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Callus mediated regeneration through cotyledonary and hypocotyl explants in chilli (*Capsicum annuum* L.)

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Abstract

Chilli is recalcitrant to *in vitro* regeneration and many factors such as genotypes, explants and growth regulators play important role in *in vitro* indirect organogenesis. The present study was undertaken to standardize callus mediated regeneration protocol through indirect organogenesis in four Indian chilli cultivars namely, KtPL-19, Pusa Sadabahar, ArCH-001 and Salem. Cotyledonary and hypocotyls explants were excised from 21-day old *in vitro* raised seedlings and subjected to different treatments for callusing and subsequent regeneration. Full-strength Murashige and Skoog (MS) medium was found better than half strength MS medium (MS/2) and B_5 medium for culture initiation. Callus induction in both cotyledonary leaf (98.11%) and hypocotyl (97.97%) explants was the maximum on MS medium containing 5.0 mgL^{-1} 2, 4 dicholorophenoxy acetic acid (2, 4-D) + 1.0 mgL^{-1} kinetin (Kn). For callus multiplication, the combination consisting of MS+ 3.0 mgL^{-1} 2,4-D+0.5 mgL^{-1} kinetin was found to be the best. Shoot bud induction was achieved on hypocotyl derived callus, when thidiazuron (TDZ) (1.5 mgL^{-1}) was used as a source of cytokinin in MS medium.

Kew words: Indirect organogenesis, *in vitro* regeneration, *Capsicum annuum* L., Cotyledonary explant, hypocotyl explant

Introduction

Plant tissue culture has been widely used as an experimental system not only to study the basic aspects in the physiology of cell growth and differentiation, but also in applied areas like micro-propagation, *in vitro* mutagenesis and development of transgenics. The technique is also utilized to increase the number of desirable germplasm available to the plant breeders (Kothari et al. 2010). Indirect organogenesis (callus mediated) is considered as an important tool for the selection of useful somaclonal variants and recovery of transformed plant (Phillip and Hubstenberg 1985). The efficient system for induction of embryogenic callus and multiple shoot regeneration from this callus is considered to be the basic requirement in obtaining transgenics. Thus, the importance of developing callus mediated regeneration has increased over the years. Unlike many Solanaceous species, chilli plant (*Capsicum annuum* L.; 2n = 2x = 24), is a recalcitrant in terms of *in vitro* cell tissue and organ differentiation and plant regeneration, which makes it difficult to apply recombinant DNA technologies for improvement against biotic and abiotic stresses (Kothari et al. 2010, Ochoa-Alejo and Ramirez-Malagon 2001). There are also intervarietal differences in chilli explants responding to various rates and combinations of plant growth regulators (Fortunato and Tudisco 1991). The existence of strong genotype specificity in the regeneration capacity of the different cultivars represents an important limiting factor in the development of regeneration protocol (Kothari et al. 2010) and therefore, specific regeneration protocol is required for each cultivar. Hence, the present investigation was undertaken to study the effect of genotypes and various combinations of growth regulators on callus mediated regeneration.

Materials and Methods

Four chilli cultivars namely, KtPL-19 (V_1), Pusa Sadabahar (V_2), ArCH-001 (V_3) and Salem (V_4) were selected for the present investigation. Out of these four cultivars, KtPL-19 and Pusa Sadabahar are released from Indian Agricultural Research Institute, New Delhi, ArCH-001 from Ankur Hybrid Seed Ltd. India, and Salem is a local cultivar from Tamil Nadu, India. Seeds were germinated under *in vitro* conditions to excise cotyledonary leaf segment and hypocotyl explants. For

