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Direct regeneration protocols of five *Capsicum annuum* L. varieties

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The bud induction obtained is a simple and efficient protocol developed for *in vitro* propagation of five varieties of cultivars. Seeds of *Capsicum annuum* L. of five varieties red, yellow, green, purple and white were decontaminated and placed in a culture bottle containing a Murashige and Skoog medium, supplemented with 6-benzylaminopurine (BAP, 5 mg/l) and naphthalene acetic acid (NAA, 1 mg/l) or indole-3- acetic acid (IAA, 0.5 mg/l) and then were incubated in the dark for 10 - 12 days for germination. Leaf explants excised from 4 weeks -old aseptic seedlings were cultured on a MS medium supplemented with hormones BAP, kinetin (Kin), the combination of BAP + Kin, BAP with NAA (0.1 or 0.01 mg/l) and BAP with IAA (0.5 mg/l). The 2.0 mg/l BAP with 0.1 mg/l NAA media was observed to be more suitable for callus formation. The highest number of regenerated shoot buds was obtained when shoot explants were cultured on a MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA. The mean number of shoot per explants was obtained in red (6.3), yellow (3.6), purple (3.3), and white (3.0) variety of *C. annuum* whereas 3.0 mg/l BAP and 0.5 mg/l IAA were observed to be more suitable for green (6.6) variety of *C. annuum.* Plantlets were successfully acclimatized in greenhouse.

Key words: *Capsicum*, auxin, cytokinin, micropropagation, organogenesis, sweet pepper.

INTRODUCTION

Capsicum is a genus of the flowering plant family Solanaceae. Its species have been cultivated in America since thousands of years, and are now cultivated worldwide. *Capsicum* consists of approximately 20-27 species from which five are domesticated - *Capsicum annuum, Capsicum baccatum, Capsicum chinense, Capsicum frutescens* and *Capsicum pubescens*. The fruits of capsicum have a variety of names like chilli pepper, red or green pepper, sweet pepper, bell pepper, miniature paprika, among others. Although, some members of family Solanaceae can easily undergo morphogenesis, red pepper was found to be recalcitrant and these reports suggest a strong influence of genotype on micropropagation and a low regeneration rate of many cultivars (Kothari et al., 2010). The various colours exhibited in *Capsicum* are due to mixture of esters of capsorubin, zeaxanthine, crytoxanthine, capsanthin and other carotenoids. These various and extractable colours

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Abbreviations: BAP, 6-Benzylaminopurine; **IAA,** indole-3- acetic acid; **Kin,** kinetin; **TDZ,** thidiazuron; **GA_3,** gibberellic acid.

of *Capsicum* fruits is extensively used in the food processing industry in wide range of products.

Capsicum is an excellent source of vitamins A, B, C and E and also rich in minerals like molybdenum, potassium, manganese and thiamine. β Carotenoids and vitamins C and A are powerful antioxidants that destroy free radicals (Simonne et al., 1997; Howard et al., 2000; Marin et al., 2004). The total antioxidants is completed by phenolic compounds, which occur in peppers in connection with sugars (Materska et al., 2003a, b). Even chilli contains seven times more vitamin C than orange. It also contains bioactive nutrients, such as violaxanthin, lutein, β-cryptoxanthin and β-carotene (Levy et al., 1995). The therapeutic properties and pungency exhibited in *Capsicum* contain capsaicinoids ($C_{18}H_{27}O_3N$) alkaloids specific for *Capsicum* genus, which show many pharmacological properties (Szolcsanyi, 2004). As medicine, it is used as counter irritant in lumbago, neuralgia, rheumatic disorders, non-allergic rhinitis, among others, thus their importance is widely known as a wellbeing food.

As propagation of plants through seeds is restricted due to low germination rates and short span of viability of seeds (Sanatombi and Sharma, 2006) and low productivity, the price of seeds are very high so the establishment of plant regeneration methods of these cultivars will reduce the dependence of nursery plant production and help to reduce seed prices and produce uniform young plants. Plant regeneration system by organogenesis in *Capsicum* has been reported from diverse explants (Gunay and Rao, 1978; Agrawal et al., 1989; Ebida and Hu, 1993; Ramírez-Malagón and Ochoa-Alejo, 1996). In this report, an efficient system for direct regeneration of bell pepper from stem and leaf explants using 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) for reducing seed prices and providing uniform young plants is reported.

