

Micropropagation of Two Varieties of Bell pepper (*Capsicum annuum* L.)

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Abstract

Cotyledonary leaf and node of two varieties, namely red and yellow of Bell pepper (*Capsicum annuum* L.) were cultured in MS with different concentrations and combinations of plant growth regulators. The cotyledonary leaf was found to be most responsive towards multiple shoot regeneration. The leaf explants of red variety showed best result towards shoot regeneration in MS with BAP (8.0 mg/l), NAA (0.02 mg/l) and IAA (1.0 mg/l). Eighty per cent explants responded within 13 - 14 days, and the mean number of shoot per explant was found to be 5.67. The cotyledonary leaf explants of yellow variety showed promising result when the cotyledonary leaf explant was cultured in MS with BAP (8.0 mg/l) and NAA (0.5 mg/l) being the mean number of shoot per explants 5.13 and 70% of them responded within 14 - 15 days. Maximum root induction was found in MS with IBA (0.5 mg/l) in both capsicum varieties. After successful root formation plantlets were transplanted and acclimatized to the soil.

Introduction

Capsicum annuum is a herbaceous flowering plant and belongs to Solanaceae. The genus *Capsicum* contains about 20 species. Of them, five are recognized widely (Sanatombi and Sharma 2007). *C. annuum* is one of the most important vegetable crops globally and commonly known as pepper, sweet pepper or bell pepper. Different colored bell peppers, namely red, green, yellow, brown, purple, etc. are available as indispensable vegetable kitchen item as well as in other form condiment and spice, adding colour, flavor, zest, and piquancy various foodstuffs. It is an excellent source of vitamins A, B-complex, C, E, and is rich in minerals like molybdenum, manganese, phosphorus, and potassium (Saleh et al. 2018).

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Capsicum is an essential summer crop of temperate regions, but nowadays, the effort is being made to grow sweet pepper in Bangladesh (Mahmud *et al.* 2017). It has great demand all over the country. Some advanced farmers grow capsicum sporadically to meet the need of the periphery of cities (Saha and Salam 2004) which is not enough to meet the increasing demand. Moreover, pepper seeds characteristically have short viability and rapidly lose germination capacity. The crop is susceptible to environmental factors, and its yield is affected significantly (Halim and Islam 2013). Micropropagation is a unique tool that is very convenient for maintaining the genetic homogeneity of the elite plant materials and preserving heterozygous genotypes and genetically transformed forms (Dabauza and Pena 2001). The genus *Capsicum* is recalcitrant regarding its *in vitro* regeneration potential, which makes it difficult or inefficient to apply recombinant DNA technologies via genetic transformation aimed at genetic improvement against pests and disease (Ashrafuzzaman *et al.* 2009). A number of studies have been carried out on the regeneration of bell pepper globally and in Bangladesh. A number of experiments were followed to demonstrate the effectiveness of plant micropropagation in different species of pepper (*Capsicum* spp.) varies depending on the plant genotype, the type of initial explants, and the factors and conditions of *in vitro* cultures (Dabauza and Pena 2001). Multiple shoot induction and plant regeneration from nodal explants of *C. annuum* were reported by Kumar *et al.* (2011), Hegde *et al.* (2017).

To overcome the major constraints of recalcitrant response of bell pepper (*C. annuum*), the present investigation was carried out. This work presents an efficient and reproducible *in vitro* regeneration protocol for two varieties, namely red and yellow bell pepper in Bangladesh.

Materials and Methods

The seeds of both red and yellow varieties of bell peppers were collected from the local market, and used for germinating plantlets which were further used as explants. The cotyledonary leaf and node were used as explants. The same sterilization procedures described by Khan *et al.* (2018) was followed. This work was conducted in the Tissue Culture Section, Biological Research Division of Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka.

