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In vitro morphogenesis and plant regeneration response from hot pepper (*Capsicum annuum* L.) cultivar Punjab Guchhedar

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ABSTRACT

An efficient and reproducible *in vitro* plant regeneration protocol was developed in hot pepper (*Capsicum annuum* L.) cv. Punjab Guchhedar. Effects of explant type, age and plant growth regulators were investigated on direct shoot regeneration. The explants *viz*., cotyledon and cotyledonary node of ages 8, 16, 24 and 32-days were cultured on Murashige and Skoog (MS) basal medium supplemented with cytokinins and auxins. The highest shoot regeneration was obtained from 24-day-old cotyledons cultured on MS basal medium containing 9.5 mg l^{-1} BAP, 3.0 mg l^{-1} Kin and 1.0 mg l^{-1} IAA. The frequency of shoot induction varied according to the age of explant and ratio of growth regulators. It is also observed that the effect of BAP was more pronounced for regeneration in hot pepper. Maximum number of shoots (11.75 per explant) was obtained on 24-day-old cotyledon explant on the same medium. Root induction was obtained on half-strength MS basal medium supplemented with 0.5 mg l^{-1} IBA. Four-week-old rooted plantlets were hardened and transferred to pots.

Key words: Hot pepper, *Capsicum annuum*, *In vitro*, cotyledon.

INTRODUCTION

Hot pepper, *Capsicum annuum* L. is an economically important vegetable and spice crop of India. It is a good source of vit. B complex, vitamins A and C (Ochoa-Alejo and Ramirez-Malagon, 7). Despite the economic importance of pungent hot pepper, efficient reproducible plant regeneration protocols have not progressed as compared to other Solanaceous crops because of their great capability to regenerate *in vitro*. In case of *Capsicum* species, it is difficult-to-regenerate whole plants from explants under *in vitro* conditions. The information on plant regeneration from established callus lines, cell suspensions and protoplasts is scarce, suggesting severe recalcitrant nature of *Capsicum* species (Ochoa-Alejo and Ramirez-Malagon 7). Some workers, (Christopher and Rajam, 3; Hira Singh *et al.*, 4) have achieved organogenesis in *Capsicum annuum* from different explants using various cultural media and *in vitro* conditions. A reproducible and highly regenerative *in vitro* protocol is a pre-requisite before a tissue culture system is used in crop improvement programmes for gene insertion and expression.

Punjab Guchhedar is one of the main cultivar grown in the state and its fruits are rich in capsaicin (0.98%) and deep red in colour, which makes it suitable for drying and powder. Fruits also have destalking habit which leaves the stalk while picking. It is tolerant to fruit rot and highly resistant to mosaic virus, and yields 150 q/ha. Hence, keeping these factors in view, this study was undertaken to develop rapid and efficient plant regeneration protocol for hot pepper cv. Punjab Guchhedar.

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MATERIALS AND METHODS

The cultures were started with aseptic germination of Punjab Guchhedar seeds on MS basal medium solidified with 0.8% agar and pH was adjusted to 5.8 before gelling with agar. Cotyledon and cotyledonary node explants were excised from *in vitro* grown seedlings of different age (8, 16, 24 and 32 days) cultured on modified MS medium with different concentrations and combinations of plant growth regulators, *i.e.* cytokinins and auxins. The modified media were supplemented with sucrose (3.0%) and gelled with agar (0.8% w/v). Eight MS based media supplemented with different combinations of 6-benzyl-aminopurine (BAP), kinetin (Kin), α-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) (Table 1) were used for shoot bud induction. All the media were sterilized in autoclave at 121°C for 20 min. All cultures were incubated in the growth room at temperature 25 ± 2°C with 16/8 h light/dark cycle. Sub-culture was carried out with an interval of three weeks.

Well developed shoots were excised and transferred to basal MS medium to omit the effect of growth regulators. The *in vitro* elongation of shoots and induction of rooting was obtained as per method standardized by Hira Singh *et al.* (9). Plantlets having well developed shoot and root system were transferred to liquid half-strength MS medium and were kept under high light intensity (about 5000

Table 1. Various MS based medium compositions used for plant regeneration in hot pepper.

Medium	Hormonal level (mg l^{-1})			
	BAP	Kin	NAA	IAA
MR_0	0.00	0.00	0.00	0.00
MR_1	3.00	0.00	0.00	0.50
MR_2	3.00	0.00	0.50	0.00
MR_3	5.00	2.00	0.00	1.00
MR_4	6.00	3.00	0.00	1.00
MR_5	7.50	3.00	0.00	1.00
MR_6	9.50	3.00	0.00	1.00
MR_7	11.00	3.00	0.00	1.00

lux) for 3-4 days followed by washing of roots to remove adhering agar. Plantlets were then placed on cotton moistened with sterilized water. The hardened plantlets were transferred to the soil in polythene bags and kept in glasshouse. To check fungal infection, the plants were initially watered with Bavistin® solution (0.1%). After 10-15 days, the plants were transferred to earthen pots. Each treatment consisted of 30 cultures and replicated thrice. The data recorded on per cent survival / subsequent regeneration and average number of shoots per explant were subjected to analysis of variance (ANOVA) to test the statistical significance (Panse and Sukhatme, 8).

