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Full Length Research Paper

Adventitious shoot regeneration from leaf explants of miniature paprika (*Capsicum annuum*) 'Hivita Red' and 'Hivita Yellow'

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A simple and efficient protocol was developed for *in vitro* propagation of two miniature paprika cultivars. Seeds of miniature paprika (*Capsicum annuum*) 'Hivita Red' and 'Hivita Yellow' were decontaminated and placed in a petri dish containing a half-strength MS medium and then were incubated in the dark for 7̰̃ 10 days for germination. Leaf explants excised from one month-old aseptic seedlings were cultured on a MS medium supplemented with TDZ (0.1, 0.5, 1.0, 2.0, or 3.0 mgL^{-1}) alone or in a combination with NAA (0.1 or 0.01 mgL^{-1}) for four weeks. The highest number of regenerated shoot buds was obtained when leaf explants were cultured on a MS medium supplemented with 2.0 mgL^{-1} TDZ and 0.1 mgL^{-1} NAA with an average shoots per explant of 8.0 in 'Hivita Red' and 5.6 in 'Hivita Yellow'. Regenerated shoot buds were separated and transferred onto a MS medium without growth regulators for shoot growth and rooting. Plantlets were successfully acclimatized in a greenhouse and cultivated for three months. After about two months, they started to produce flowers and continuously produced fruits. Morphology and fruit shape of regenerated plants were normal and plants set seeds as the same as to the seed-raised plants.

Key words: cytokinin, micropropagation, organogenesis, sweet pepper.

INTRODUCTION

Fruits of miniature paprikas (*Capsicum annuum* 'Hivita Red' and 'Hivita Yellow') are used as a spice with commercial importance in many counties and popular as fresh fruits due to their high contents of water and sugars and a crispy masticating sense. There are popular hybrid sweet peppers, small in size, which ranges from 4 up to 10 cm in length. Their contents of bioactive nutrients, such as violaxanthin, capsanthin 5, 6- epoxide, zeaxanthin, lutein, β-cryptoxanthin, β-carotene (Minguez-Mosquera and Hornero-Mendez, 1993; Levy et al., 1995) and vitamins C and E (Daood et al., 2006), are three times as high as the original paprikas, thus, their importance is widely known as a well-being food. Although fruits of miniature paprikas have become very popular products, *Capsicum* spp. shows high levels of cross pollination, thus, it leads to heterogeneity in a seed population, which is also a constraint in propagation of the cultivars with desirable agronomic traits and for commercial seed production. As the propagation through seeds is further restricted by a short span of viability, low germination rates of seeds (Sanatombi and Sharma, 2006) and low productivity, the price of seeds is so high that an establishment of a plant regeneration method of these cultivars will reduce the dependence of nursery plant production on seed propagation and will contribute in both reducing seed price and producing uniform young plants. Moreover, adventitious regeneration of plants could help in the development of disease resistant

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Abbreviations: MS, Murashige and skoog medium; **BAP,** 6-benzylaminopurine; **IAA,** indole-3- acetic acid; **IBA,** indole-3-butyric acid; **TZD,** thidiazuron; **GA$_3$,** gibberellic acid; **NAA,** α-naphthalene acetic acid.

Table 1. Composition of a nutrient solution used for the culture of miniature paprika in the greenhouse.

Chemical	Concentration (mg L^{-1})		Chemical	Concentration (mg/L)
$Ca(NO_3)_2{\cdot}4H_2O$	472		Fe-EDTA	15.0
$MgSO_4{\cdot}7H_2O$	246		H_3BO_3	1.4
KNO_3	202		$CuSO_4{\cdot}5H_2O$	0.2
NH_4NO_3	80		$MnSO_4{\cdot}4H_2O$	2.1
KH_2PO_4	272		$Na_2MoO_4{\cdot}2H_2O$	0.1
			$ZnSO_4{\cdot}7H_2O$	0.8

cultivars against bacteria, fungi, virus and pest.

Plant regeneration via organogenesis in *Capsicum* spp. has been reported from diverse explants (Gunay and Rao, 1978; Agrawal et al., 1989; Valera-Montero and Ochoa-Alejo, 1992; Ebida and Hu, 1993; Ramirez-Malagon and Ochoa-Alejo, 1996; Steinitz et al., 1999; Vinod et al., 2005; Sanatombi and Sharma, 2006) and by various plant growth regulators (PGRs) (Robledo-Paz and Carrillo-Castañeda, 2004; Soniya and Nair, 2004; Golegaonkar and Kantharajah, 2006). However, regeneration in miniature paprikas using the leaf as the explant has not been reported so far. Thus, a regeneration protocol is needed by controlling PGRs to be supplemented to the medium.

