International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 4, Issue 3, 2013, pp 391-396
http://www.bipublication.com

EFFICIENT *IN VITRO* REGENERATION FROM COTYLEDON EXPLANTS IN BELL PEPPER (*CAPSICUM ANNUUM* L. CV. CALIFORNIA WONDER)

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[Received-23/08/2013, Accepted-20/09/2013]

ABSTRACT

Present study was carried out to standardize a protocol for high efficiency *in vitro* plant regeneration system in *Capsicum annuum* L. cv. California Wonder using cotyledon explants. Various concentrations of 6-benzylaminopurine (BA), indole-3-acetic acid (IAA) and thidiazuron (TDZ) were used for shoot regeneration. Highest percentage of shoot regeneration (80.95%) was obtained on Murashige and Skoog (MS) medium supplemented with 6.0 mg/l BA and 0.3 mg/l IAA. Initiation of shoots was observed after about 20 days on this concentration of hormones. The regenerated shoots were in form of profuse rosettes which underwent fair elongation of 2-2.5 cm on transfer to MS medium supplemented with 2.25 mg/l BA and 2 mg/l gibberellic acid (GA_3). *In vitro* induction of roots occurred after 10-12 days of transfer of shoots to root regeneration medium. Half-strength MS medium containing 0.25 mg/l IAA gave the highest percentage (50%) of *in vitro* root induction. Plantlets showed successful acclimatization on hardening. Standardization of an efficient *in vitro* regeneration protocol could be helpful in carrying out various genetic modifications in this economically important crop.

Keywords: *Capsicum, in vitro regeneration, cotyledon, plant growth hormones, callus*

INTRODUCTION

Capsicum has been a part of human diet since about 7500 BC [1]. Apart from being used as a basic ingredient in a great variety of food all over the world, it serves as an indispensable spice in various cuisines [2]. Vivid colours exhibited by this crop are extensively used in the food processing industry [3]. Therapeutic properties of *Capsicum* to treat various disorders is attributed to the presence of a group of alkaloids called capsaicinoids [4].

Besides being an important economic crop, the propagation of agronomic traits in *Capsicum* suffers from many constraints due to cross-pollinating behaviour of this crop. Also, short viability span and low germination rate pose limitations for propagation through seeds [5].

Genetic engineering for obtaining desirable traits in bell pepper is dependent upon reliable means of *in vitro* plant regeneration. There are some available reports on *in vitro* regeneration of pepper plants from different explants [6-13]. Earlier studies have vividly indicated various inherent problems associated with *in vitro* regeneration of *Capsicum* for e.g recalcitration with respect to morphogenesis, formation of rosette shoots or ill-defined shoot buds and genotye/explant dependence. These problems alone or together jeopardize the whole tissue culture efforts [14]. So, it gets necessary to optimize *in vitro* propagation protocols for specific cultivars to apply recombinant DNA technologies aimed at genetic improvement against pests, diseases and abiotic stresses.

The present work was undertaken to obtain efficient *in vitro* plant regeneration using cotyledon explants of *Capsicum annuum* L. cv. California Wonder.

[II] MATERIALS AND METHODS

2.1 Plant material

Seeds of bell pepper (*Capsicum annuum* L. cv. California Wonder) were procured from the Department of Vegetable Science, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan.

2.2 Culture medium

Murashige and Skoog (MS) medium supplemented with 100 mg/l myo-inositol, 3% sucrose and 0.8% agar-agar was used as basal medium [15]. The pH of the medium was adjusted to 5.8.

2.3 *In vitro* germination of seeds

The seeds were firstly given a brief wash with teepol and then imbibed for an hour in water. Thereafter, seeds were kept in 0.1 % $HgCl_2$ solution for 2-3 min and then thoroughly washed (3-4) times with sterilized distilled water. The seeds were inoculated on half-strength MS basal medium for *in vitro* germination. 15 days old aseptically grown seedlings were used as source of explant.

2.4 *In vitro* plantlet regeneration

MS basal medium supplemented with different combinations and concentrations of auxins and cytokinins, viz., 6-benzylaminopurine (BA), indole-3-acetic acid (IAA), thidiazuron (TDZ) and gibberellic acid (GA_3) was used for *in vitro* regeneration studies (Table 1, 2, 3 and 4). For *in vitro* shoot induction, cotyledon explants were excised from 15 days old aseptically grown seedlings. These explants were cut into small pieces of 1-1.5 cm size and inoculated on shoot regeneration medium (Table 1 and 2). For proper growth, all cultures were kept in 16 h of light/8 h of dark period at 25±2 ˚C. After induction of shoots, the cultures were evaluated for percentage shoot regeneration. For adequate elongation, the shoots were transferred to shoot elongation medium (Table 3). The elongated shoots were transferred to root regeneration medium (Table 4) and the percentage root regeneration was recorded.

