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Studies on *in vitro* propagation in hot pepper

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ABSTRACT

***In vitro* regeneration of two commercially important varieties of hot pepper (*Capsicum annuum* L.) *viz.*, Punjab Surkh and Punjab Guchhedar was achieved by direct organogenesis. The epicotyl explants excised from aseptically grown 16, 24 and 32-day-old seedlings were utilized. In both the varieties, 24-day-old epicotyl explant was found relatively more responsive to the initiation of multiple shoots. Multiple average shoots per explant (1.48) were produced by Punjab Guchhedar with MS medium supplemented with 5.0 mgl^{-1} BAP, 2.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA and Punjab Surkh (1.53) on MS medium supplemented with 6.0 mgl^{-1} BAP, 3.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA. For elongation, shoot buds were excised and transferred to shoot elongation medium (MS medium supplemented with 2.0 mgl^{-1} BAP, 0.5 mgl^{-1} IAA and 1.0 mgl^{-1} GA_3). After 12-16 days of culture, elongated shoots were cultured on rooting medium *i.e.*, MS medium (half strength) supplemented with 0.5 mgl^{-1} IBA for 8 days followed by 6 days on MS (half strength) basal medium. About 60-65 per cent rooted plants were hardened, transferred in the polythene bags and then earthen pots.**

Key words: *Capsicum annuum*, epicotyl, *in vitro* regeneration, hot pepper.

INTRODUCTION

Hot pepper (*Capsicum annuum* L.) is one of the main solanaceous crop which is indispensable vegetable and spice crop grown for its green and red ripe fruits. Presently, it is gaining more importance in the global market because of its value added products like chilli powder, oleoresin and colouring matter. Pungency in hot pepper is due to an alkaloid called capsaicin, which has high medicinal value. Besides this, hot pepper fruits are low in sodium and calories, free from cholesterol and have high vitamin A&C and good source of minerals (Ochoa-Alejo and Ramirez-Malagon, 8; Danise, 4). India stands first in pepper cultivation with an area of 9.40 lakh hectares with a total production of 10.30 lakh metric tonnes (Anon, 1) with 8.80 q/ha productivity as compared to world's productivity (82.92 q/ha). So, there is an ample scope to increase the yield of hot pepper, which can be enhanced by eight times (Singh, 13; Madhavi *et al.,* 5). Using various conventional breeding approaches like selection, hybridization and mutation, widely adapted and disease resistant varieties have been developed in this crop. However, further improvement in these aspects through conventional approaches appears to be only marginal. So, it is the need of the hour to develop new technologies, which can bridge the gap between the production and actual demands of peppers.

The rise of biotechnology has also catalyzed renewed emphasis on the importance of biological and genetic diversity and their conservation. It is useful in many areas like *in vitro* culture techniques, genome mapping, marker assisted selection and development of transgenic plants. So to realize the potential of tissue culture techniques for genetic improvement and pre-requisite for genetic transformation of pepper, it is essential to have reliable, efficient and reproducible protocols for high frequency of plant regeneration. Although, hot pepper is considered as recalcitrant species with respect to *in vitro* regeneration. Among all solanaceous crops, it is most difficult to regenerate under *in vitro* conditions (Ranjan *et al*., 10). Hence keeping this in view, we conducted experiment on two commercially grown varieties, *viz*. Punjab Surkh and Punjab Guchhedar of hot pepper by taking epicotyl as explant for *in vitro* plant regeneration.

MATERIALS AND METHODS

Experiments were carried out at the Department of Vegetable Crops and Tissue Culture Laboratories, Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University, Ludhiana. Seeds of two commercially important varieties of hot pepper *viz*., Punjab Surkh and Punjab Guchhedar were procured from Department of Vegetable Research Farm. Seeds were washed thoroughly with distilled water. This was followed by surface sterilization with 4 per cent (v/v) sodium hypochlorite for 15 min. duration and several washes with sterile double distilled water. Surface sterilized seeds were cultured on basal MS (Murashige and Skoog, 7) medium for germination. Epicotyl explants were derived from 16-, 24- and 32-day-old *in vitro* grown seedlings. Explants

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were cultured on modified MS medium containing MS salts with 3 per cent (w/v) sucrose. The pH of the medium was adjusted to 5.8 with 1N NaOH and 1N HCl before adding 0.8 per cent (w/v) agar and different concentrations and combinations of growth regulators. The media were then dispensed in culture tubes (150 × 25 mm, Borosil) or jars (400 ml capacity) and sealed prior to autoclaving at 121°C for 21 min.

Epicotyl segments (0.5-1.0 cm) were aseptically cut and cultured in culture tubes containing 35 ml bud induction medium. 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.