In the preliminary experiments, leaf, petiole, stem and root explants were cultured on a MS medium supplemented with different types and concentrations of cytokinins, such as 2iP, BAP, kinetin and thidiazuron (TDZ). Of the different explants and cytokinins tried, only leaf explants produced shoots when cultured on a MS medium supplemented with TDZ. The thidiazuron, a substituted phenyl urea (N-phenyl-1,2,3-thidiazol-5-yl urea), is a potent plant growth regulator which exhibit cytokinin-like activity in various culture systems (Khan et al., 2006). It was successfully applied to induce organogenesis from different explants such as wounded seedlings, intact seedlings, leaf, cotyledonary nodes and shoot tips of pepper (Szasz et al., 1995; Hyde and Phillips, 1996; Dabauza and Pena, 2001; Venkataiah et al., 2003). In this study, we report an efficient system for regeneration of miniature paprikas from leaf explants using TDZ for contributing in both reducing seed price and producing uniform young plants.

MATERIALS AND METHODS

Plant materials, culture media and experimental conditions

Seeds of miniature paprikas (*C. annuum*) 'Hivita Red' and 'Hivita Yellow' were obtained from Seminis Korea Inc. Seeds were thoroughly washed in running tap water and subsequently were surface sterilized with a 70% (v/v) ethanol solution for 30 s and a 1.5% (v/v) sodium hypochlorite solution for 10 min, followed by three time rinses with sterilized water.

Seeds were germinated in a petri dish containing a 25 mL half-strength MS medium with 3%(w/v) sucrose and 0.8% (w/v) agar. Seeds were maintained for 10 - 15 days for germination, at 25 ± 2°C in darkness and then were exposed to light of 45 µmol m^{-2} s^{-1} with a light/dark cycle of 16/8 h.

Shoot induction

Leaves without petioles were excised from seedlings and cut into 1 - 2 cm long segments and explants were inoculated immediately in order to prevent drying of cut edges of explants. The abaxial sides of explants were placed down to contact the regeneration medium. For shoot regeneration, two experiments were conducted. In experiment 1, explants were cultured on a MS medium supplemented with TDZ (0, 0.1, 0.5, 1.0, 2.0, or 3.0 mgL^{-1}) alone and in experiment 2, explants were cultured in combinations of TDZ (0, 0.1, 0.5, 1.0, 2.0, or 3.0 mgL^{-1}) and NAA (0.1 or 0.01 mgL^{-1}) for four weeks. For one experiment, 25 explants were used and the experiment was repeated thrice. The percentage of explants with regenerated shoot buds and number of shoots regenerated per explant were record after four weeks.

Shoot elongation and rooting

Explants with multiple shoots and 1 - 2 mm long shoot buds were separated and transferred onto a MS medium for growth and shoot differentiation after two weeks. For one experiment, 25 shoots were used and the experiment was repeated thrice. Single shoots (about 1 cm in length) were removed from the culture and were transferred onto a fresh MS medium for rooting. All culture media used were adjusted to pH 5.8 using 1N HCl or NaOH and solidified with 0.8% (w/v) agar before autoclaving at 121°C for 15 min.

Acclimatization and fruiting

After one month, rooted plantlets were removed from the culture medium and agar was washed off gently and thoroughly under running tap water. Plantlets were then planted in acclimatization boxes containing a plug medium (Tosilee medium, Shinan Grow Co., Korea) for two weeks. The potted plants were gradually acclimatized to an *ex vitro* condition for 1 - 2 weeks after transferring to the greenhouse. A nutrient solution was supplied daily through an overhead irrigation system. The composition of nutrient solution was based on the formulation used by commercial plug greenhouses (Table 1).

Statistical analysis

A completely randomized design with three replications was used. The experimental results were subjected to an analysis of variance (ANOVA) by using the SAS program (Statistical Analysis System, V. 9.1, Cary, NC, USA).

