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BRIEF COMMUNICATION

In vitro plant regeneration in six cultivars of *Capsicum* spp. using different explants

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Abstract

In vitro regeneration from leaf, cotyledon and hypocotyl explants of six cultivars belonging to three species of *Capsicum* was achieved by direct organogenesis. The cultivar Umorok showed the best response while Meiteimorok, Haomorok, Mashingkha and Uchithi showed intermediate response and the cultivar Chiengpi was the least responsive. Leaf and cotyledon explants regenerated more shoots than hypocotyl explants and the maximum number of shoots were produced on Murashige and Skoog (1962) medium containing 8.8 μM 6-benzylaminopurine (BAP) with 11.4 μM indole-3-acetic acid (IAA). Elongation of shoot buds derived from different explants was achieved on medium containing 2.8 μM IAA and the elongated shoots were rooted on medium containing 2.8 or 5.7 μM IAA and 2.4 or 4.9 μM indole-3-butyric acid (IBA). Four-week old rooted plantlets were hardened and transplanted to the soil. The plantlets showed 90 % survival during transplantation.

Additional key words: bud induction, *Capsicum annuum*, *C. chinense*, *C. frutescens*, direct organogenesis, micropropagation.

Chillies are the fruits of the genus *Capsicum* which account for about 22 % of total global spices trade composition. Besides being a source of hot spice and natural plant colour, chillies are also good sources of capsaicin and are used in pharmaceutical industries. In Manipur (23º47′ - 25º41′ NL; 93º61′- 94º47′ EL; 750-3 600 m asl; 1 600 - 3 430 mm annual rainfall), six cultivars of *Capsicum* are cultivated in different agro-climatic areas. The fruits of *Capsicum annuum* L. are generally mild and used in curried dishes while those of *C. chinense* Jacq. and *C. frutescens* L. are highly pungent and used in hot sauces. A major constraint facing the crop in the region is its seed storage due to high atmospheric humidity and the high incidence of seed-borne fungal diseases. Propagation of plants through tissue culture offers a unique advantage over conventional propagation methods such as rapid multiplication of valuable genotypes, expeditious release of improved cultivars, production of disease-free plants, non-seasonal production, germplasm conservation and facilitating their easy exchange. In chilli, several procedures are available for inducing *in vitro* plant regeneration using leaf, cotyledon and hypocotyl explants (Gunay and Rao 1978, Phillips and Hubstenberger 1985, Agrawal *et al*. 1989, Ochoa-Alejo and Ireta-Moreno 1990, Arroyo and Revilla 1991, Szasz *et al*. 1995, Christopher and Rajam 1996, Ramirez-Malagon and Ochoa-Alejo 1996, Ramage and Leung 1996, Franck-Duchenne *et al*. 1998, Husain *et al*. 1999, Venkataiah *et al*. 2003, Khan *et al.* 2006, Sanatombi and Sharma, 2007a,b). However, several of these reports suggest a strong influence of genotype on the regeneration process (Ochoa-Alejo and Ireta-Moreno 1990, Szasz *et al*. 1995, Christopher and Rajam 1996, Ramirez-Malagon and Ochoa-Alejo 1996). The present research, involving culture of leaf, cotyledon and hypocotyl explants of six chilli cultivars was undertaken to determine the regeneration potential of the genotypes and to develop efficient *in vitro* plant regeneration protocols for the six economically important cultivars.

Seeds of the six cultivars of *Capsicum* belonging to three species (*Capsicum annuum* L. cvs. Meiteimorok and Haomorok, *Capsicum chinense* Jacq. cvs. Umorok and Chiengpi, *Capsicum frutescens* L. cvs. Mashingkha and Uchithi) were extracted from fresh ripe fruits

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Abbreviations: BAP - 6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid.