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

Medium	Hormonal level (mg l^{-1})			
	BAP	Kin	NAA	IAA
M-0	0.00	0.00	0.00	0.00
M-1	3.00	0.00	0.00	0.50
M-2	3.00	0.00	0.50	0.00
M-3	5.00	2.00	0.00	1.00
M-4	6.00	3.00	0.00	1.00
M-5	7.50	3.00	0.00	1.00
M-6	9.50	3.00	0.00	1.00
M-7	11.00	3.00	0.00	1.00

The adventitious shoot buds induced on epicotyl were excised and then cultured on bud elongation medium consisting of MS medium supplemented with low doses of auxin (IAA), cytokinin (BAP) and gibberrellic acid (GA_3) combinations. The elongated shoot buds (1.5-2.0 cm long) were excised and cultured on rooting medium consisting of MS medium fortified with 0.5 mg l^{-1} indole-3-butyric acid (IBA). The rooted plants were transferred to liquid half-strength MS medium and kept under high light intensity (5000 lux) for 3-4 days followed by washing of roots under running tap water to remove adhering medium. The plantlets were then kept on wet soil for 4-5 days and then transferred to polythene bags containing soil:sand:compost (1:1:1) under high humidity conditions and finally transferred to earthen pots.

All cultures were incubated in incubation room under 16 h light and 8 h dark cycle with a light intensity of 2500-3000 lux at 25 ± 2°C temperature. The data were analyzed statistically according to completely randomized block design (CRBD) as described by Panse and Sukhatme (9).

RESULTS AND DISCUSSION

The regeneration percentage from epicotyl of two varieties of hot pepper is represented in Figs. 1 and 2. The epicotyl explants produced shoot buds directly on shoot induction medium within 13-18 days of culture. The regeneration from epicotyls varied from no regeneration to 46.23 in Punjab Surkh and no regeneration to 48.31 in Punjab Guchhedar. Per cent shoot regeneration in Punjab Surkh from epicotyl explants was poor depicting maximum regeneration only 34.07 per cent on M-4 followed by M-5 media. Most of the explants showed callus at cut end that later on turned brownish-black in colour followed by drying (Fig. 3H).

Likewise, in Punjab Guchhedar, trend and response was similar but maximum regeneration (Fig. 3A) occurred on M-3 medium followed by M-4 medium. In this case also, only callus formation occurred without any regeneration of shoots in M-2 medium having NAA. The maximum per cent average regeneration was recorded in 24-days-old explants in both genotypes (22.57 per cent in Punjab Surkh and 16.20 per cent in Punjab Guchhedar). BAP, Kin and IAA combination showed more effectiveness in plant regeneration than other combinations. NAA was found very less effective towards shoot regeneration as it caused highly proliferating callus formation.

Epicotyls excised from 24-day-old *in vitro* grown seedlings, performed better in both the genotypes. The highest average number of shoots (1.53) in Punjab Surkh were obtained in M-4 medium (Table 2) followed by (1.16) on M-3 medium. Similarly, in Punjab Guchhedar (0.92) the highest number of shoots were obtained on M-4 (Table 3) followed by (0.88) on M-5. Average frequency of shoot regeneration was lower

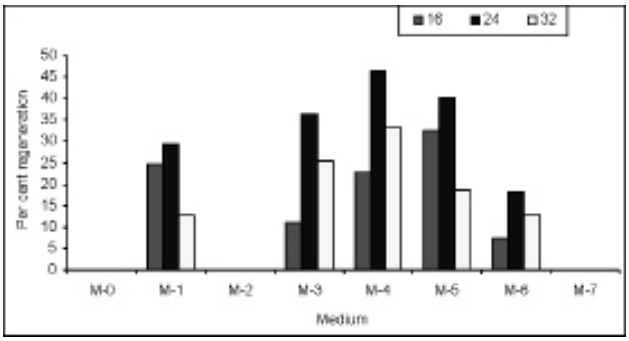

Fig. 1. Effect of medium composition and age of explant on per cent survival and subsequent shoot regeneration from epicotyl explant in hot pepper var. Punjab Surkh.

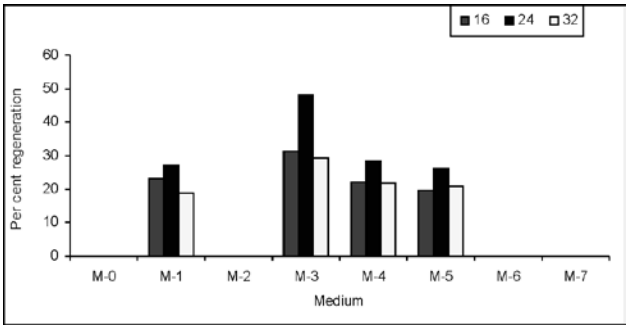

Fig. 2. Effect of medium composition and age of explant on per cent survival and subsequent shoot regeneration from epicotyl explant in hot pepper var. Punjab Guchhedar.

in both the varieties which justifies Steinitiz *et al.* (14) observations that genus *Capsicum* is highly recalcitrant for regeneration. Similarly, Mathew (6) obtained low average frequency of shoot regeneration (1.58) in Byadagi Dabbi and Arka Lohit (1.28) cultivars from hypocotyl and cotyledon explants.

