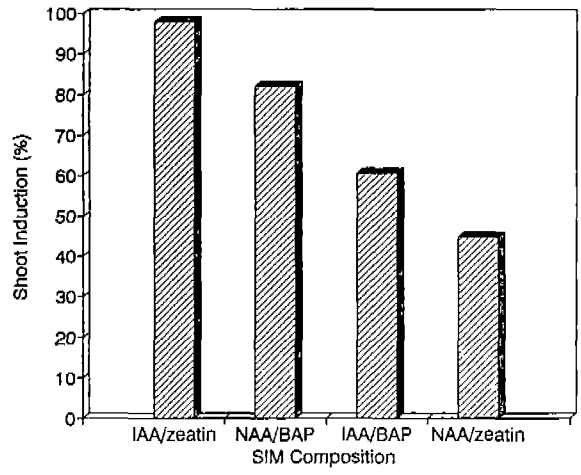

Fig. 2. Effect of shoot induction media (SIM) composition (growth regulators) on shoot induction frequency (%). The data indicates the maximum frequency among the results given from each combination of growth regulators (0.3 mg/L IAA and 3 mg/L zeatin; 0.3 mg/L NAA and 3 mg/L BAP; 0.1 mg/L IAA and 1 mg/L BAP; 0.3 mg/L NAA and 3 mg/L zeatin, from the left bar of the Figure).

In MS basal medium, the shoot induction frequency was low but there was elongation. With growth regulators the frequency was considerably higher but the petioles didn't differentiate, and elongation was retarded. As a new approach, the explants were cultured with growth regulators for a limited time and then subcultured them on MS basal medium (Table 1). As a result of that, thin shoots were induced which expanded normally, and 3-7 shoots were formed around the cut surface (Fig. 3D).

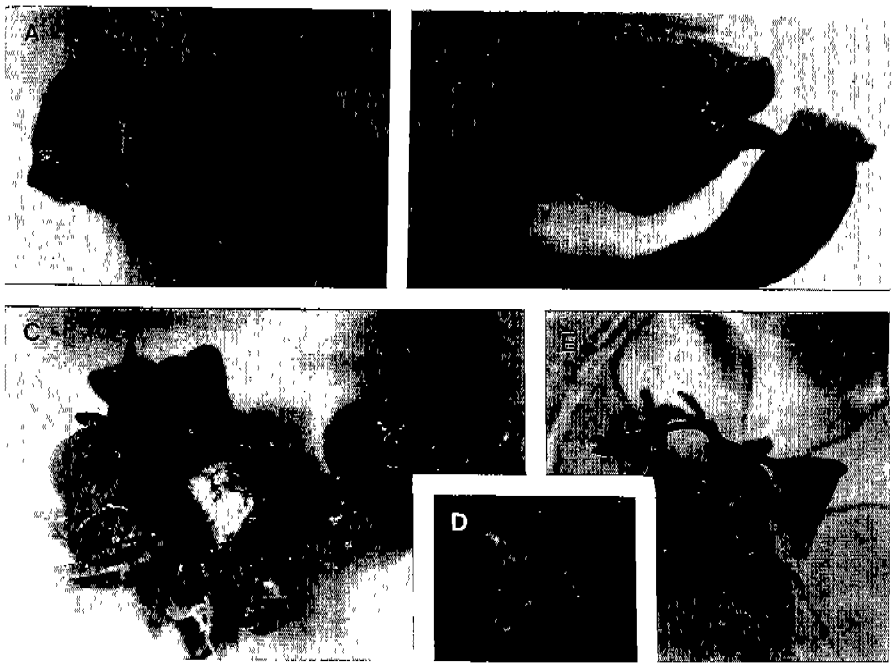

Fig. 3. Shoot induction from seed explants using NAA/BAP. A, Shoot bud induction on the cut surface of an embyro hypocotyl of seed explant after 7-day culture: B, Shoot bud growth after 10-day culture. An arrow (→) indicates regenerated shoots: C, Rosette-shaped shoots from seed explant induced using NAA/BAP after more than 3-week culture: D, Expanded shoots from seed explants. The explants were subcultured onto hormone-free MS media after culture on hormone (NAA/BAP)-containing media: E, Shoot elongation. An arrow indicates normally-formed stem.