RESULTS AND DISCUSSION

Cotyledonary explants excised of different ages were cultured in medium supplemented with various combinations and concentrations of auxins and cytokinins. No regeneration was recorded in the basal MS medium. The maximum regeneration (39.58%) was obtained on medium (MR-6) having 9.5 mgl^{-1} BAP, 3.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA (Fig. 1). Maximum plant regeneration was observed in explant collected from 24-day-old seedlings (Fig. 3). In almost all the media, profuse callus formation was observed. The combination of NAA and BAP resulted in formation of large, compact and green coloured callus on whole cotyledon which failed to regenerate into plantlets. Replacement of NAA with IAA resulted in less callus formation and significantly more regeneration. Combination of BAP, Kin and IAA was observed best for hot pepper regeneration. Earlier studies also showed that the optimal shoot regeneration medium varied with cultivar and growth regulators (Husain *et al.*, 5; Ochoa-Alejo and Ramirez-Malagon, 7).

It was also reported that the effect of BAP was more pronounced in hot pepper *in vitro* regeneration. Hence, with increase in BAP concentration upto 9.5 mgl^{-1}, a significant increase in plant regeneration was recorded. This may be due to increase in the activity of cell division. Further increase in the concentration, led to significantly decline in regeneration. The explant collected from 24-day-old seedlings showed

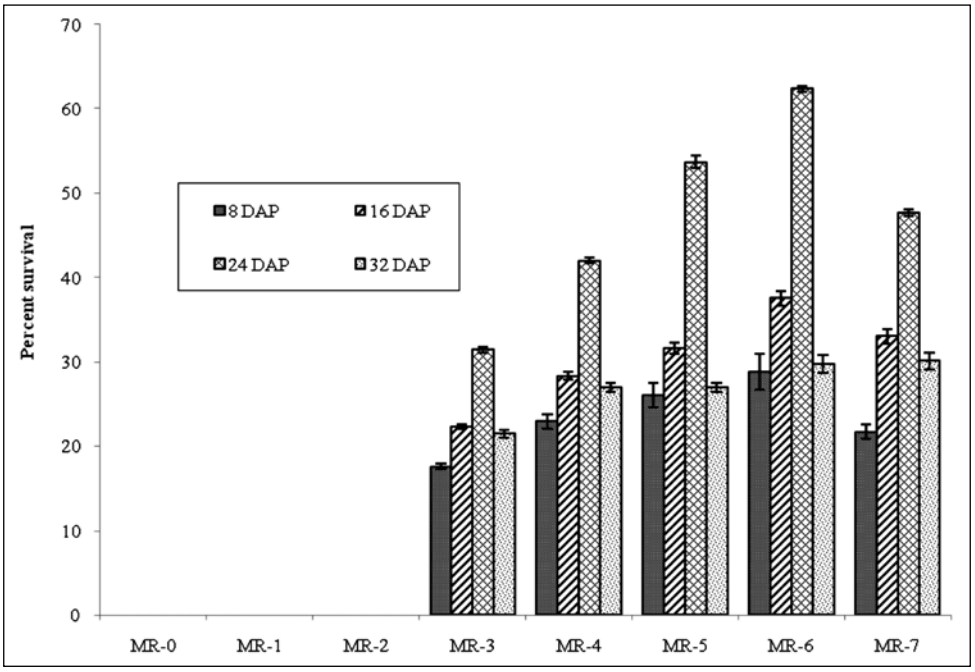

Fig. 1. Effect of medium composition and age of explant on per cent survival and subsequent shoot regeneration from cotyledon explant.

significantly better response than other seedling ages. The per cent shoot regeneration recorded from 24-day-old seedlings was 29.66 followed by 16-day-old seedlings (19.09). Similar, studies by Christopher and Rajam (3) have shown that 21-day-old cotyledon explant gave the best response.