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in vitro seed germination, dry seeds were soaked in distilled water for 12 h. Surface sterilization was done using 0.1% $HgCl_2$ with agitation for 2 min followed by three rinses in sterile double distilled water. Seeds were then inoculated onto MS (Murashige and Skoog 1962) medium with 30 gL^{-1} sucrose, gelled with 7.0 gL^{-1} agar and kept in dark at 25±1°C. After germination, seedlings were exposed to 16 h photoperiod. Explants excised from 21 d seedlings were used for callusing and regeneration. Different media such as M_1=Full MS; M_2=1/2 MS + Full micro and organics of MS medium; M_3= Full strength B_5; were used for screen medium for further regeneration. All the media were supplemented with 30 gL^{-1} sucrose and gelled with 7.0 gL^{-1} agar. After screening the medium, they were also supplemented with different growth regulator combinations (as per treatment mentioned in respective tables) for callusing, callus multiplication, organogenesis and shoot regeneration. Effect of growth regulators and sucrose level in MS medium on fresh and dry weight of callus was also examined. All the media were autoclaved at 120°C for 20 min. The heat-labile phyto-hormone thidiazuron (TDZ) was added to the medium after autoclaving using 0.20 µm size microfilter (Millipore®, USA). The culture room was maintained at 25±2°C, 16/8 h of light and dark cycle by cool-white fluorescent tubes (47 µmol $m^{-2}s^{-1}$). Subcultures were carried out at 30 days interval and 15 samples were maintained in each treatment. All the experiments were laid out in factorial completely randomized design (CRD) with three replications. Percentage data were subjected to Arc Sin transformation before analysis. The analyses of data were done by using SPSS 13.0 Software.

Results and Discussion

Basal media (Full MS, 1/2 MS + full micro and organics, and full strength B_5) at different strengths were screened for optimum culture establishment of cotyledonary leaf and hypocotyl explants. Among the three media tested, full-strength MS medium was found the best for culture establishment (Table 1). Among the cultivars studied, the best response was registered in KtPL-19. The results are in agreement with earlier findings of Gunay and Rao (1978). Later several workers (Christopher et al. 1986, Ezura 1993, Binzel et al. 1996) also found full-strength MS medium ideal for culture initiation. However, Gupta et al. (1990) observed low salt medium that is, B5 medium was better than other media. Different concentration of 2, 4-D and Kn, and their combinations were tried for callus induction on cotyledonary and hypocotyl explants. It was observed that with the increase in concentration of 2, 4-D up to 5.0 mgL^{-1}, percent callus induction increased irrespective of the varieties and explants explored, except in case of KtPL-19, wherein 6.0 mgL^{-1} 2,4-D was found the best (Table 2). Gupta et al. (1990) also found 5.0 mgL^{-1} of 2, 4-D was effective for callus induction. Callus induction was also observed, when media were devoid of 2, 4-D. This might be due to the presence of endogenous growth hormones, which may have promoted callus induction on medium devoid of growth regulators (Gupta et al. 1990). It was also observed that with the increase in concentration of 2,4-D, the days required for callus induction also decreased significantly in both cotyledonary leaf and hypocotyl explants. This might be due to the fact that 2,4-D is very effective for early induction and multiplication of callus (Bhojwani and Razdan 1996). However, Ashrafuzzaman et. al. (2009) reported the highest callus induction from hypocotyl in a combination of BAP (5.0 mgL^{-1}) with IAA (1.0 mgL^{-1}) with NAA (0.1 mgL^{-1}) and aso the callus induction as well as shoot initiation was higher in hypocotyls than cotyledon.

Effect of growth regulators and sucrose level in MS medium on fresh and dry weight of callus was examined. Fresh weight and dry weight of callus increased as days of sub culturing elapsed (Figure 1). Maximum fresh and dry weight was recorded at 45 days of subculture. The increase in callus fresh and dry weight was found to increase with the increase in 2,4-D concentration up

Table 1: Effect of media on culture establishment (survival) on cotyledonary leaf and hypocotyl explant.

Medium	Cotyledonary leaf explants survival (%)					Hypocotyl explants survival (%)				
	V_1	V_2	V_3	V_4	Mean	V_1	V_2	V_3	V_4	Mean
M_1	88.25 (69.99)*	85.20 (67.41)	83.23 (65.85)	81.24 (64.37)	84.48 (66.90)	87.23 (69.09)*	84.28 (66.67)	80.25 (63.65)	80.25 (63.65)	83.00 (65.76)
M_2	62.54 (52.29)	60.23 (50.93)	60.15 (50.88)	58.55 (49.95)	60.37 (51.01)	65.25 (53.91)	60.23 (50.93)	60.11 (50.86)	59.23 (50.34)	61.21 (51.50)
M_3	58.52 (49.93)	58.52 (49.93)	57.25 (49.19)	56.55 (48.79)	57.71 (49.46)	60.25 (50.94)	59.80 (50.68)	56.15 (48.56)	56.10 (48.53)	58.07 (49.67)
Mean	69.77 (57.40)	67.98 (56.09)	66.88 (55.31)	65.45 (54.36)		70.91 (57.98)	68.10 (56.09)	65.50 (54.35)	65.19 (54.35)	
$CD_{0.05:}$	Medium0.99; Cultivar1.15; Interaction 1.99					Medium 1.44; Cultivar 1.66; Interaction 2.88				