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collected from local cultivation fields or market outlets and washed in distilled water containing 0.02 % *Tween-80*. Seeds were then treated with 0.1 % *Dhanustin-50* (fungicide) for 10 - 15 min and rinsed three times with distilled water. This was followed by surface sterilization with 0.1 % $HgCl_2$ (m/v) solution for 5 min and several washes with sterile distilled water. The surface-sterilized seeds were then inoculated in flasks containing sterile filter paper soaked in sterile distilled water and incubated in the dark for 7 - 10 d at 25 ± 2 °C. After germination, the seeds were transferred to culture tubes containing MS basal medium. Hypocotyl, cotyledon and leaf explants were derived from four-five week-old *in vitro* germinated seedlings.

Explants were cultured on MS medium containing MS salts with 3 % (m/v) sucrose. The pH of the medium was adjusted to 5.8 with 1 M NaOH and 1 M HCl before adding 0.8 % (m/v) agar and different concentrations or combinations of growth regulators. The media were then dispensed in culture tubes or flasks respectively and sealed with cotton plugs made of non-absorbent cotton prior to autoclaving at 121 °C for 20 min. All cultures were maintained in a growth chamber at a temperature of 25 ± 2 °C and 16-h photoperiod provided by white fluorescent tubes (irradiance of 30 μM $m^{-2}s^{-1}$).

Hypocotyl segments (1 cm long) and whole leaf or cotyledon including sometimes part of petiole, were aseptically cut and cultured in culture tubes containing bud induction medium. The leaf and cotyledon explants were placed adaxial side down on the culture media. Six bud induction media consisting of MS medium supplemented with different combinations of 6-benzyl-aminopurine (BAP) and indole-3-acetic acid (IAA) were used for shoot bud induction: *1*) 8.8 μM BAP + 5.7 μM IAA; *2*) 8.8 μM BAP + 11.4 μM IAA; *3*) 22.2 μM BAP + 5.7 μM IAA; *4*) 22.2 μM BAP + 28.5 μM IAA; *5*) 44.4 μM BAP + 5.7 μM IAA; and *6*) 44.4 μM BAP + 28.5 μM IAA.

The adventitious shoot buds induced on hypocotyl, cotyledon and leaf explants were excised from the remaining parts of the explants, cut into smaller pieces, and then cultured in flasks containing bud elongation medium consisting of MS medium supplemented with low doses of auxins, 0.3 μM IAA or 0.2 μM IBA and 2.8 μM IAA or combination of 0.3 μM IAA with 0.2 μM BAP. The number of shoots obtained from a particular explant was counted after 6 weeks.

The elongated shoot buds (about 2 cm long) were excised and cultured in flasks containing rooting media consisting of MS medium supplemented with different concentrations of auxins, 2.8 and 5.7 μM IAA; 2.4 and 4.9 μM IBA or 2.6 and 5.3 μM NAA. The number of roots (including the main roots and their branches) and the length of the roots were recorded after four weeks of culture.

The rooted plantlets were gently removed from the flasks and the roots were washed in tap water to remove traces of agar. The plantlets were then transplanted in perforated paper cups containing sand:soil (1:1) and kept covered with clear polythene bags having a few holes for the initial 10 d. The plantlets were kept in a 50 % shaded net-house and watered daily with tap water to maintain high humidity. After 10 d, humidity was gradually decreased by increasing the size of holes in the polythene bags and the polythene bags were finally removed. Four week-old hardened plants were then transplanted to bigger earthen pots or to the field.

All the experiments were repeated thrice and each treatment for shoot bud induction and rooting of the shoot buds consisted of ten replicates. Data on the number of plants obtained from the different explants and the number and length of roots were analyzed by analysis of variance (*ANOVA*) followed by Duncan's multiple range test for the number and length of roots.

The leaf, cotyledon and hypocotyl explants produced shoot buds directly on the shoot induction medium within two weeks of culture. Leaf and cotyledon explants produced buds mainly from the petiolar end while the hypocotyl explants produced buds initially from the cut ends and later from all over the surface. Initially, the buds appeared as small green bulges but later on developed into small leafy structures which elongated into shoots on the elongation medium. Analysis of variance showed a complex pattern of variation with all the effects including second and third order interactions showing significance at 0.01 % probability and the type of explant contributing maximum (63.5 %) to the total variation observed in the study followed by medium and genotype. Christopher and Rajam (1996) also reported a similar result with strong inter-explant variability (56.3 %) followed by genotype and medium in a study involving leaf, cotyledon and hypocotyl explants of seven chilli genotypes.