2.5 Hardening

After *in vitro* development of the complete plantlets, they were taken out of the culture vessels. Taken proper care, the roots of the plantlets were washed to remove the adhering medium. The plantlets were planted in cocopeat mixture and high humidity was maintained for 8-10 days by covering them with transparent polybags. When the plantlets showed initial signs of establishment after 8-10 days of transfer to plastic pots, polythene bags were punctured. After 13-14 days, plantlets were left uncovered overnight and after 21 days the polythene bags were removed. Subsequently, the plantlets were transferred to potting mixture comprising of sand, soil and farm yard manure after 25 days.

2.6 Statistical analysis

The data recorded on different parameters were subjected to completely randomized design (CRD) [16]. The statistical analysis based on

mean value per treatment was made using analysis of variance of CRD.

[III] RESULTS AND DISCUSSION

Cotyledon segments taken from 15 days old aseptically grown seedlings showed growth of cells on the cut edges after 8-9 days, marking callus initiation. Initially, calli were greenish in colour, but as they grew old without induction of shoot buds, the colour changed from green to cream (Fig. 1A). Also, it was observed that if the shoot buds developed during initial stages of callus proliferation the overall percentage of shoot regeneration was more but the chances of shoot regeneration decreased during later stages, as the callus grew old. As a control experiment, cotyledon explants were cultured on the medium containing no growth regulators. No shoot regeneration was observed in control.

Induction of adventitious shoot buds took place after about 20 days on MS medium supplemented with 6.0 mg/l BA and 0.3 mg/l IAA (Fig. 1B) and after 20-25 days on MS medium containing 3.5 mg/l BA and 0.2 mg/l IAA. It was observed that shoot bud induction took place in nearly all the concentrations of BA and IAA which were tested (with major variations in average number and percent shoot regeneration) but regeneration of shoots was best observed (80.95%) on MS medium containing 6.0 mg/l BA and 0.3 mg/l IAA (Table 1). As compared to TDZ-IAA, the combination of BA-IAA gave better results. BA has earlier been used by Szasz *et al.* (1995) for shoot induction [17].

Shoot induction was observed after 30-35 days in culture on MS medium supplemented with TDZ-IAA. For this combination, the highest percentage of regeneration (53 %) was obtained on MS medium containing 2.20 mg/l TDZ and 0.25 mg/l IAA (Table 2).

Very little elongation of the shoots was observed after 3 weeks on MS medium supplemented with 5.0 mg/l BA, 0.3 mg/l IAA and 2.0 mg/l GA_3 and the shoots were still profuse rosettes. In the absence of IAA from the elongation media (i.e MS medium containing 2.25 mg/l BA and 2.00 mg/l GA_3), a fair shoot elongation of upto 1-1.5 cm was observed after about 15 days and upto 2-2.5 cm after about 25 days (Fig. 1D, Table 3).

Table:1. Effect of different concentrations of BA and IAA (in MS medium) on shoot regeneration from cotyledon explants of bell pepper (*Capsicum annuum* L. cv. California Wonder).

S. No.	MS medium containing BA + IAA (mg/l)	Percentage of shoot regeneration
1.	2.0 + 0.3	31.25 (33.99)*
2.	2.5 + 0.3	46.25 (42.85)
3.	3.5 + 0.2	76.47 (60.98)
4.	4.0 + 0.2	68.06 (55.59)
5.	4.5 + 0.1	77.39 (61.61)
6.	5.0 + 0.3	71.67 (57.84)
7.	6.0 + 0.3	80.95 (64.12)

$CD_{0.05}$ (0.24)

SE± (0.11)

* The figures in the parenthesis are arc sine transformed values

In the rest of concentrations, a poor elongation response was observed. In contrast to the results obtained by Qin *et al.* (2005), extensive callusing was obtained on medium containing 5.0 mg/l BA,

0.3 mg/l IAA and 1.0 mg/l GA_3 which suppressed the growth of already emerged shoots [19]. It was observed that if the shoot obtained after indirect organogenesis was a profuse rosette right from its emergence, its elongation was difficult to achieve but if the shoot emerged bearing well-defined leaves, it showed a fair elongation in the presence of GA_3 in the MS medium. Ranjan *et al.* (2010) have reported an increase in length of shoot with increase in BA and GA_3 levels [20].

Table: 2. Effect of different concentrations of TDZ and IAA (in MS medium) on shoot regeneration from cotyledon explants of bell pepper (*Capsicum annuum* L. cv. California Wonder).