M_1= Full MS; M_2=1/2 MS + full micro and organics; M_3= Full strength $B_{5;}$ *Data in parenthesis are Arc SinÖ% transformed value. V_1= KtPL-19; V_2 = Pusa Sadabahar; V_3 = ArCH-001; V_4 = Salem.

Table 2: Effect of growth regulators on callus induction (%) from cotyledenary leaf and hypocotyl explants.

Treatment number	Cotyledonary leaf					Hypocotyl				
	V_1	V_2	V_3	V_4	Mean	V_1	V_2	V_3	V_4	Mean
C_1	10.21 (18.64)*	8.25 (16.70)	8.11 (16.55)	9.11 (17.58)	8.92 (17.37)	8.11 (16.55)	8.04 (16.48)	9.25 (17.72)	4.18 (11.80)	7.40 (15.64)
C_2	30.56 (33.58)	15.21 (22.97)	22.29 (28.19)	48.12 (43.94)	29.92 (32.17)	35.11 (36.36)	19.61 (26.30)	30.11 (33.30)	22.18 (28.11)	26.75 (31.02)
C_3	31.32 (34.02)	25.11 (30.09)	41.65 (40.21)	51.14 (45.68)	37.31 (37.51)	48.25 (44.02)	35.52 (36.60)	61.24 (51.52)	25.26 (30.19)	42.57 (40.58)
C_4	58.11 (49.69)	75.19 (60.16)	56.35 (48.67)	68.39 (55.82)	64.51 (53.59)	60.58 (51.13)	81.54 (64.59)	64.11 (53.22)	40.11 (39.32)	61.59 (52.07)
C_5	61.48 (51.66)	80.32 (63.70)	80.34 (63.64)	75.11 (60.10)	74.31 (59.78)	78.31 (62.27)	85.11 (66.34)	78.36 (62.31)	61.13 (51.46)	75.73 (60.85)
C_6	95.23 (53.89)	96.24 (78.86)	98.44 (82.87)	97.24 (80.48)	96.79 (74.03)	95.12 (77.28)	96.24 (78.86)	98.32 (82.59)	97.45 (80.85)	96.78 (79.89)
C_7	96.21 (78.81)	98.46 (82.91)	98.52 (83.05)	99.24 (85.04)	98.11 (82.45)	96.35 (79.03)	98.36 (82.68)	98.50 (83.01)	98.69 (83.47)	97.97 (82.05)
C_8	98.85 (83.89)	97.68 (81.28)	97.23 (80.46)	98.90 (84.02)	98.17 (82.41)	98.20 (85.33)	98.14 (82.20)	95.51 (77.81)	96.66 (79.51)	97.13 (80.46)
Mean	60.25 (50.53)	62.06 (54.58)	62.87 (55.46)	68.41 (59.08)		65.00 (56.12)	65.32 (56.88)	66.93 (57.69)	55.71 (50.59)	
	$CD_{0.05}$: Treatment: 1.45; Cultivar: 1.02; Interaction 2.90					Treatment: 1.85 Cultivar: 1.31; Interaction 1.70				

V_1 = KtPL-19; V_2 = Pusa Sadabahar; V_3 = ArCH-001; V_4 = Salem. *Data in parenthesis are Arc SinÖ% transformed value. C_1: MS+0.0 2,4-D + 0.0 KIN; C_2: MS+1.0 2,4-D + 0.0 KIN; C_3: MS+2.0 2,4-D + 0.25 KIN; C_4: MS+3.0 2,4-D + 0.50 KIN; C_5: MS+4.0 2,4-D + 0.50 KIN; C_6: MS+5.0 2,4-D + 0.50 KIN; C_7: MS+6.0 2,4-D + 1.0 KIN; C_8: MS+7.0 2,4-D + 1.0 KIN.