The cultivars Meiteimorok and Umorok responded better than Haomorok, Chiengpi, Mashingkha and Uchithi (Table 1). Difference in regeneration potential of different *Capsicum* genotypes observed in the present study is similar to earlier reports (Ochoa-Alejo and Ireta-Moreno 1990, Christopher and Rajam 1996, Ramirez-Malagon and Ochoa-Alejo 1996, Venkataiah *et al*. 2003). The effectiveness of different combinations of BAP and IAA in inducing shoot buds from various explants of *Capsicum* has been reported in several studies (Gunay and Rao 1978, Phillips and Hubstenberger 1985, Agrawal *et al*. 1989, Arroyo and Revilla 1991, Ezura *et al*. 1993, Christopher and Rajam 1994, Szasz *et al*. 1995, Ramirez-Malagon and Ochoa-Alejo 1996). Based on these reports, we tested the effectiveness of six media containing combinations of BAP and IAA in bud induction from the three explants. Among the bud induction media tested, medium 2 containing 8.8 μM BAP with 11.4 μM IAA produced the maximum number of shoot buds while media 1 and 5 produced the least number of shoot buds from all the explants of the six genotypes. Some of the regeneration systems of *Capsicum* reported so far show the critical effect of BAP alone in shoot bud induction (Sripichitt *et al*. 1987, Christopher and Rajam 1994, Szasz *et al*. 1995, Christopher and Rajam 1996). However, in the present study, medium containing BAP

Table 1. Number of shoots regenerated from different explants of *Capsicum annuum* cvs. Meiteimorok and Haomorok, *C. chinense* cvs. Umorok and Chiengpi, and *C. frutescens* cvs. Mashingkha and Uchithi on MS medium supplemented with *1*) 8.8 μM BAP + 5.7 μM IAA; *2*) 8.8 μM BAP + 11.4 μM IAA; *3*) 22.2 μM BAP + 5.7 μM IAA; *4*) 22.2 μM BAP + 28.5 μM IAA; *5*) 44.4 μM BAP + 5.7 μM IAA; and *6*) 44.4 μM BAP + 28.5 μM IAA. Mean ± SE, $n = 10$. Among the three factors (explant, genotype and media), the type of explant had the maximum (63.5 %) effect on the regeneration potential.

Cultivar	Explant	1	2	3	4	5	6
Meiteimorok	leaf	1.2 ± 0.49	14.4 ± 0.31	4.3 ± 0.85	10.2 ± 0.40	2.2 ± 0.73	6.5 ± 1.01
	cotyledon	0.3 ± 0.20	5.1 ± 1.49	3.0 ± 0.93	6.3 ± 1.08	1.0 ± 0.40	1.5 ± 0.78
	hypocotyl	-	-	0.8 ± 0.40	0.3 ± 0.20	0.2 ± 0.19	-
Haomorok	leaf	2.2 ± 0.42	3.0 ± 0.93	5.2 ± 1.43	3.3 ± 0.49	1.2 ± 0.49	-
	cotyledon	1.9 ± 0.93	1.3 ± 0.85	3.2 ± 0.47	2.0 ± 0.99	1.9 ± 0.94	1.1 ± 0.70
	hypocotyl	-	-	-	-	-	-
Umorok	leaf	1.2 ± 0.42	10.7 ± 1.92	3.8 ± 0.68	9.2 ± 0.47	2.0 ± 0.71	5.1 ± 1.49
	cotyledon	1.5 ± 0.78	8.9 ± 0.81	2.6 ± 1.05	7.8 ± 0.97	0.6 ± 0.32	2.7 ± 0.74
	hypocotyl	0.9 ± 0.37	0.1 ± 0.09	2.1 ± 0.59	1.0 ± 0.53	1.1 ± 0.61	-
Chiengpi	leaf	0.3 ± 0.20	1.7 ± 0.47	1.5 ± 0.35	4.3 ± 0.38	1.4 ± 0.43	2.0 ± 0.45
	cotyledon	-	1.4 ± 0.43	0.3 ± 0.20	1.9 ± 0.44	-	1.2 ± 0.42
	hypocotyl	-	-	-	-	-	-
Mashingkha	leaf	1.9 ± 0.56	8.3 ± 0.66	1.2 ± 0.44	3.2 ± 0.47	0.8 ± 0.34	2.2 ± 0.42
	cotyledon	0.2 ± 0.19	2.7 ± 0.74	1.0 ± 0.40	2.1 ± 0.59	0.5 ± 0.25	1.4 ± 0.47
	hypocotyl	-	-	1.4 ± 0.47	-	1.0 ± 0.40	-
Uchithi	leaf	4.0 ± 0.75	6.6 ± 0.38	2.9 ± 0.69	3.8 ± 0.68	1.5 ± 0.74	2.2 ± 0.73
	cotyledon	1.2 ± 0.42	3.3 ± 0.49	0.8 ± 0.40	1.9 ± 0.44	0.7 ± 0.15	1.2 ± 0.49
	hypocotyl	-	-	0.5 ± 0.25	-	-	-