Preparation of the explants from *in vitro* growing seedling was carried out. Different concentrations and combinations of plant growth regulators (PGRs) such as BAP, IAA, NAA and Kn were used in MS to develop multiple shoots. For induction and development of root, about 3 - 4 cm long shoots were separated and cultured on freshly prepared full and half strength of MS with different concentrations (0.25 - 1.0 mg/l) of IBA. All media contained 3% sucrose and 0.8% agar or gel rite with 5.8 pH adjusted before autoclaving. All cultures were maintained in 16 hrs photoperiod at 25 ± 2°C.

The plantlets with sufficiently developed roots were taken out from the culture vessels, and the roots were washed under running tap water. The plantlets were then

transplanted to small pots containing sterilized soil. Three weeks after transplantation, when the regenerated plants were fully established in the small pots, they were transferred to larger pots for further growth and development.

Result and Discussion

The plant growth regulators, namely auxin and cytokinin are critical for plant regeneration in tissue culture (Hill and Schaller 2013). Usually, high cytokine in to auxin ratio is beneficial for shoot regeneration (Phillips and Hubstenberger 1985). Therefore, the influence of various concentrations of cytokinin (BAP) alone or with auxin (IAA or NAA) in MS was studied on shoot induction and their development in bell pepper. The fundamental goal of *in vitro* culture of bell pepper was to develop a reliable protocol for achieving plant regeneration from both varieties using explants cotyledonary leaf and cotyledonary node. Bhutia et al. (2016) observed that among the explants (hypocotyl, cotyledon, nodal, and shoot tip), shoot tip and cotyledon explants performed the best of shooting frequency and shoot number. Similar observations were also reported in five varieties of Capsicum (Sawmy et al. 2014). In this study, the best response was observed from the cotyledonary leaf explants of both types (Tables 1 and 2).

Table 1. Responses of explants towards shoot regeneration on MS with different combinations of BAP, NAA and IAA on red variety of *C. annum*.

Name of explants	PGRs supplements (mg/l)			No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoot / explants after 3 months)
	BAP	NAA	IAA				
CL	4.0	0.02	0.5	20	60	16-18	3.93
	4.0	0.02	1.0	20	65	19-20	3.47
	6.0	0.02	0.5	20	60	20-21	4.60
	6.0	0.02	1.0	20	70	20-21	4.93
	8.0	0.02	0.5	20	75	12-13	5.33
	8.0	0.02	1.0	20	80	11-12	5.67
CN	4.0	0.02	0.5	20	60	17-18	1.47
	4.0	0.02	1.0	20	60	16-18	1.93
	6.0	0.02	0.5	20	70	20-21	1.73
	6.0	0.02	1.0	20	65	20-21	2.26
	8.0	0.02	0.5	20	60	12-13	2.67
	8.0	0.02	1.0	20	75	12-13	3.06

CL = Cotyledonary leaf, CN = Cotyledonary node.

Different concentrations of BAP, IAA and NAA were used in MS to determine the optimum media composition for the initiation and development of multiple shoots from both explants of the red variety. The results of the experiments are shown in Table 1. MS containing 8.0 mg/l BAP, 0.02 mg/l NAA, and 1.0 mg/l IAA exhibited the best response

towards shoot initiation and their multiplication from both the explants (Table 1). The best response was recorded from cotyledonary leaf where direct shoot induction started within 11 - 12 days from the base of leaf explants (Fig. 1a) followed by shoot bud