MATERIALS AND METHODS

Seeds of five varieties of *C. annuum* L. (red, yellow, white, purple and green) from Allahabad seed market were properly washed in running tap water and 1.5 ml tween 20 for 10 min and a final rinse four times with sterilized water. Seeds were inoculated on MS medium with 3% sucrose which was supplemented with BAP and kinetin (Kin, 5 mg/l) in culture bottles. The pH of the culture media was adjusted to 5.8 using 1N HCl or NaOH and solidified with 0.8% (w/v) agar before autoclaving at 121°C for 15 min. Each experiment was repeated three times. After inoculation seeds were maintained in dark for 10 to 12 days for germination, at 25°C and later they were exposed to 12 h photoperiod.

Explants were collected after 4 weeks from contamination of free healthy plantlets grown in the culture bottle in the laboratory. Explants used for the experiment included leaf, shoot and root segments. Leaves without petioles, shoot and root explants were excised from seedling and cut into 1.5 cm long segments and explants were inoculated immediately in order to prevent drying of cut ends of explants. The abaxial sides of leaf explants were placed down in contact of medium. For shoot, different concentrations and combinations of BAP (0.5, 1.0, 2.0, 3.0 mg/l) and Kin (0.5, 1.0, 2.0, 3.0 mg/l) alone or in combination with NAA (0.1 or 0.01 mg/l) and IAA (0.5 mg/l) were supplemented in MS medium. The number of regenerated shoots was recorded after four weeks. The multiple shoots were separated and transferred onto MS medium supplemented with IAA (0.5 mg/l) and BAP (2.0 - 3.0 mg/l) for shoot and root differentiation. The rooted plantlets were removed from the culture medium after 1 month and agar was washed off properly and thoroughly under running tap water. Plantlets were then planted in acclimatization culture bottles containing a plug medium for two weeks. For acclimatization, the bottled plants were first covered with polythene bags to maintain humidity and holes were punctured on them every other day to decrease the humidity in the chamber. The polythene was removed completely and the pots were transferred to the green house for further acclimatization. A nutrient solution was supplied daily through an irrigation system. The composition of nutrient solution was based on the formulation used by commercial plug greenhouses (Table 1). The results were statistically analysed by Duncan's multiple range test (DMRT) in which three replications was used and found to be significant.

RESULTS AND DISCUSSION

In the preliminary experiment, explants were collected from the plantlets which were contamination of free healthy plants for direct regeneration after 4 weeks of seed inoculation. MS media were used with different combinations of hormones. The effect of the cytokinins BAP and Kin alone was studied and it was observed that the cytokinins alone did not have a significant effect on the shoot regeneration potential of the capsicum plants (Table 1). The effect of combinations of NAA and BAP or IAA and BAP was tested. Addition of IAA (0.5 mg/l) significantly enhanced the frequency of shoot induction and number of shoot buds regenerated per explant in all five cultivars. When MS medium was supplemented with 2.0 mg/l BAP alone, explants did not produce shoot buds in all five cultivars; in contrast, the addition of the auxin IAA (0.5 mg/l) induced shoots and roots in all cultivars. MS medium supplemented with 0.5 mg/l IAA and 2.0 mg/l BAP was found to be most effective for direct regeneration in the red, yellow, purple and white cultivars and the percentage of shoot induction was observed to be 88.3, 78.0, 60.2 and 52% respectively where as for the green bell pepper MS medium supplemented with 0.5 mg/l IAA and 3.0 mg/l 1 BAP gave a shoot induction percentage of 92%.

The above media combinations also had a similar effect on the number of shoots developed per explant. The mean number of shoots induced per explant in Red (Figures 1 and 2), yellow, purple and white cultivars were 6.3, 3.6, 3.3 and 3.0, respectively, whereas in green bell pepper, MS medium supplemented with 0.5 mg/l IAA and 3.0 mg/l BAP gave 6.6 mean number of shoots per explant (Table 2). Successful rooting was also obtained in the same media combination (Figures 1 and 2). The results were statistically analysed and found to be significant. The shoot explants was observed to be more amenable for regeneration of adventitious shoots and

Table 1. Effect of different concentrations of hormones on direct shoot formation after four weeks of culture.