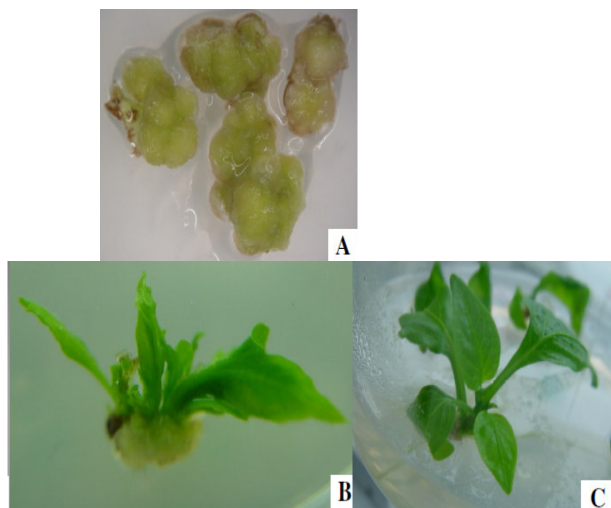

Figure 1. The established protocol of plant regeneration using leaf explants in two miniature paprika cultivars. (A) Induction of a friable greenish-yellow callus after 2 weeks culture of leaf explants; (B) induction of shoot buds after 3 weeks culture of leaf explants and (C) elongated shoot.

RESULTS AND DISCUSSION

In the preliminary experiments, various explants obtained from one month-old seedlings, such as leaf, petiole, stem and root, were tested for the shoot induction on a MS medium containing different concentrations of TDZ. Among the test, only the leaf explant showed shoot induction and other explants formed only callus (data no shown). Similar observations were also made in *Capsicum* spp. indicating that the leaf explant was more amenable to regeneration of adventitious shoots than other explants (Agrawal et al., 1989; Christopher and Rajam, 1996; Venkataiah and Subhash, 2001). The TDZ, a compound that has a high cytokinin-like activity, has been widely used to regenerate adventitious shoots of many *capsicum* spp. (Linden and Riikonen, 2006; Jones et al., 2007; Wang and Bao, 2007). This behavior is believed to be due to the potency of TDZ to increase the biosynthesis of endogenous adenine-type cytokinins (Huetteman and Preece, 1993), thus, making TDZ an effective cytokinin for stimulation of shoot bud proliferation.

In experiment 1, callus formation was observed from cut surfaces of explants in all treatments except a MS medium without TDZ. After two weeks, the entire explants were covered with mass of a friable greenish-yellow callus (Figure 1A) and then the tiny multiple shoot buds were induced from the callus after one week of culture when the medium was supplemented with 0.5 - 3.0 mgL^{-1} TDZ. However, concentration of TDZ influenced the frequency of shoot bud formation and the number of shoot buds formed per explant. A maximum numbers of shoot buds of the two cultivar 'Hivita Red' and 'Hivita Yellow' were produced in a MS medium supplemented with 2.0 mgL^{-1} TDZ (Table 1). The highest percentage shoot induction (83.3%) with a mean of 2.6 shoot per explant was obtained in 'Hivita Red', whereas in 'Hivita Yellow' only 53% of explants developed shoots with a mean of 1.5 shoots per explant. At 3.0 mgL^{-1} TDZ number of shoot buds decreased in both explants, while shoot induction percentage increased in 'Hivita Yellow'.

In general, a combination of an auxin and a cytokinin is frequently used to obtain high ratios of shoot induction. Therefore, the effect of combinations of NAA and TDZ was tested in experiment 2. Addition of NAA either at 0.01 or 0.1 mgL^{-1} significantly enhanced frequency of shoot induction and number of shoot buds regenerated per explant in both cultivars. When the MS medium was supplemented with 0.1 $mg{\cdot}L^{-1}$ TDZ, explants did not produced shoot buds in both cultivars, in contrast to that, the addition of 0.1 mgL^{-1} NAA induced shoots in both cultivars. The MS medium supplemented with 0.1 mgL^{-1} NAA and 2.0 mgL^{-1} TDZ was found to be most effective for both cultivars regarding to both number of shoots per explant (8.0 and 5.6 in 'Hivita Red' and 'Hivita Yellow', respectively), and percentage of shoot induction (93.3 and 80.0% in 'Hivita Red' and 'Hivita Yellow', respectively).

Our data showed that, although TDZ produced some numbers of shoots when used alone at low concentrations, its combination with the auxin (NAA) was far more effective in shoot induction. Furthermore, a combination of TDZ with 0.1 mgL^{-1} NAA was more effective than a combination with 0.01 mgL^{-1} NAA. Reports on the effect of NAA in combination with cytokinin in cranberry have been contradictory. Marcotrigiano et al. (1996) described a medium with 10 mM TDZ and 1 mM NAA as being the most suitable for shoot induction, whereas Qu et al. (2000) described the effect of NAA in low concentrations (0.1 mM) as being negative. However, in this experiment, a positive effect of NAA on the shoot bud induction was demonstrated. Our investigations have also revealed the determining role of the genotype in the efficiency of adventitious shoot bud formation (Tables 2 and 3). In this study, 'Hivita Red' has shown a higher shoot forming capacity than 'Hivita Yellow'.