Table 1. Frequency (%) of shoot induction from seed explants of pepper (*Capsicum annuum*) according to culture periods using NAA and BAP

NAA (mg/L)	BAP (mg/L)	Culture periods (days) on growth regulator-containing medium		
		4	7	14
0.05	1	70.6 (none)[a]	-	-
	2	5.0 (none)	-	-
	3	26.3 (none)	-	-
	5	15.0 (none)	-	-
0.1	1	20.0 (none)	-	-
	2	25.0 (none)	62.5 (13.3)	63.3 (none)
	3	42.1 (none)	51.7 (13.3)	69.4 (2.3)
	5	21.1 (none)	35.7 (none)	82.0 (2.0)
0.3	1	30.0 (none)	-	-
	2	25.0 (none)	84.4 (8.0)	41.9 (none)
	3	29.6 (none)	43.2 (none)	76.7 (10.0)
	5	42.1 (none)	39.4 (15.0)	38.7 (none)
0.6	1	-	-	-
	2	-	24.0 (16.7)	15.0 (18.2)
	3	-	39.2 (30.0)	32.0 (27.3)
	5	-	17.9 (none)	44.0 (11.1)

[a] indicates shoot elongation frequency on MS media. The data were the averages from 20-60 seed explants. The explants were subcultured onto MS medium for 2 weeks after 4, 7 or 14-day culture of growth regulator-containing media under 27°C and light condition. Standard variations for the shoot induction and elongation frequency were about ±2.8-5.4.

Table 2. Frequncy (%) of the shoot induction from seed explants of pepper (*Capsicum annuum*) using IAA and zeatin. The data were the averages ± standard variation from 80-100 seed explants after 2-week culture under 27°C and light condition

Zeatin (mg/L)	IAA (mg/L)		
	0.05	0.1	0.3
1	84.1±0.0	85.7±0.0	-
2	89.5±0.0	94.3±3.4	96.3±3.7
3	81.3±0.0	95.1±2.6	98.0±0.2
5	-	96.1±1.8	96.6±3.4

The best results were with the IAA/zeatin combination. Shoots began to differentiate 6-7 days after treatment with 0.1-0.3 mg/L IAA and 2-5 mg/L zeatin. Leaflike shoots about 10 mm-long grew out from more than 90% of the explants after an additional 1-week culture. Distinct petioles were also found (Table 2, Fig. 4A).

When the explants were cultured with 0.3 mg/L IAA, 3 mg/L zeatin and then subcultured on MS basal medium for 4 days, only 34.8% of the explants produced shoots and their growth was slow. As in the NAA/BAP combination, short term treat-

Fig. 4. Shoot induction and plant regeneration from seed explants using IAA/zeatin. A, Normally grown and differentiated shoots from seed explants. The explants were subcultured onto hormone-free media for 2 weeks after 7-day culture on hormone (IAA/zeatin)-containing media. Arrows (→) indicate well-formed petioles: B, Shoot elongation from seed explant using GA. Arrows indicate normally-formed stems: C, Rooting in MS medium containing 0.3 mg/L NAA: D. Acclimated plant: E, Regenerated plants in a greenhouse.

ment with growth regulators was not good for shoot induction. In this study, we treated the explants with growth regulators for 14 days.

Shoot elongation

When the explants with shoots induced using NAA/BAP were subcultured on MS basal medium, shoots elongated at a frequency of 8.0-30.0% within 3-4 weeks according to the concentration of growth regulators (Table 1, Fig. 3E). This was still higher than culture in only MS basal medium. Peculiarly, the shoots elongated rather better in 0.6 mg/L NAA which had caused shoot induction frequency to be low.

We tested the effects of GA on shoot elongation. After a 7-day culture with NAA/BAP, the shoots were subcultured on MS supplemented with 0.5 mg/L GA, but GA seemed to have no significant effect on shoot elongation (Table 1). To investigate GA effects in the case of IAA/zeatin, 14-day cultured shoots were subcultured on MS basal medium or MS supplemented with 0.2, 0.5, or 1 mg/L GA. In the MS basal medium, regardless of the concentration of growth regulators, none of the shoots elongated very well, but in the media containing GA, they elongated quite dramatically (Figs. 4B, 5). When the IAA concentrations were 0.1 and 0.3 mg/L, the addition of 0.5 and 1 mg/L GA, showed prominent elongation efficiencies (each frequency of elongation: 100% and 91.7%, respectively). It was also found that 2-4 shoots elongated from one explant. This indicated that the use of the IAA/zeatin combination was very effective both on shoot induction and on elongation.

Root induction and acclimatization

The elongated shoots tried to root on the MS media with 0.1-1 mg/L NAA, IAA and that without hormones (Fig. 4C). Rooting took 10-14 days. NAA induced thick roots but IAA promoted long thin

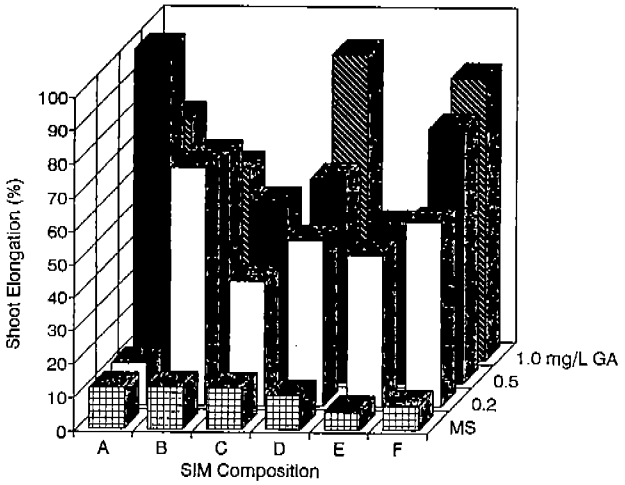

Fig. 5. Effects of shoot induction media (SIM: IAA/zeatin) and elongation media (MS or MS supplemented with 0.2, 0.5 and 1 mg/L GA) on shoot elongation. SIM (IAA, zeatin) -- A, (0.1, 2): B, (0.1, 3): C, (0.1, 5): D, (0.3, 2): E, (0.3, 3): F, (0.3, 5) (mg/L).