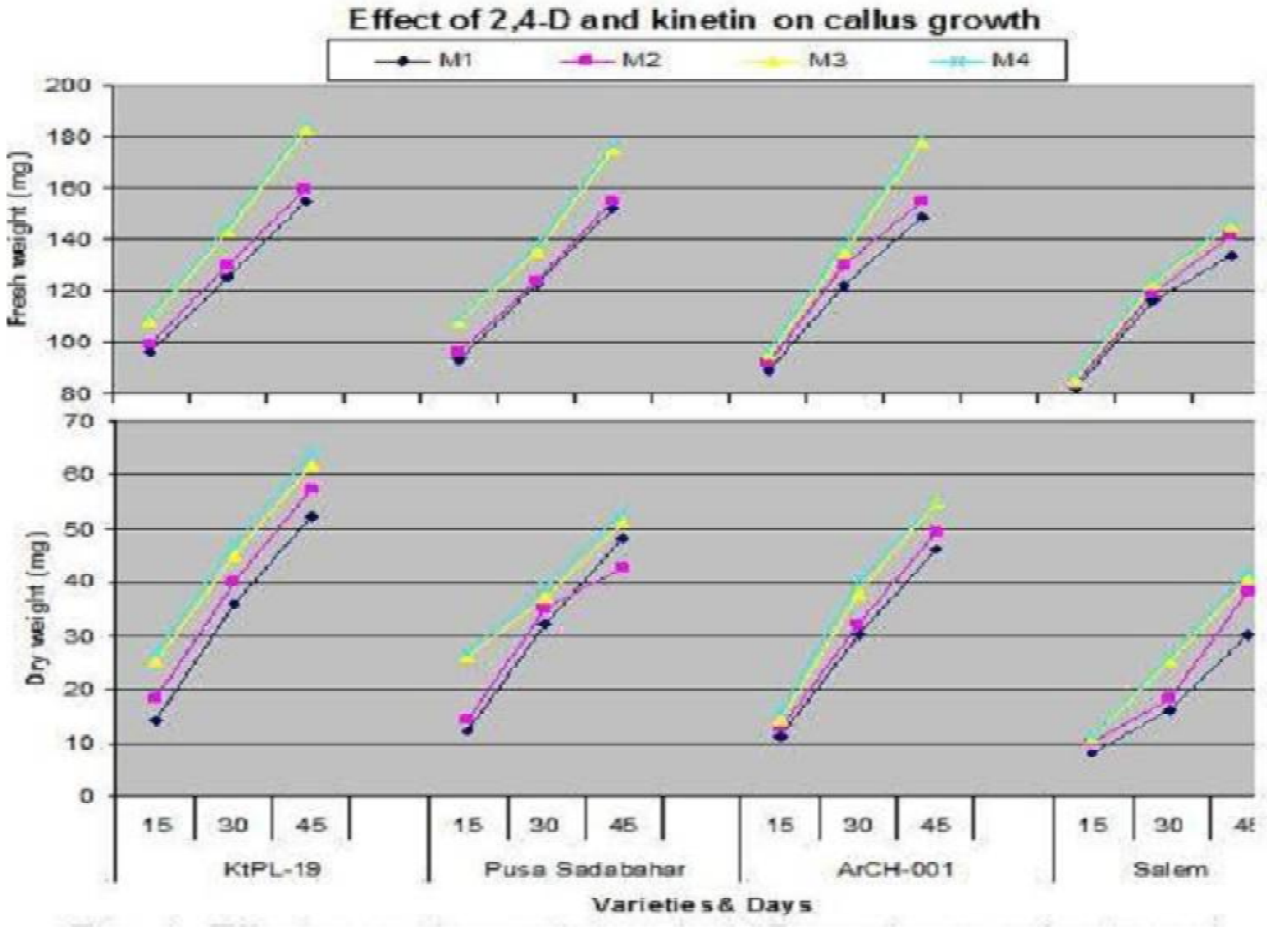

Figure 1. Effect of growth regulators in MS medium on fresh and dry weight of callus (M1: 1.0 mg/l 2, 4-D+0.5 mg/l KIN; M2: 2.0 mg/l 2, 4-D + 0.5 mg/l KIN; M3 : 3.0 mg/l 2, 4-D + 0.5 mg/l KIN; M4 : 4.0 mg/l 2, 4-D + 0.5 mg/l KIN).