Table 2. Effect of auxins on rooting of shoot buds (pooled from leaf and cotyledon explants) of the six chilli genotypes (*Capsicum annuum* L. cvs. Meiteimorok and Haomorok, *Capsicum chinense* Jacq. cvs. Umorok and Chiengpi, *Capsicum frutescens* L. cvs. Mashingkha and Uchithi) after four weeks of culture. Mean ± SE, $n = 10$. Means followed by the same letters are not significantly different at $P < 0.01$ according to Duncan's multiple range test.

	Auxins [μM]	Meiteimorok	Haomorok	Umorok	Chiengpi	Mashingkha	Uchithi
Root number	2.8 IAA	23.0 ± 1.10c	46.0 ± 1.08a	45.2 ± 1.69c	17.5 ± 1.29a	10.5 ± 0.77c	17.2 ± 1.40c
	5.7 IAA	24.3 ± 1.80c	29.8 ± 1.57b	24.8 ± 0.86e	5.7 ± 1.53bc	4.4 ± 0.60d	10.3 ± 1.10d
	2.4 IBA	35.8 ± 0.76b	30.8 ± 1.45b	33.2 ± 1.17d	19.1 ± 0.85a	13.9 ± 1.58c	25.1 ± 1.04b
	4.9 IBA	45.9 ± 1.92a	20.4 ± 1.03c	20.5 ± 0.93e	7.5 ± 1.70b	23.8 ± 1.73b	65.9 ± 1.91a
	2.6 NAA	19.1 ± 1.40c	25.4 ± 1.14bc	51.4 ± 1.88b	7.7 ± 0.79b	26.5 ± 1.80b	10.1 ± 1.04d
	5.3 NAA	25.6 ± 0.75b	28.0 ± 1.37b	73.0 ± 1.45a	2.1 ± 1.05c	53.5 ± 1.63a	24.7 ± 0.95b
Root length [cm]	2.8 IAA	5.4 ± 0.32b	8.0 ± 0.55a	3.8 ± 0.54b	6.3 ± 0.34a	5.5 ± 0.45a	6.8 ± 0.49a
	5.7 IAA	5.0 ± 0.45b	7.1 ± 0.52ab	6.8 ± 0.69a	2.5 ± 0.66b	4.1 ± 0.27b	4.8 ± 0.53b
	2.4 IBA	6.4 ± 0.32ab	6.7 ± 0.49ab	5.6 ± 0.38a	5.6 ± 0.38a	4.9 ± 0.19ab	4.3 ± 0.62b
	4.9 IBA	7.8 ± 0.54a	5.5 ± 0.47b	7.1 ± 0.44a	2.6 ± 0.60b	5.1 ± 0.44ab	3.8 ± 0.31b
	2.6 NAA	1.8 ± 0.15c	1.2 ± 0.08c	1.5 ± 0.6c	1.2 ± 0.19bc	1.5 ± 0.14c	0.6 ± 0.12c
	5.3 NAA	1.0 ± 0.08c	0.8 ± 0.06c	1.0 ± 0.10c	0.2 ± 0.08c	0.8 ± 0.05c	0.4 ± 0.25c