The data pertinent to the number of shoots regenerated per explant are presented in Table 2. Shoots were obtained on all the media except MR-O, MR-1 and MR-2 having low BAP concentration (0-3.0 mgl^{-1}) and without Kin. The maximum number of shoots per cotyledon explant (11.75) was recorded on MR-6 medium (Fig.3 b & c) having 9.5 mgl^{-1} BAP, 3.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA. The shoot bud induction from cotyledons was strongly dependent on the addition of BAP and Kin in the culture medium. The cytokinins play a crucial role in the induction of organogenesis as observed in many pepper tissue culture experiments. Cytokinins in the culture media dramatically increased both the percentage of explants forming buds as well as number of buds per explant, and also hastened the rate of bud production (Binzel *et al.*, 2; Ochoa-Alejo and Ramirez-Malagon, 7). Shoot bud induction from cotyledons of pepper required higher concentration of cytokinins as reported by many workers (Husain *et al.*, 5; Ochoa-Alejo and Ramirez-Malagon, 7; Kumar *et al.*, 6). It is clearly indicated that colyledonary explant was the best explant for regeneration.

Plant regeneration from cotyledonary node explant was observed in all the media except MR-2 supplemented with 3.0 mgl^{-1} BAP and 0.50 mgl^{-1} NAA (Fig. 2). This medium led to formation of compact and green callus at the base of explant which failed to regenerate. Similar observations had already been reported by Husain *et al.* (5). Maximum plant regeneration (69.30%) was obtained on MR-6 having 9.5 mgl^{-1} BAP, 3.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA followed by MR-5 medium having 7.5 mgl^{-1} BAP, 3.0 mgl^{-1}Kin and 1.0 mgl^{-1} IAA. The concentration of BAP >9.5 mgl^{-1} was found detrimental for plant regeneration along with 3.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA. Maximum regeneration was observed in 16-day-old seedling explants followed by 24-day-old seedlings. Likewise, other workers have reported that 15-day-old seedlings are the best for collection of explants like cotyledonary nodes (Arous *et al.*, 1), hypocotyl and cotyledonary leaf (Kumar *et al.*, 6) in pepper.

Shoot bud regeneration appeared in about 10-18 days of culture in case of cotyledonary node explant. All the media elicited shoots except MR-0 (MS Basal) and MR-2 (Table 3). Combination of BAP-Kin-IAA has enhanced significantly their number. Further increase in cytokinins concentration above the optimum level resulted in reduction of the number of shoots owing to inhibiting effects of higher doses of cytokinins. In contrast to this, Arous *et al.* (1) obtained buds from cotyledonary nodes with low cytokinin concentration,

Table 2. Number of shoots regenerated from cotyledon explants of hot pepper cv. Punjab Guchhedar (values mentioned as replication Mean ± SE).

Explant	Age of explant (days)	MR_0	MR_1	MR_2	MR_3	MR_4	MR_5	MR_6	MR_7
Cotyledon	8	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	2.4 ± 0.10	3.7 ± 0.11	4.6 ± 0.12	7.0 ± 0.14	3.8 ± 0.12
	16	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	2.6 ± 0.06	4.7 ± 0.11	5.8 ± 0.13	8.1 ± 0.11	4.7 ± 0.16
	24	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	3.6 ± 0.07	5.6 ± 0.12	6.2 ± 0.07	11.7 ± 0.12	7.1 ± 0.11
	32	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	2.3 ± 0.13	3.5 ± 0.13	3.7 ± 0.14	7.8 ± 0.10	3.1 ± 0.12

Table 3. Number of shoots regenerated from cotyledonary node explant of hot pepper cv. Punjab Guchhedar on MS medium supplemented with different combinations and concentrations of cytokinins and auxins (values mentioned as replication Mean ± SE).

Explant	Age of explant (days)	MR_0	MR_1	MR_2	MR_3	MR_4	MR_5	MR_6	MR_7
Cotyledonary node	8	0.0 ± 0.00	1.5 ± 0.10	0.0 ± 0.00	2.0 ± 0.08	2.5 ± 0.11	3.1 ± 0.08	3.9 ± 0.10	2.2 ± 0.09
	16	0.0 ± 0.00	2.1 ± 0.15	0.0 ± 0.00	3.3 ± 0.20	4.2 ± 0.08	5.2 ± 0.08	6.6 ± 0.23	3.3 ± 0.08
	24	0.0 ± 0.00	2.0 ± 0.10	0.0 ± 0.00	2.3 ± 0.09	3.2 ± 0.07	4.0 ± 0.07	4.1 ± 0.12	2.8 ± 0.09
	32	0.0 ± 0.00	1.8 ± 0.08	0.0 ± 0.00	2.1 ± 0.10	3.5 ± 0.09	3.6 ± 0.09	5.2 ± 0.11	2.5 ± 0.13