to 3 mgl^{-1}. The results are in agreement with the earlier findings of Gupta et al. (1990). Both auxins and cytokinins are required for callus growth and it has earlier been demonstrated in different species by Singh et al. (1985). In combined application also, increase in 2,4-D and kinetin levels increased callus fresh and dry weight. In the present study callus growth was recorded the best at 3.0 mgL^{-1} 2,4-D, while Dodds and Roberts (1985) indicated 2 mgL^{-1}2,4-D level as most effective for callus proliferation. Linear increase in fresh and dry weight was observed with increment in sucrose levels as well as subculture duration (Figure 2). With the increase in sucrose level callus fresh and dry weight increased in all cultivars, and the maximum fresh and dry callus weight was recorded at 30 gL^{-1}level. The results are in close conformity with the results obtained

Table 3: Effect of different growth regulators on *in vitro* formation of shoot bud on callus clump derived from cotyledonary leaf and hypocotyls.

Treatment number	Growth regulator (mgL^{-1})	Number of shoot buds per callus mass									
		Cotyledonary leaf					Hypocotyl				
		V_1	V_2	V_3	V_4	Mean	V_1	V_2	V_3	V_4	Mean
T_1	2.0 BAP + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_2	3.0 BAP + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_3	4.0 BAP + 0.5 NAA	0.89	1.52	0.79	0.59	0.95	0.90	1.67	0.83	0.62	1.01
T_4	5.0 BAP + 0.5 NAA	0.90	1.60	0.79	0.59	0.97	0.92	1.70	0.85	0.64	1.03
T_5	6.0 BAP + 0.5 NAA	0.90	1.61	0.80	0.61	0.98	0.92	1.75	0.88	0.65	1.05
T_6	1.0 KIN + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_7	2.0 KIN + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_8	3.0 KIN + 0.5 NAA	0.65	0.78	0.62	0.55	0.65	0.70	0.80	0.70	0.57	0.69
T_9	0.5 TDZ	0.75	0.80	0.64	0.58	0.69	0.75	0.85	0.74	0.59	0.73
T_{10}	1.0 TDZ	1.50	1.82	0.95	0.75	1.26	1.69	1.85	0.97	0.79	1.33
T_{11}	1.5 TDZ	1.54	1.85	0.98	0.80	1.29	1.75	1.87	0.99	0.85	1.37
T_{12}	2.0 TDZ	1.40	1.60	0.78	0.75	1.13	1.60	1.69	0.90	0.80	1.25
	Mean	1.07	1.45	0.79	0.65		1.15	1.52	0.86	0.69	
$CD_{0.05}$:	Treatment: 0.013; Cultivar: 0.019; Interaction : 0.037						Treatment: 0.02 Cultivar: 0.03;		Interaction 0.06		

V_1 = KtPL-19; V_2 = Pusa Sadabahar; V_3 = ArCH-001; V_4 = Salem.