alone induced shoot buds only in leaf explants of Haomorok and hypocotyls explants of Umorok and were not used for further studies. Leaf explants were also found to be more responsive than cotyledon and hypocotyl of *Capsicum* in earlier studies (Agrawal *et al*. 1989, Christopher and Rajam 1996, Venkataiah *et al*. 2003). Similarly, better responsiveness of leaf explants than other explants has been reported in several genotypes of *Solanum* spp. (M'Ribu and Veilleux 1990, Carputo *et al*. 1995).

The shoot buds induced in the bud induction media were rosettes of small leaf-like structures that failed to elongate into normal shoots on the same medium. Chilli is considered to be recalcitrant in tissue culture at the shoot elongation stage (Liu *et al*. 1990, Steinitz *et al*. 1999) and several methods were employed for elongating the rosettes like the use of low doses of BAP and IAA (Phillips and Hubstenberger 1985, Venkataiah *et al*.

2003), phenyl acetic acid (Husain *et al*. 1999), gibberellic acid (Szasz *et al.* 1995), silver nitrate (Hyde and Phillips 1996), 24-epi-brassilinode (Frank-Duchenne *et al*. 1998) and achieving *ex vitro* elongation after transplantation (Arroyo and Revilla 1991, Ebida and Hu 1993, Hyde and Phillips 1996). In the present study, elongation of shoots was achieved on medium containing 2.8 µM IAA within two weeks of culture. Initially, the bud masses grew in size followed by rooting and elongation of shoots. The bud masses derived from a particular explant produced 2 - 16 elongated shoots in the elongation medium. MS medium containing 0.3 µM IAA with 0.2 µM BAP was found to be more effective for shoot elongation from buds induced in chilli tissue cultures (Phillips and Hubstenberger 1985, Venkataiah *et al*. 2003). However, in the present case, it resulted in callus formation around the bud masses and the buds failed to grow in size. Whereas in the medium containing 0.3 µM IAA or 0.2 µM IBA, the growth of the shoot buds was very slow and only 1 - 2 buds elongated after about ten weeks.

The excised elongated shoots (about 2 cm long) rooted in the rooting medium. Rooting of the shoot buds derived from all the three explants of the six cultivars was achieved on medium containing 2.8 or 5.7 µM IAA and 2.4 or 4.9 µM IBA. Roots induced on these media were fine and long, sometimes with branches accompanied by further elongation of the shoots (Table 2). The effectiveness of IBA and IAA on rooting of *in vitro* regenerated chilli plantlets 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 the regenerated shoots in *Capsicum*. However, in the present study, the roots induced on medium containing NAA were short, thick and numerous with fine root hairs and little growth of the shoots (Table 2).

The plantlets rooted on medium containing only IAA or IBA were used for transplantation since plantlets rooted on medium containing NAA developed poor rooting and elongation. When four-week old rooted plantlets were transplanted to paper cups, these hardened within 10 - 15 d. New apical leaves appeared and the plantlets established well when transferred to bigger earthen pots or in the field. The plantlets showed 90 % survival during transplantation.

Our study, thus, shows the critical effect of the type of explant, the genotype and the culture medium on *in vitro* regeneration of *Capsicum* species. The leaf and cotyledon explants are found to be more responsive than hypocotyl explants and MS medium containing 8.8 µM BAP with 11.4 µM IAA was the best medium for shoot bud induction. Medium containing 2.8 µM IAA was found to be the best medium for elongation of the shoot buds while media containing 2.8 - 5.7 µM IAA or 2.5 - 4.9 µM IBA induced efficient rooting of the shoot buds.