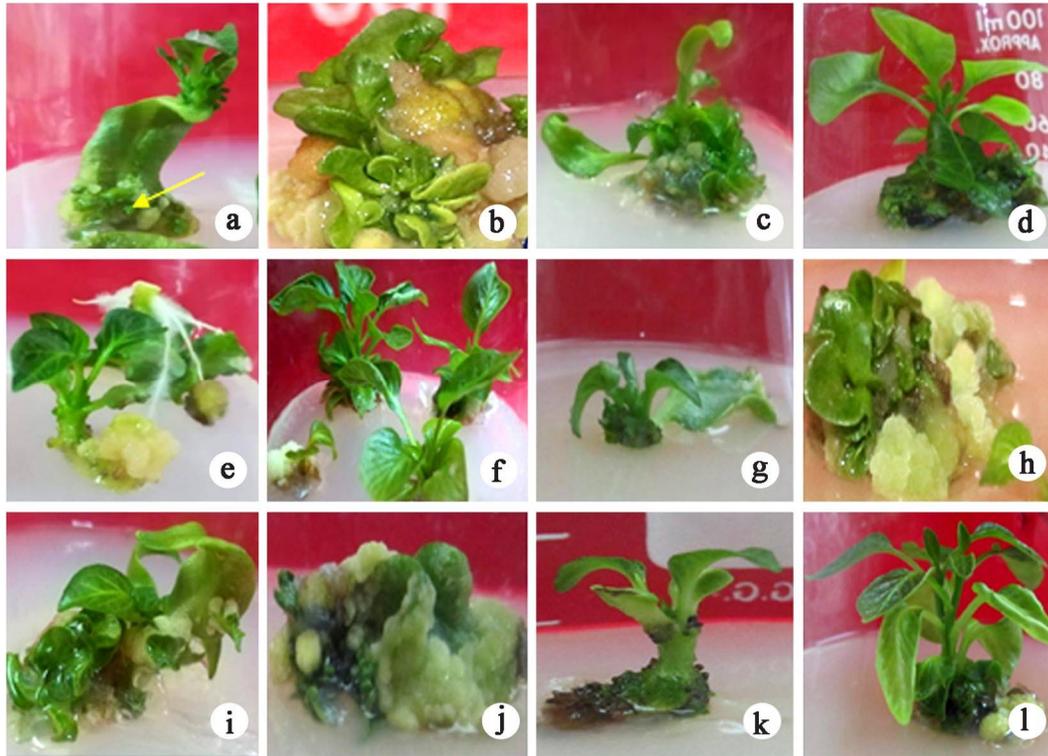


Fig. 1. Different stages of regeneration from cotyledonary leaf and nodal explants of both red and yellow variety of *C. annuum*. (a) Initiation of direct shoots from cotyledonary leaf of red variety. (b) Formation of sessile leaves from leaf explant on same media combination and variety as in Fig. a. (c) Proliferation of a few shoots from leaf explants. (d) Elongated shoots on MS with 0.5 mg/l IAA. (e) Shoot regeneration from nodal explants of red variety on the same media combination as mention in Fig. a. (f) Elongated shoots from nodal explants of red variety. (g) Initiation of shoots from the base of leaf explants of yellow variety. (h) Formation of leaves from leaf explant on same media combination and variety as in Fig. g; (i) Multiple shoots on MS with 0.5 mg/l IAA. (j) Formation of callus rather than shoot induction on same media as in Fig. g. (k) Formation of shoot from nodal explant of yellow variety on same media as in Fig. g. (l) Elongated shoots from nodal explants of yellow variety.

induction and shoot regeneration. It required 10 - 12 weeks to grow fully mature shoots. Sometimes callus was produced from the cut end of the leaf, but the callus did not show any sign towards shoot formation (Fig. 1a). Similar observations were also made in *Capsicum* spp. by Venkatiyan *et al.* 2001 indicating that the leaf explant was more amenable to the regeneration of adventitious shoots than the other explants.

Table 2. Responses of explants towards shoot regeneration on MS with different combinations of BAP and NAA on yellow variety of *C. annuum*.

Name of explants	PGRs (mg/l)		No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoot / explants (after 3 months)
	BAP	NAA				
CL	4.0	0.5	20	20	16-17	3.20
	4.0	1.0	20	15	16-18	3.66
	6.0	0.5	20	15	17-16	3.93
	6.0	1.0	20	25	17-16	4.33
	8.0	0.5	20	70	15-16	5.13
	8.0	1.0	20	55	15-16	4.80
CN	4.0	0.5	20	20	16-17	1.27
	4.0	1.0	20	15	16-17	1.40
	6.0	0.5	20	10	17-16	1.80
	6.0	1.0	20	15	17-16	2.33
	8.0	0.5	20	55	15-16	2.40
	8.0	1.0	20	60	14-15	2.67

CL = Cotyledonary Leaf, CN = Cotyledonary Node.