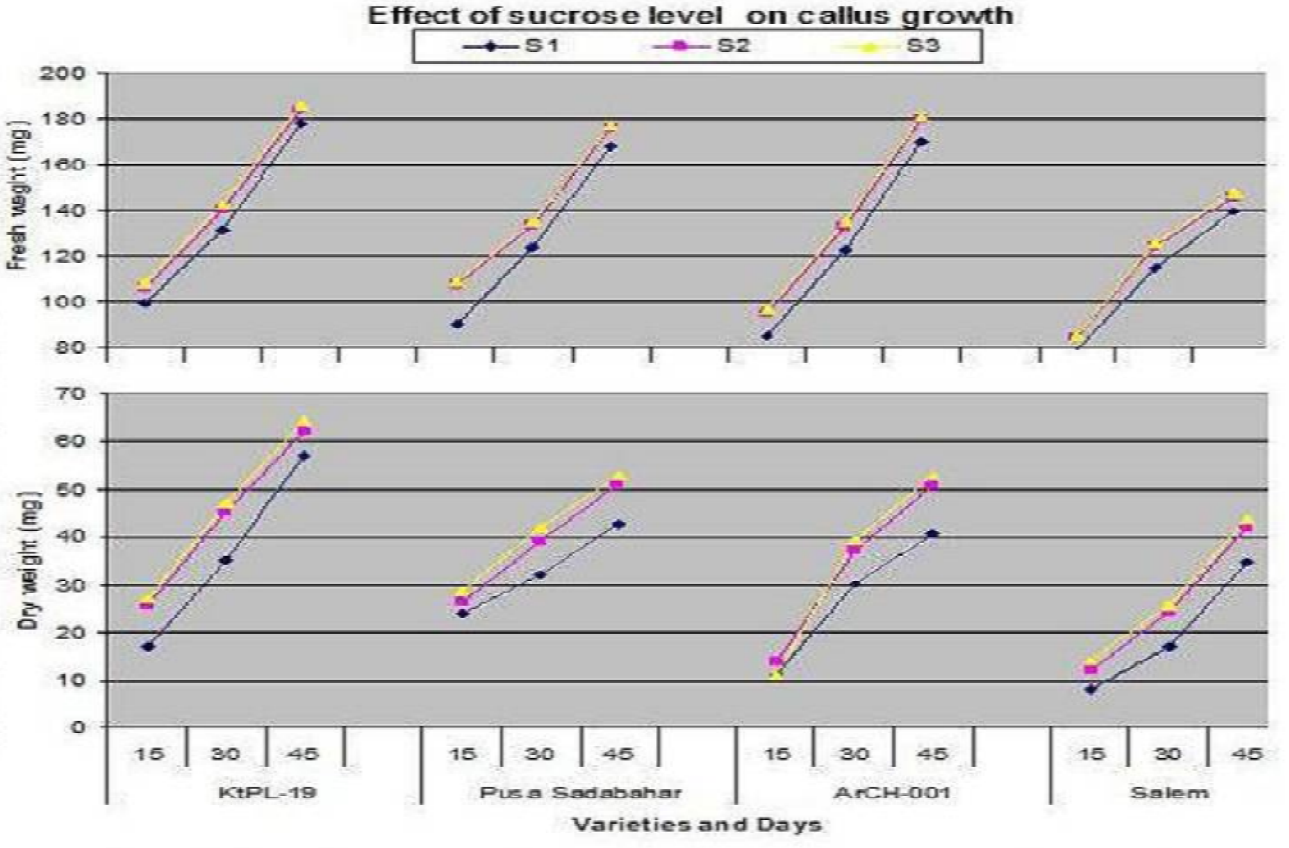

Figure 2. Effect of sucrose level in MS medium on fresh and dry weight of callus (S1 : 20 g/l; S2 : 40 g/l; S3 : 50 g/l).

by Gupta et al. (1990). Several workers also reported addition of 30 gL^{-1}sucrose for better callusing (Mohamed, 1979). It has also been proposed that sucrose apart from being an active carbon source, helps in osmotic adjustment of the medium thus causing rapid callusing (Doley and Leyton 1970).

Among the three cytokinins *viz*., Benzyl Amino Purine (BAP), Kinetin (Kn) and Thidiazuron (TDZ), TDZ was found to be most effective for shoot bud organogenesis in the callus obtained from both cotyledonary leaf and hypocotyl explants. No shoot bud was recorded in treatments containing 2.0 or 3.0 mgL^{-1} BAP and 1.0 or 2.0 mgL^{-1} kn. However, with the increase in BAP or kn levels, the frequency of shoot bud induction increased and 5.0 mgL^{-1} BAP was found better than other levels (Table 3, Plate-1). Similar trend was also observed in TDZ and the effective concentration was 1.5 mgL^{-1} In general, shoot bud organogenesis from callus was low (maximum 1.87/callus mass) and of the two callus sources, hypocotyl derived callus showed better organogenesis potential than the cotyledonary leaf explant. However, Grozeva and Todorova (2015) advocated that the in vitro organogenesis and regeneration in pepper is affected by genotype, explants and concentration of basal medium. Cotyledons are more susceptible to regeneration than hypocotyls. Szasz et al. (1995) also reported TDZ to be most effective cytokinin for shoot bud organogenesis. Thidiazuron is a substituted phenyl urea developed primarily as cotton defoliant. Thidiazuron has a high efficiency in stimulating cytokinin-dependent shoot regeneration from a wide variety of plants (Malik and Saxena 1992). The high concentration of cytokinin (BAP and kn) induced shoot bud formation and this was also observed by Gupta et al. (1990). The number of elongated shoots from induced shoot bud was also higher in the medium containing 1.5 mgL^{-1} TDZ. With the increase in dose of cytokinin, the number of regenerated shoots increased in both cotyledonary leaf and hypocotyl explant. The results are in agreement with that of Gupta et al. (1990) and Malik and Saxena (1992). The number of regenerated shoots was lower than the number of shoot buds observed. This may be due to the formation of ill-defined buds or shoot-like structures, which do not