Variety	Red capsicum	Yellow capsicum	Purple capsicum	White capsicum	Green capsicum
Treatments (mg/l)	**No. of shoots induced**				
BAP					
0.5	0.6^{f}	0.3^{g}	0.3^{fg}	0.3^{ef}	0.3^{j}
1.0	0.6^{f}	0.3^{g}	0.6^{efg}	0.3^{ef}	1.0^{hij}
2.0	1.3^{def}	1.3^{defg}	1.0^{defg}	1.0^{cdef}	1.6^{fghi}
3.0	0.6^{f}	0.6^{fg}	0.6^{efg}	0.3^{ef}	1.3^{ghij}
Kin					
0.5	0.6^{f}	0.3^{g}	0.0^{g}	0.0^{f}	0.6^{ij}
1.0	0.6^{f}	0.3^{g}	0.6^{efg}	0.6^{def}	0.6^{ij}
2.0	0.6^{f}	1.0^{efg}	0.6^{efg}	0.6^{f}	1.6^{fghi}
3.0	0.6^{f}	0.6^{fg}	0.3^{fg}	0.0^{def}	0.6^{ij}
BAP + Kin					
0.5 + 0.5	1.3^{def}	1.0^{efg}	0.6^{efg}	0.6^{def}	1.6^{fghi}
1.0 + 0.5	1.6^{def}	1.3^{defg}	1.0^{defg}	0.6^{f}	2.0^{efgh}
2.0 + 0.5	2.0^{cdef}	1.3^{defg}	1.3^{cdef}	1.6^{def}	2.6^{def}
3.0 + 0.5	1.3^{def}	2.0^{bcde}	1.3^{cdef}	1.0^{def}	1.6^{fghi}
0.5 + 1.0	1.3^{def}	1.0^{efg}	0.6^{efg}	1.3^{bcd}	1.3^{ghij}
1.0 + 1.0	1.6^{def}	1.6^{cdef}	1.3^{cdef}	1.0^{cdef}	2.0^{efgh}
2.0 + 1.0	2.3^{cde}	2.3^{bcd}	1.6^{bcde}	1.6^{bcd}	2.6^{def}
3.0 + 1.0	1.3^{def}	1.6^{cdef}	1.0^{defg}	1.3^{bcde}	2.0^{efgh}
0.5 + 2.0	1.0^{ef}	0.6^{fg}	0.6^{efg}	1.0^{cdef}	1.3^{ghij}
1.0 + 2.0	1.6^{def}	1.0^{efg}	1.0^{defg}	1.0^{cdef}	2.0^{efgh}
2.0 + 2.0	2.0^{cdef}	1.6^{cdef}	1.6^{bcde}	1.6^{bcd}	2.3^{defg}
3.0 + 2.0	1.6^{cdef}	1.6^{cdef}	1.3^{cdef}	1.6^{bcd}	1.6^{fghi}
0.5 + 3.0	1.6^{def}	1.3^{defg}	0.6^{efg}	0.6^{def}	2.0^{efgh}
1.0 + 3.0	1.6^{def}	1.0^{efg}	0.6^{efg}	0.6^{def}	2.3^{defg}
2.0 + 3.0	2.0^{cdef}	1.6^{cdef}	1.3^{cdef}	1.0^{cdef}	3.0^{de}
3.0 + 3.0	1.6^{def}	1.3^{defg}	1.3^{cdef}	1.0^{cdef}	2.3^{defg}
BAP + NAA					
0.5 + 0.01	1.3^{def}	1.0^{defg}	1.0^{defg}	1.0^{cdef}	1.6^{fghi}
1.0 + 0.01	2.3^{cde}	1.6^{cdef}	1.3^{cdef}	1.3^{bcde}	2.0^{efgh}
2.0 + 0.01	2.3^{cde}	2.0^{bcde}	1.6^{bcde}	1.3^{bcde}	3.3^{cd}
3.0 + 0.01	1.6^{def}	1.3^{defg}	1.3^{cdef}	1.0^{cdef}	2.6^{def}
0.5 + 0.1	2.3^{cde}	2.0^{bcde}	1.6^{bcde}	1.6^{bcd}	3.0^{de}
1.0 + 0.1	2.6^{bcd}	2.3^{bcd}	1.6^{bcde}	1.6^{bcd}	3.3^{cd}
2.0 + 0.1	3.3^{bc}	2.6^{abc}	2.3^{abc}	2.0^{abc}	4.3^{bc}
3.0 + 0.1	2.6^{bcd}	2.3^{bcd}	2.0^{bcd}	1.6^{bcd}	3.3^{cd}
BAP + IAA					
2.0 + 0.5	6.3^{a}	3.6^{a}	3.3^{a}	3.0^{a}	5.0^{b}
3.0 + 0.5	4.0^{b}	3.0^{ab}	2.6^{ab}	2.3^{ab}	6.6^{a}

Values are means of 3 replicates in all 5 varieties. Mean values followed by the same letters are not significantly different at p≥ 0.05 DMRT (varieties individually).

roots than hypocotyl and root explants.