In combination of BAP, NAA and IAA cotyledonary leaf explants of red variety showed 80% shoot initiation with maximum (5.67) mean number of shoots as against 3.06 in node explants (Table 1). Borychowski et al. (2002), Ashrafuzzaman et al. (2009) observed the highest number of shoot regeneration on MS in the combination with 5.0 mg/l BAP with 1.0 mg/l IAA from cotyledon and hypocotyl explants, respectively.

However, Raj et al. (2015) and Kumar et al. (2011) found best result by using MS with BAP (5 mg/l) and IAA (0.1 mg/l) from leaf and cotyledons explants, respectively. In the present experiment, calli were found to develop from the leaf explants. Only adventitious leaves were often formed from the explants that were not appropriately elongated (Fig. 1b). Orłinska and Nowaczyk (2014) found the formation of clusters of sessile leaves during organogenesis induction. Very often, only a few shoots were elongated even if enough multiple shoots were formed (Fig. 1c). For proper elongation GA₃ was added in the subcultured of multiple explants, but no significant elongation was observed. But, when the multiple shoots were subcultured on MS with 0.5 mg/l IAA better elongation was observed compared to regular media combinations of BAP, NAA, and IAA (Fig. 1d). On the other hand, MS with BAP (8.0 mg/l), NAA (0.02 mg/l) and IAA (1.0 mg/l) also showed the best response in cotyledonary node explants. In node explants of red variety lower number of shoots was found to form than leaf explants. Seventy five per cent of explants responded towards regeneration within 13 - 14 days (Fig. 1e), and the

mean number of shoot per explants was 3.06 (Fig. 1f). Ashrafuzzaman *et al.* (2009) found the highest number of shoots from cotyledon in MS with BAP (5.0 mg/l) and IAA (1.0 mg/l).

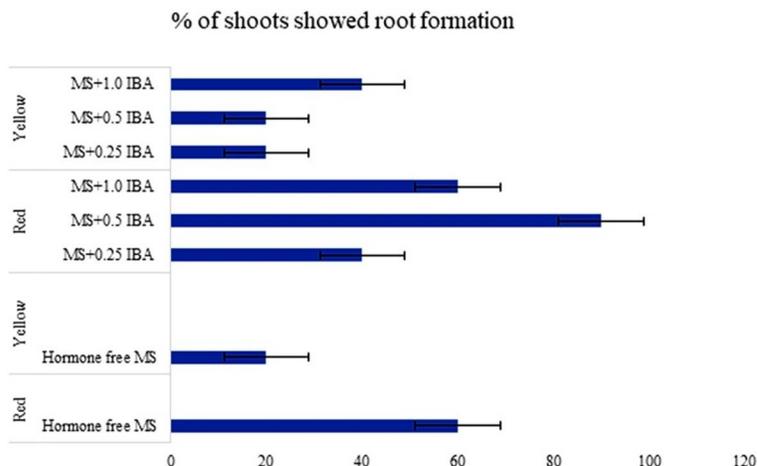


Fig. 2. Effect of the different media and PGR (IBA) conditions on root induction from the *in vitro* regenerated shoots of red and yellow variety of *C. annuum*.