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BRIEF COMMUNICATION

Micropropagation of *Ailanthus altissima* and *in vitro* heavy metal tolerance

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Abstract

Ailanthus altissima, a fast-growing and contamination-resistant species is investigated for its use in areas contaminated by heavy metals. A micropropagation protocol for *A. altissima* was developed, cultured shoots were tested for *in vitro* heavy metals tolerance. Proliferation rate and shoot length were affected by 6-benzylaminopurine (BAP) and Murashige and Skoog's (MS) salt concentrations, best results were obtained in full strength MS medium supplemented with 1.32 or 2.64 μM BAP. Rooting percentage was strongly influenced by indole-3-butyric acid. Cultures of *A. altissima* exposed to heavy metals demonstrated a tolerance comparable to species already utilized in phytoremediation.

Additional key words: shoot culture, copper, zinc, manganese, phytoremediation.

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Ailanthus altissima Swingle is a world-wide spread tree with rapid growth rate and substantial reproductive ability that frequently out-competes native vegetation (Knapp and Canham 2000). The species is also described as a characteristic woody pioneer of disturbed sites, being able to grow in hard packed soils and polluted atmospheres (Pan and Bassuk 1986). The above observations suggest that *Ailanthus* could be employed in the reforestation of polluted areas where the introduction of other pioneer species has failed or in phytoremediation techniques.

Some species of the genus *Ailanthus* has already been used for tissue culture, *e.g. A. triphysa* (Natesha and Vijayakumar 2004), *A. malabarica* (D'Silva and D'Souza 1992) and for callus culture *A. altissima* (Zenkteler and Stefaniak 1991), but as yet no reports are available on the shoot culture of *A. altissima.*

Plant tissue culture techniques have previously been found useful in the selection of metal tolerant plants (Watmough and Dickinson 1995, Rout *et al.* 2005)

Explants obtained from shoots of a vigorous *Ailanthus altissima* Swingle tree, were washed for 10 min in water containing liquid detergent, surface sterilized by immersion (10 min) in a 50 % solution of commercial bleach, then rinsed 3 times in sterile distilled water. Explants were placed into test tubes containing Shenk and Hildebrandt (1972; SH) medium supplemented with 0.5 μM indole-3-butyric acid (IBA), 0.44 μM 6-benzylaminopurine (BAP), 100 mg dm^{-3} myo-inositol, 0.4 mg dm^{-3} thiamine, 30 g dm^{-3} sucrose, 5.5 g dm^{-3} agar (*B&V*, Parma, Italy). Medium pH was adjusted to 5.7 with 0.1 M KOH and autoclaved for 20 min at 121°C and 138 kPa. Cultures were incubated in walk-in chambers at 23 ± 1 °C and 16-h photoperiod. Light was provided by cool white fluorescent lamps at photon fluence rate of 90 ± 10 μmol m^{-2} s^{-1} at plant height.

After an initial growth, sterile shoots were transferred to *G7 Magenta* boxes (*Magenta Corporation*, Chicago, IL, USA.) containing 70 cm^3 of basal medium (BM) consisting of full strength Murashige and Skoog (1962; MS) salts supplemented with 0.5 μM IBA, 4.4 μM BAP, 100 mg dm^{-3} myo-inositol, 0.4 mg dm^{-3} thiamine, 30 g dm^{-3} sucrose, 5.5 g dm^{-3} agar. Subcultures were taken at monthly intervals.

One-node stem segments, 15 mm long, were placed horizontally in *G7 Magenta* boxes containing media derived from BM with different concentrations of MS salts (full, half and quarter strength), BAP (0, 1.32, 2.64 and 5.28 μM) and IBA (0 and 0.5 μM). After three weeks the proliferation rate and the length of new shoots were recorded.

To determine rooting ability, shoots were transferred to half strength MS media without cytokinins. Media were supplemented with 0, 1.5, 3, 6, 12 μM IBA. *Phytagel* (*Sigma*, St. Louis, USA) at a concentration of 2 g dm^{-3} was used as gelling agent. Microcuttings,

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Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; IAA - 3-indoleacetic acid; NAA - naphthalene-1-acetic acid; MS - Murashige and Skoog's (1962) medium; SH - Shenk and Hildebrandt's (1972) medium.
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