roots. As the unelongated shoots were tried to root, most shoots came to die and some rooted only without elongation. The most effective concentration was 0.3 mg/L NAA, and the explants with more than two shoots elongated from one explant were easily induced to root separately. New individuals which had rooted well were acclimatized and transferred to greenhouse to get F_1 seeds (Fig. 4D, E). As a result, it took only 5-6 weeks to go from seed explants to whole plants (2 weeks for shoot induction, 1-2 weeks for elongation and 2 weeks for rooting).

DISCUSSION

Very rapid plant regeneration has been achieved *via* organogenesis from seed explants in red pepper (*Capsicum annuum* L.). The promotive effects of the growth regulators are in the following descending order: IAA/zeatin > NAA/BAP > IAA/BAP > NAA/zeatin. This differs from previous reports where the IAA/BAP combination was the most effective on shoot induction (Phillips and Hubstenberger, 1985; Agrawal *et al.*, 1989; Arroyo and Revilla, 1991). Of the auxins, IAA was better than NAA and zeatin was better than BAP of the cytokinins.

Agrawal *et al.* (1989) concluded that the NAA/BAP combination was not suitable because rooting and callus formation were active only in 0.5-1.0 mg/L NAA and 0.5-5.0 mg/L BAP. However, as mentioned above, if the concentration of NAA is lowered, shoots can be induced. But, because NAA induces callus as well as shoots even at low concentrations, and later elongation is difficult, it can be suggested that IAA is a more effective auxin.

Shoot buds from seed explants were considered to be newly formed adventitious buds for the following reasons: normal shoot induction took 2 weeks, rather than 3-5 days, within which shoots could be induced from axillary buds; several shoots were induced simultaneously and after shoot induction elongation didn't appear at once, unlike axillary buds.

Shoot induction and elongation reached good levels using IAA/zeatin. In cotyledons, ill-defined stems had been formed by NAA/BAP which died shortly after (data not shown). Others, using NAA/BAP (Ebida and Hu, 1993), reported that only after the roots had first been induced, did the shoots elongate. Thus, we can conclude that shoot elongation by NAA/BAP is difficult. In the IAA/zeatin combination GA promoted shoot elongation with 0.5 or 1 mg/L GA being the most effective. Arroyo and Revilla (1991) reported that shoots grew into rosette shapes with numerous well developed leaves but did not elongate in spite of several attempts, including the addition of GA. Therefore, we think that the growth regulator composition at the time of shoot induction has much influence on shoot elongation, and that rosette-shaped shoots are hard to elongate and normal petiole formation is very important to shoot elongation. This problem of shoot elongation is critical, because there is a report saying that transformed shoots which did not elongate could not produce whole transformed plants (Liu *et al.*, 1990).

In the present experiment, we uncovered that for normal shoot induction and elongation subculturing on MS basal medium or GA-containing media after a limited time instead of continuous treatment was essential. This method is parallel to Valera-Montero and Ochoa-Alejo's (1992) using rooted hypocotyls as explants. With this approach, once shoots were formed in the inverted hypocotyls elongation was stimulated by creating a condition that mimicked the behavior of young seedlings in culture medium. That is, it can be said that the conditions conductive to root development also stimulates shoot elongation. Therefore, we can expect that if the radicles were removed from the seed explant, elongation would be inhibited. In this experiment also, the radicles were essential to shoot induction and elongation (Ezura *et al.*, 1993).

Ebida and Hu's report (1993) saying that even shoots which had not elongated, once rooted, could elongate somewhat, supports the thoughts that the

condition for root formation can stimulate the shoot elongation. The fact that shoots elongated better in 0.6 mg/L NAA in which shoot induction frequency had been low, may have been due to the high concentration of NAA remaining after subculture, which in turn, may have stimulated radicle development and concomitantly promoted shoot elongation. Putting it all together, the kinds and concentration of growth regulators, treatment duration, radicle existence and preculture greatly influence shoot differentiation in pepper plant regeneration using seed explants. This new method using seed explants has several advantages as follows; 1) explants can be easily and rapidly obtained, 2) the regenerated plants can be obtained after only 5-6 weeks of culture, which is currently the most rapid regeneration system reported, 3) the shoot induction frequency is up to 100%. With this method, we can overcome the difficulty of shoot elongation.

We feel that this pepper regeneration system can be used efficaciously in furthering pepper research including its genetic transformation.

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