Callus was formed in M-2 medium but failed to regenerate into plantlets or shootlets. At very high level of hormones (6 mgl^{-1} BAP and higher), explants showed mortality with change in callus colour from green to brownish black. In both the varieties callus formation was observed in epicotyl explant but Punjab Guchhedar showed more callus formation than Punjab Surkh. Some explants, showed regeneration in the earlier stage but later on turned brown to black coloured mass resulting in mortality.

For elongation two MS basal media supplemented with 2.0 mgl^{-1} BAP, 0.5 mgl^{-1} IAA (R1) and other medium supplemented with extra 1.0 mgl^{-1} GA_3 (R2) were used. It can be inferred that 1.0 mgl^{-1} GA_3 increased the elongation of *in vitro* grown shootlets (Fig. 3B). After 15 days of sub culture, all the cultures were shifted to basal MS medium to reduce the endogenous level of cytokinins (Fig. 3C). Arous *et al.* (2,3) reported the elongation of shoots on MS medium containing 1.0 mgl^{-1} BAP, 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} GA_3. Many workers reported that GA_3 is the best growth regulator for shoot elongation (Zhou *et al.*, 16; Szasz *et al.*, 15; Shivegowda *et al.*, 12; Ranjan *et al.*, 11). For rooting induction, i*n vitro* raised shoots were initially cultured on half-strength MS medium containing 0.5 mgl^{-1} IBA for eight days (Fig. 3D) then again cultured for six days on half-strength MS basal medium which also helped in elongation of roots (Fig. 3E).

Plantlets having well developed shoot and root system were transferred to liquid MS (half-strength) medium and plants were kept under high intensity of about 5000 lux for 3-4 days followed by washing of roots under running tap water to remove adhering medium and agar. This was done to acclimatize the *in vitro* grown plants slowly and slowly preparing for photosynthesis and autotrophic nature. Transferring micro-propagated plants directly from culture vessels to *ex vitro* conditions is generally difficult as the transpiration rate is higher in such plants. The *in vitro* grown plants have poor stomatal control and abnormally high cuticular water loss resulting in wilting and necrosis of leaves or even senescence of leaves and plantlets (Ranjan *et al.*, 11). To minimize transplanting shock the *in vitro* grown plants were first put on liquid medium and then placed on cotton moistened with sterilized

Table 2. Average number of shoots regenerated from epicotyl explant of hot pepper var. Punjab Surkh on MS medium.

Medium	Age of seedlings (days)			Mean
	16	24	32	
M-0	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
M-1	0.73 (1.40)	1.06 (1.43)	0.88 (1.37)	0.89 (1.40)
M-2	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
M-3	1.10 (1.45)	1.34 (1.53)	1.05 (1.43)	1.16 (1.47)
M-4	1.40 (1.55)	1.89 (1.69)	1.31 (1.52)	1.53 (1.59)
M-5	0.92 (1.38)	1.30 (1.51)	1.03 (1.42)	1.08 (1.44)
M-6	0.72 (1.31)	1.13 (1.37)	0.68 (1.29)	0.84 (1.32)
M-7	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Mean	0.61 (1.26)	0.84 (1.32)	0.62 (1.25)	
CD (P = 0.05)	Age : 0.01	Medium : 0.01	Age × Medium : 0.02	

Figures in parenthesis indicate square root transformation of values

Table 3. Average number of shoots regenerated from epicotyl explant in hot pepper var. Punjab Guchhedar on MS medium.

Medium	Age of seedling (days)			Mean
	16	24	32	
M-0	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
M-1	0.54 (1.24)	1.10 (1.45)	0.81 (1.35)	0.82 (1.34)
M-2	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
M-3	1.04 (1.43)	1.96 (1.72)	1.46 (1.57)	1.48 (1.57)
M-4	0.83 (1.35)	1.03 (1.41)	0.94 (1.39)	0.92 (1.38)
M-5	0.68 (1.29)	1.08 (1.44)	0.88 (1.37)	0.88 (1.37)
M-6	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
M-7	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Mean	0.38 (1.16)	0.64 (1.25)	0.51 (1.21)	
CD (P = 0.05)	Age : 0.01	Medium : 0.01	Age × medium : 0.02	