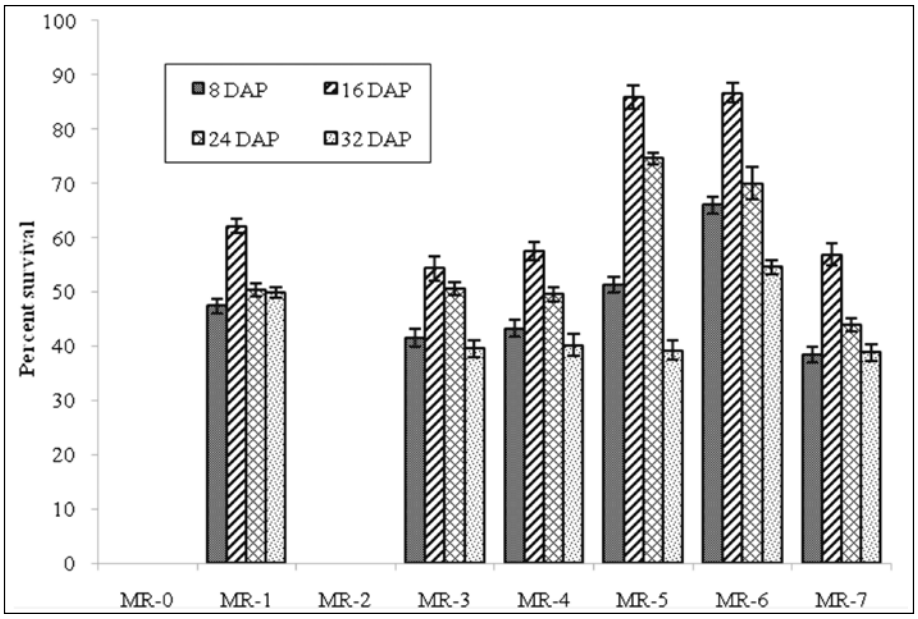

Fig. 2. Effect of medium composition and age of explant on per cent survival and subsequent shoot regeneration from cotyledonary node explant.

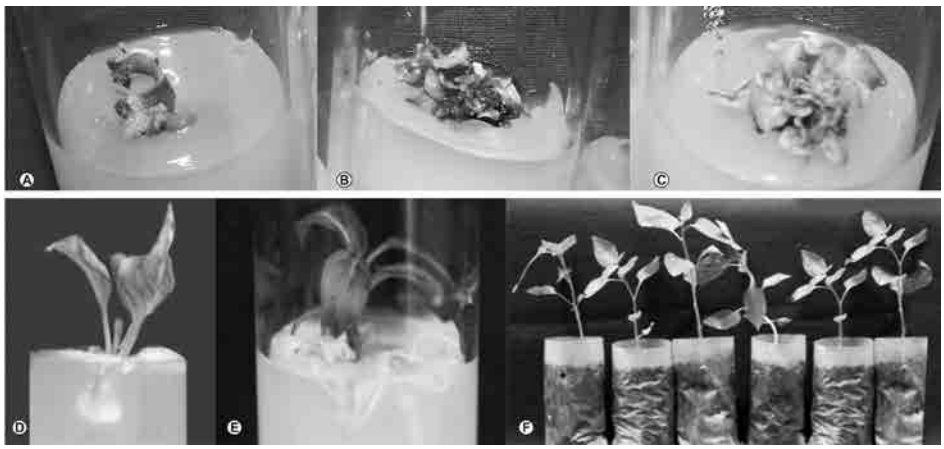

Fig. 3. (a-f) Various stages of *in vitro* plant regeneration in hot pepper cv. Punjab Guchhedar; (a) Survival of explant on regeneration medium; (b & c) Induction of multiple shoots regeneration from explant cotyledon; (d) Elongation and root induction; (e) Plantlets with well developed shoot and root system; (f) *Ex-vitro* establishment of micro-propagated plants in polythene bags.

i.e. 3.0 mgl^{-1} BAP. The average number of shoots per explant was highest (6.58) in medium MR-6 followed by MR-5 (5.20) from 16-day-old seedlings followed by 24-day-old seedlings.

The regenerated shoots were excised and transferred on to the shoot elongation (Fig. 3d) MS medium fortified with BAP 2.5 mgl^{-1}, IAA 0.5 mgl^{-1} and 1.0 mgl^{-1} GA_3. However, *in vitro* root induction was obtained from half-strength MS medium containing 0.5 mgl^{-1} IBA for eight days followed by six days on half-strength MS basal medium as standardized by Hira Singh *et al.* (4). The hardened plants were transferred to the normal field soil in polythene bags and kept in the glasshouse.

The present investigations have clearly indicated the potential of cotyledon explant to form *in vitro* shoot buds and regenerate into well developed automorphic plants. The optimum number of shoot buds were obtained on MS medium fortified with 9.5 mg l^{-1} BAP, 3.0 mg l^{-1} in and 1.0 mg l^{-1} IAA. Cotyledon explants excised from 24-day-old *in vitro* raised seedlings was the best for organogenesis and produced the highest

number of shoots per explant. The success of *in vitro* regeneration will enable the use of appropriate genetic transformation programmes.

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