The effects of different combination of plant growth regulators on *in vitro* micro propagation of capsicum annuum L. was examined by Otroshy et al. (2011). They involved culturing of nodal segment in MS media. Among the different concentrations of cytokinins, the best result was observed on medium containing 2 mg/l BAP and 0.5 mg/l IBA. The addition of an auxin IAA (0.5 mg/l) and NAA (0.1 mg/l) along with the cytokinin BAP (2.0 mg/l) gave maximum shoot induction when compared to cytokinin alone containing media. The addition of auxins along with the cytokinins also caused rhizogenesis from

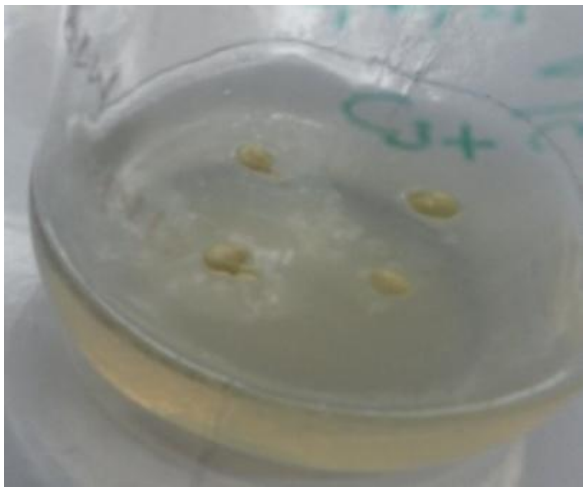

Figure 1. Seeds of capsicum inoculated on M.S medium.

Figure 2. Seed germination after 18 days.

Table 2. Composition of a nutrient solution used for the culture of miniature paprika in the greenhouse.

Solution A		Solution B	
Chemical	**Concentration (mg/l)**	**Chemical**	**Concentration (mg/l)**
$Ca(NO_3)_2 \cdot 4H_2O$	465	Fe-EDTA	16.0
$MgSO_4 \cdot 7H_2O$	246	H_3BO_3	1.3
KNO_3	202	$CuSO_4.5H_2O$	0.2
NH_4NO_3	78	$MnSO_4.4H_2O$	2.4
KH_2PO_4	277	$Na_2MoO_4.2H_2O$	0.1
		$ZnSO_4.7H_2O$	0.8

the explants after three weeks of inoculation. Literature shows that the addition of an auxin, either IAA or NAA, enhanced shoot formation (Thomas, 2002). Marcotrigiano et al. (1996) described a medium with 10 m M thidiazuron

Figure 3. Germination of contamination free plant after 4 weeks.

Figure 4. Shoot and root formation from shoot explants after 2 weeks of culture of Red *Capsicum* variety in 0.5 mg/l IAA and 2.0 mg/l BAP media.

Figure 5. Shoot and root elongation from shoot explants after 3 weeks of culture of Red *Capsicum* variety in 0.5 mg/l IAA and 2.0 mg/l BAP media.

Figure 6. Plantlet transferred to high humidity bottles for acclimatization.

(TDZ) and 1 m M NAA as being the most suitable for shoot induction. Dabauza and Pena (2001) reported adventitious shoot bud formation and shoot elongation by culturing cotyledons and leaves on a medium supplemented with TDZ alone or in combination with gibberellic acid (GA_3). Also, Eapen et al. (1998) transferred shoot buds to a medium supplemented with BAP, GA_3 and IAA for elongation of shoots and rooting. Though they successfully elongated the shoots, 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; Szasz et al., 1995). Husain et al. (1999) reported effectiveness of NAA in inducing rhizogenesis of regenerated shoots in *Capsicum* spp. However, in the present study, 5 roots per shoot were induced in BAP (3.0 mg/l) + IAA (0.5 mg/l) in green variety of *C. annuum* whereas 2.0 mg/l BAP + 0.5 mg/l IAA was good for all the other four cultivars (red, yellow, purple and white) of *C. annuum* plant. In the present study, shoot elongation and rooting were achieved on the same media and this can reduce the labour and cost as well as the culture period. Plantlets with well-developed shoots and roots were removed from the culture and were transplanted into culture bottles containing a plug medium with a nutrient solution supplied daily (Figures 3 to 6). Plants were successfully transferred to the field with 80% survival (Figure 7). Thus this regeneration protocol can be applied for the mass production of paprika plants

Figure 7. Potted *Capsicum* plant.

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 in off season also by providing them with a suitable *in vitro* environment from various explants of the five paprika cultivars.

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