Such genotypic differences for shoot regeneration have been reported in pepper (Christopher and Rajam, 1996; Dabuaza and Pena, 2001; Hyde and Phillips, 1996; Szasz et al., 1995; Venkataiah et al., 2003). Inhibition of shoot elongation is a common problem with TDZ and it may be consistent with its super-optimal cytokinin activity, whereas the presence of a phenyl group in TDZ may be the possible cause of shoot-bud fasciation (Huetteman and Preece, 1993; Steinitz et al., 2003). In the present study, shoot buds induced from explants on a TDZ-containing medium did not elongate and resulted in a rosette of shoots when continued to be cultured on the same medium. The TDZ sometimes stimulates shoot proliferation and inhibits their elongation. Thus, in order to elongate the shoot, regenerated shoot buds needs to be transferred to a 'secondary medium' lacking TDZ. Hence, shoot buds were separated from shoot induction medium

Table 2. Effect of different concentrations of TDZ on adventitious shoot formation from leaf explants after four weeks of culture.

TDZ (mgL^{-1})	'Hivita Red'		'Hivita Yellow'	
	No. of shoots formed per explant	Shoot induction (%)	No. of shoots formed per explant	Shoot induction (%)
0	0.0 ± 0.0c[z]	0	0.0 ± 0.0d	0
0.1	0.0 ± 0.0c	0.0	0.0 ± 0.0d	0.0
0.5	0.4 ± 0.2c	36.7	0.3 ± 0.1d	16.7
1.0	1.2 ± 0.2b	73.3	0.6 ± 0.2bc	40.0
2.0	2.6 ± 0.8a	83.3	1.5 ± 0.7a	53.0
3.0	1.4 ± 1.0b	80.0	0.9 ± 0.2b	63.3

[z]Means in columns were separated by DMRT at P = 0.05.

Table 3. Effect of different concentrations of TDZ and NAA on adventitious shoot formation from leaf explants after four weeks of culture.

PGRs (mgL^{-1})		'Hivita Red'		'Hivita Yellow'	
TDZ	NAA	No. of shoots formed per explant	Shoot induction (%)	No. of shoots formed per explant	Shoot induction (%)
0	0	0.0±0.0f[z]	0	0.0±0.0d	0
0.1	0.01	0.0±0.0f	0.0	0.4±0.1cd	20.0
0.5	0.01	0.9±0.2f	40.0	0.5±0.2cd	26.7
1.0	0.01	2.3±0.1de	76.7	1.2±0.4c	43.3
2.0	0.01	5.9±0.4b	90.0	3.5±0.2b	80.0
3.0	0.01	3.4±0.4cd	83.3	2.5±0.2b	66.7
0.1	0.1	0.3±0.1f	13.3	0.7±0.1cd	40.0
0.5	0.1	1.3±0.7ef	43.3	1.1±0.5e	43.3
1.0	0.1	3.0±0.2cd	63.3	2.9±0.5b	73.3
2.0	0.1	8.0±0.8a	93.3	5.6±0.4a	80.0
3.0	0.1	3.8±0.9c	90.0	3.5±0.8b	66.7

[z]Means in columns were separated by DMRT at P = 0.05.

and cultured on a MS medium without TDZ. After two weeks of culture elongated shoots (Figure 1B) were separated and transferred onto a fresh medium for root induction. Most frequently, however, root formation is inhibited by cytokinins which are used to induce shoots. Therefore, we transferred shoots within two weeks of culture. In general, shoots to be rooted are transferred, from a high strength medium to a low strength medium. In the present study, growth of shoot and rooting were achieved simultaneously. Therefore, we used a full strength medium. Roots initiated from the shoot base within a week and a well developed shoot (> 10 cm in length) with a root system was achieved after four weeks of culture (Figure 1C). Roots induced on a MS medium were fine and long, sometimes with branches accompanied by further elongation of shoots. Dabauza and Pena (2001) reported adventitious shoot bud formation and shoot elongation were achieved by culturing cotyledons and leaves on a medium supplemented with TDZ alone or in a combination with GA_3. Also, Eapen et al. (1998) transferred shoot buds to a medium supplemented with BA, GA_3 and IAA for elongation of shoots and rooting. Though they successfully elongated the shoot, they used auxins (IAA or NAA) for rooting; therefore, it required two different media in order to obtain complete plantlets. The effectiveness of IBA and IAA on rooting of *in vitro*-regenerated shoots of *Capsicum* spp. has been reported earlier (Agrawal et al., 1989; Christopher and Rajam, 1994, 1996; Szasz et al., 1995). Husain et al. (1999) reported higher effectiveness of NAA in inducing rhizogenesis of regenerated shoots in *Capsicum* spp. However, in the present study, number of roots induced on a MS medium without PGRs was 6 per shoot. In the present study, however, shoot elongation and rooting were achieved on a MS medium at the same time and this can reduce the labor and cost as well as a culture period.