In yellow variety of bell pepper, among the various combinations of BAP (4.0 - 8.0 mg/l) and NAA (0.5 - 1.0 mg/l) cotyledonary leaf explants showed better response than cotyledonary node (Table 2). Song *et al.* (2010) also got the best result from leaf explants of the "Hivita Yellow" variety of Capsicum. In this experiment, the most promising results towards the shoot regeneration were recorded from cotyledonary leaf explants when they were cultured in MS supplemented with BAP (8.0 mg/l) and NAA (0.5 mg/l). In this combination of BAP and NAA 70% explants responded towards shoot initiation within 14 - 15 days (Fig. 1g) and the mean number of shoots per explants was recorded 5.13 within three months of culture. Like the red variety leaf explants produced huge number of sessile leaf rather than uniform shoots (Fig. 1h). The multiple shoots were subcultured in MS with IAA (0.5 mg/l) for their elongation. On IAA (0.5 mg/l) supplemented media, moderate elongation was observed (Fig. 1i). Occasionally huge callus was formed from the leaf explants of yellow variety (Fig. 1j). But from the callus, no shoots were found to regenerate. Song *et al.* (2010) reported that the highest number (5.6) of regenerated shoot buds was obtained when leaf explants were cultured on an MS with TDZ (2.0 mg/l) and NAA (0.1 mg/l N) in 'Hivita Yellow' variety. The combination of BAP (8.0 mg/l) and NAA 0.5 mg/l on MS cotyledonary node also exhibited better response than all other combinations (Table 2). By using the cotyledonary node, as explants 60% of explants showed shoot initiation (Fig. 1k). Cotyledonary node explants

produced a comparatively lower mean number (2.67) of shoots than leaf explants. Generally, no callus was found to have formed from cotyledonary node.



Fig. 3. Formation of roots and transplantation of plantlets of *C. annuum* from *in vitro* regenerated shoots. (a) Formation of roots on MS with 0.5 mg/l IBA of red variety; (b) same as Fig. (a) but in yellow variety; (c) transplantation of red variety in plastic pot; (d) acclimatization of plantlets of yellow variety; (e) mature plants of red variety in soil.

Occasionally a small amount callus was produced at the base of shoots (Fig. 11). Arous et al. (2001) observed maximum regeneration in *C. annuum* with 5.0 mg/l BAP and 1.0 mg/l NAA using cotyledon explants. Otroshy et al. (2011) obtained best results on MS containing BAP (6.0 mg/l) and IBA (1.0 mg/l) from cotyledon explants of *C. annuum*. Swamy et al. (2014) also obtained a satisfactory result when they cultured cotyledon in MS with BAP (2.0 mg/l) and NAA (0.5 mg/l) with an average shoots/explant of 3.6 in yellow variety. Further they reported an average of 6.3 mean number of shoots/expants the same media combination, in red variety of Capsicum. Present study supports the observation of Swamy et al. (2014), where the red variety showed a better regeneration response than the yellow type.

For induction and development of roots, freshly prepared PGRs free MS and MS with different concentrations of IBA (0.25 - 1.0 mg/l) were used. It was observed that roots initiated in PGR-free MS and MS with all the concentrations of IBA from the two varieties of *Capsicum* (Fig. 2). In the present study, the best response towards root induction was observed on MS with IBA (0.5 mg/l) in two bell pepper varieties (Fig. 3 a,b). In contrast, Pishbin *et al.* (2014) and Hasnat *et al.* (2007) used half of the MS basal medium containing 1 mg/l IBA. Alongside, Renfiyeni *et al.* (2017) found best medium for root induction, and elongation was WPM (Woody Plant Medium supplemented with 0.4 mg/l NAA). Furthermore, Song *et al.* (2010) used MS without PGRs for rooting *C. annuum* 'Hivita Red' and 'Hivita Yellow' variety. The rate of survivability of the transplanted plantlets was found to be 85% in red variety (Fig. 3c) as against 55% in yellow variety (Fig. 3d). The plantlets matured in 2 - 2.5 months (Fig. 3e).

The present study demonstrated a simple, efficient and reproducible direct shoot regeneration system for two locally available capsicum variety using cotyledonary node and cotyledonary leaf explants. Cotyledonary leaf was found more competent towards shoot induction. This system could be recommended for future gene transformation protocols with useful candidate genes.

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