S. No.	MS medium containing TDZ + IAA (mg/l)	Percentage of shoot regeneration
1.	0.88 + 0.10	24.00 (29.33)*
2.	1.10 + 0.15	30.00 (33.21)
3.	1.76 + 0.20	28.00 (31.95)
4.	2.20 + 0.25	53.00 (46.72)
5.	3.30 + 0.30	50.00 (45.00)

$CD_{0.05}$ (1.12)

SE± (0.50)

* The figures in the parenthesis are arc sine transformed values

Table: 3. Effect of different concentrations of BA and GA_3 on *in vitro* shoot elongation of bell pepper (*Capsicum annuum* L. cv. California Wonder).

S. No.	MS medium containing BA + GA_3 (mg/l)	Shoot elongation
1.	2.00 + 1.50	+
2.	2.25 + 2.00	++
3.	3.00 + 1.00	+

\+ Elongation

++ Good elongation

In vitro root regeneration of the elongated shoots was obtained on half-strength MS medium containing 0.25 mg/l IAA after 10-12 days. On increasing the concentration of IAA to 0.5 mg/l root induction was delayed by one week. The formation of complete plantlet with well-developed root and shoot system was observed after 25-30 days of transfer of shoots to medium containing 0.25 mg/l IAA (Fig. 2A). It was observed that after *in vitro* root induction, the callus formation at the base of the shoot turned black and ultimately died with the elongation of root in due course of time.

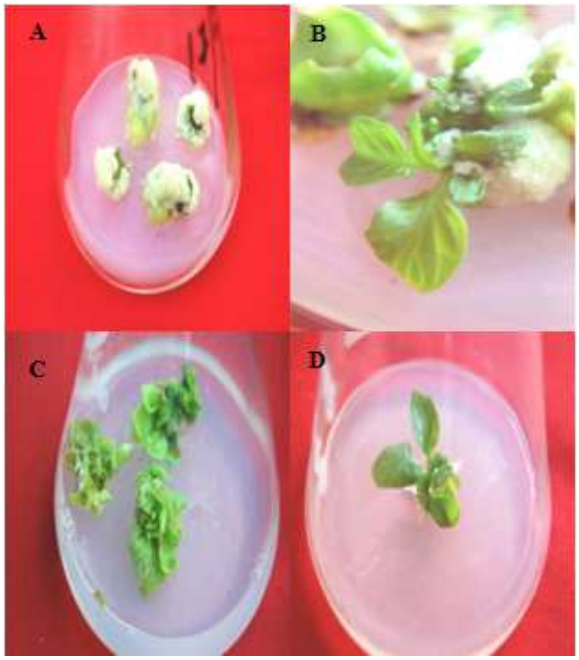

Fig: 1 (A-D). *In vitro* regeneration and elongation of shoots.

(A) Cotyledon explants showing callus proliferation after 20-25 days of culture on MS medium containing 0.88 mg/l TDZ and 0.10 mg/l IAA.

(B) Development of leaf like structures without the formation of rosette on MS medium containing 6.0 mg/l BA and 0.3 mg/l IAA after 25-30 days in culture.

(C) Development of profuse rosette like structures without clear distinction of leaves on MS

medium containing 3.5 mg/l BA and 0.2 mg/l IAA after 40-42 days in culture.

(D) E longated shoots (2-2.5 cm) on MS medium containing 2.25 mg/l BA and 2.0 mg/l GA3 after 25 days of transfer.

Table: 4. Effect of different concentrations of IAA on root induction of *in vitro* regenerated shoots of bell pepper (*Capsicum annuum* L. cv. California Wonder).

S. No.	Half-strength MS medium containing IAA (mg/l)	Percentage of root regeneration
1.	**0.50**	**20**
2.	**0.25**	**50**

Fig: 2 (A-D). *In vitro* rooting of shoots and hardening of *in vitro* regenearted plantlets.

(A) Development of plantlets with well-established root system after 25 days of transfer to half-strength MS medium containing 0.25 mg/l IAA.

(B) Fully developed plantlet of bell pepper taken out of culture medium after 25 days.

(C) *In vitro* regenerated plantlets kept for hardening in pre-sterilized cocopeat mixture covered with transparent polythene bags to maintain humidity at 0 day.

(D) Acclimatized plantlets transferred to potting mixture containing soil, sand and farm yard manure.

Successful growth and acclimatization of the *in vitro* regenerated plantlets was observed on transplantation to cocopeat mixture and subsequently to a mixture of sand, soil and farm yard manure (Fig. 2C and Fig. 2D). The acclimatized plants showed 90% survival.

A high efficiency *in vitro* plant regeneration protocol has been standardized in *Capsicum annuum* L. cv. California Wonder. The advantage of this system offers interesting perspectives for the introduction of some desirable genes in this economically important species by exploitation of recombinant DNA technology aimed at genetic improvement.

ACKNOWLEDGEMENT

The authors would like to thank Prof. and Head, Department of Vegetable Science, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan for the germplasm of bell pepper (*Capsicum annuum* L. cv. California Wonder) .

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