Plantlets with well-developed shoots and roots (Figure 2A) were removed from the culture and were transplanted into the acclimatization box (Figure 2B),

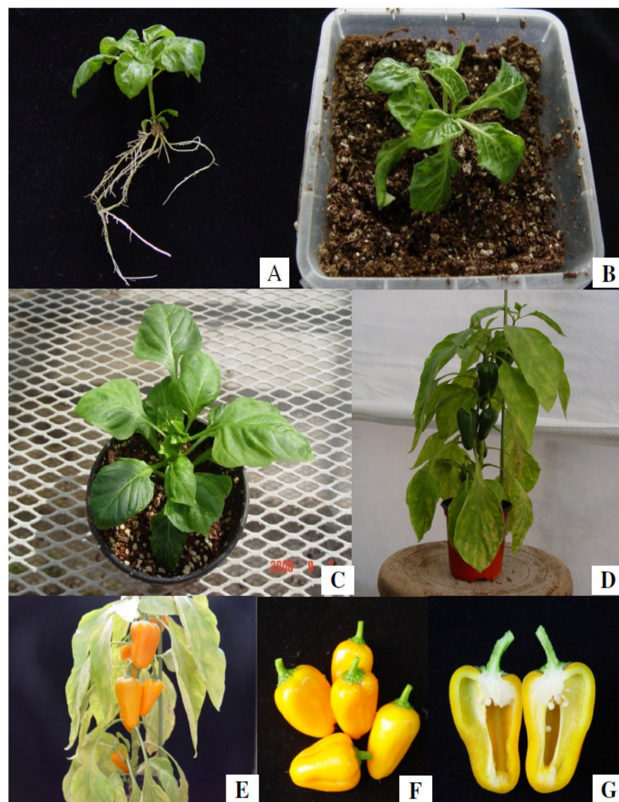

Figure 2. Regenerated plants grown in the greenhouse. (A) a plantlet having well-developed roots and shoots; (B) a plantlet transferred to a high humidity box for acclimatization; (C) a potted plant; (D) a regenerated plant with fruits; (E) colored paprika fruits; (F) fruits and (G) inside view of a fruit.

containing a plug medium and gradually acclimatized within two weeks (Figure 2C). Regenerated plantlets, after about two months, started to produce flowers and continuously produced normal green-colored miniature fruits (Figure 2D), which then changed their color to either yellow or red (Figure 2E). Morphology and fruit shape of the regenerated plant were normal and the plant set seeds as the same as compared to the seed-raised plants (Figures 2F and G). An et al. (2007) reported the annual yield of fruits in 'Hivita Red' and 'Hivita Yellow' were 10,424 kg/10a and 12,165 kg/10a, respectively, thus this regeneration protocol can be applied for the mass production of paprika plants in order to reduce the seed price and to enhance the grower's income.

The present study demonstrated a simple and efficient method for plant regeneration from leaf explants of two miniature paprika cultivars. The system was started with the initiation of a tissue culture and ended with plants fruiting in the greenhouse. The 93.3% in 'Hivita Red' and 80.0% in 'Hivita Yellow' of explants developed shoot buds on a MS medium supplemented with 0.1 mgL^{-1} NAA and 2.0 mgL^{-1} TDZ. Such a regeneration protocol would be useful for mass propagation and genetic transformation of this valuable plant species. Though, TDZ can act as an inhibitor to shoot elongation, this can be overcome by transfer of shoot cultures to a MS medium not containing TDZ. A 'primary medium' can be used to maximize shoot proliferation (Huetteman and Preece, 1993) and after a sufficient time, shoots or bud masses can lead to shoot elongation by transfer to a 'secondary medium'. After proper shoot elongation, roots developed within a week and then plantlets could be acclimatized in the green-house. The acclimatized plantlets had a normal miniature paprika fruits. This report demonstrates that adventitious shoots could be induced from leaf explants of miniature paprika cultivars. This efficient plant regeneration system via shoot organogenesis can be exploited for genetic manipulation experiments for both reducing nursery plant price and producing uniform young plants. Moreover, this protocol might be a potential system for the improvement of the genetic transformation efficiency.

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