Figures in parenthesis indicate square root transformation of values

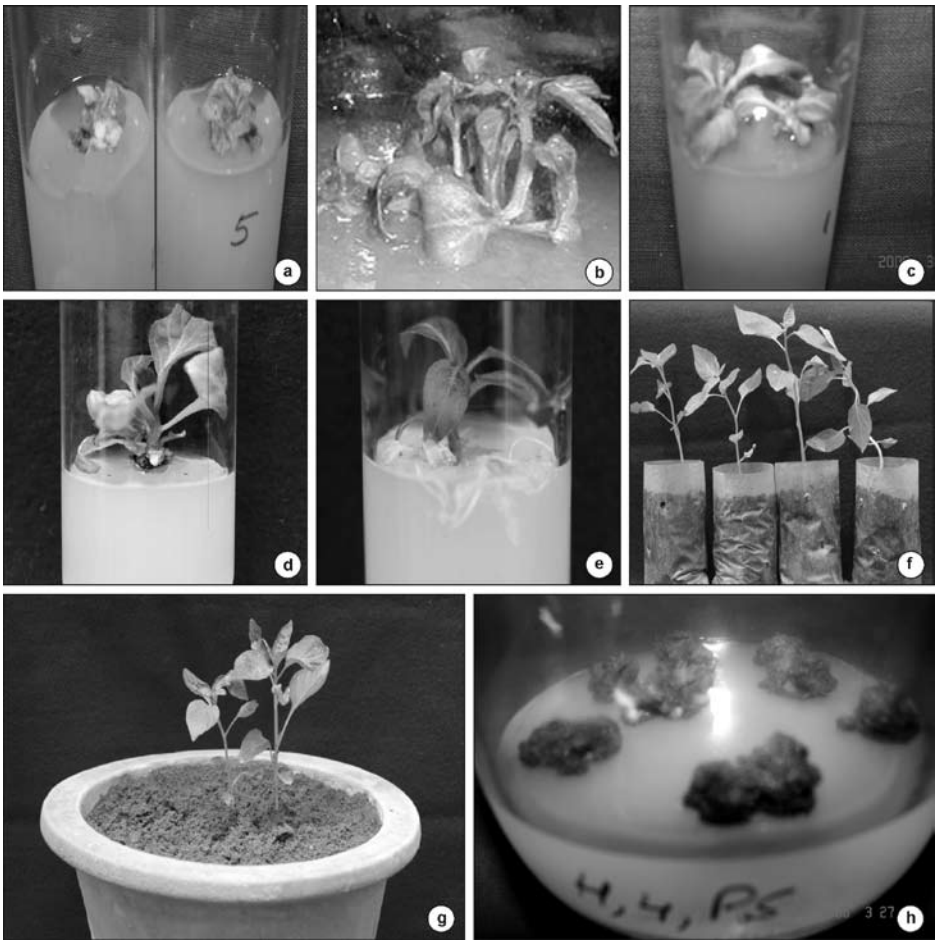

Fig. 3. (A-H) Various stages of *in vitro* plant regeneration in hot pepper; a) Induction of multiple shoot regeneration from epicotyl on shoot induction medium, b) Elongation of *in vitro* grown shootlets, c-d) Root induction on root induction medium, e) Plantlets with well developed shoot and root system, f-g) *Ex-vitro* establishment of micropropagated plants in polythene bags and earthen pots, h) Brownish callus formation on some media.

water. The processing of hardening acclimatized the plantlets for their subsequent transfer to soil. The hardened plants were transferred to polythene bags containing soil:sand:compost (1:1:1) and kept in the glasshouse (Fig. 3F&G). In the beginning, the plants were watered with 0.1 per cent Bavistin solution, which prevents all fungal growth. The plants exhibited 50-60 per cent survival in the glasshouse.

In both the varieties, 24-day-old epicotyl explant was found to be relatively more responsive to the initiation of multiple shoots. Multiple shoots per explant were produced on MS medium having 5.0 mgl^{-1} BAP, 2.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA and MS medium supplemented with 6.0 mgl^{-1} BAP, 3.0 mgl^{-1} Kin and 1.0 mgl^{-1} IAA in Punjab Guchhedar and Punjab Surkh, respectively. MS medium supplemented with 2.0 mgl^{-1} BAP, 0.5 mgl^{-1} IAA and 1.0 mgl^{-1} GA_3 found best for shoot elongation while MS (half-strenth) medium fortified with 0.5 mgl^{-1} IBA for 8 days followed by ½MS basal medium for 6 days was found the best for *in vitro* rooting. *In vitro* regeneration is a pre-requisite for the improvement of any genotype through genetic transformation. Hence, this study could be helpful towards the genetic manipulation and improvement of hot pepper cultivars.

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