Plate 1. A. Callusing on MS medium supplemented with 5.0 mg/l 2,4-D + 0.5 mg/l kinetin in hypocotyl and cotyledonary leaf explants. B. Shoot bud induction on hypocotyl derived callus mass on MS medium supplemented with 1.5 mg/l TDZ. C. Shoot multiplication on MS medium supplemented with 6.0 mg/l BAP + 1.0 mg/l kinetin + 0.5 mg/l GA_3. D. Rooting of *in vitro* regenerated microshoots on 1/2 strength MS medium + 1.0 mg/l IBA. E. Hardening of plantlets under *in vitro* condition. F. Growth of the plantlets after 30 days of transfer to glasshouse.

Table 4: Effect of different growth regulators on number of shoot regenerating from callus derived from cotyledonary leaf and hypocotyls segments.

Treatment number	Growth regulator (mgL^{-1})	Number of regenerated shoot									
		Cotyledonary leaf					Hypocotyl				
		V_1	V_2	V_3	V_4	Mean	V_1	V_2	V_3	V_4	Mean
T_1	2.0 BAP + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_2	3.0 BAP + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_3	4.0 BAP + 0.5 NAA	0.50	0.83	0.42	0.30	0.51	0.60	1.00	0.45	0.40	0.61
T_4	5.0 BAP + 0.5 NAA	0.62	0.90	0.55	0.32	0.61	0.61	1.25	0.50	0.47	0.71
T_5	6.0 BAP + 0.5 NAA	0.65	0.98	0.58	0.32	0.63	0.61	1.25	0.50	0.46	0.71
T_6	1.0 KIN + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_7	2.0 KIN + 0.5 NAA	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	0.00	-
T_8	2.0 KIN + 0.5 NAA	0.40	0.42	0.38	0.40	0.40	0.45	0.52	0.50	0.35	0.46
T_9	0.5 TDZ	0.60	0.70	0.60	0.40	0.58	0.60	0.63	0.60	0.45	0.57
T_{10}	1.0 TDZ	1.44	1.71	0.85	0.60	1.15	1.50	1.60	0.85	0.70	1.16
T_{11}	1.5 TDZ	1.45	1.80	0.90	0.75	1.23	1.70	1.67	0.85	0.77	1.25
T_{12}	2.0 TDZ	1.25	1.38	0.60	0.69	0.98	1.58	1.54	0.84	0.72	1.17
	Mean	0.87	1.09	0.61	0.47		0.96	1.18	0.64	0.54	
$CD_{0.05}$:	Treatment: 0.02; Cultivar: 0.01;	Interaction : 0.04					Treatment: 0.0.2 Cultivar: 0.01; Interaction 0.04				

V_1 = KtPL-19; V_2 = Pusa Sadabahar; V_3 = ArCH-001; V_4 = Salem.

elongate (Ochoa-Alejoet and Ireta-Moreno, 1990). There might be the case of apical dominance expressed by single growing shoot. The presented findings on callus mediated regeneration could also be important to explore the somaclonal variations, which is an alternative means of conventional hybridization.

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मिर्च को कृत्रिम परिस्थितियों (इन–विट्रो) में संजीवित करना मुश्किल होता है और इसके अप्रत्यक्ष अंग विकास में विभिन्न घटकों जैसे जीन प्ररूप, कर्तोत्तक एवं विकास नियामक इन विट्रो दशा में जीवोत्पत्ति हेतु महत्वपूर्ण भूमिका निभाते हैं। वर्तमान अध्ययन भारतीय मिर्च की चार किस्मों, केटीपीएल –19, पूसा सदाबहार, एआरसीएच–001 और सलेम, में कैलस मध्यस्थ अप्रत्यक्ष अंग विकास पुनर्जनन प्रोटोकॉल को मानकीकृत करने के लिए किया गया। कृत्रिम परिस्थितियों में उगाये हुए 21 दिन पुराने पौध के बीजपत्र एवं बीजपत्राधार को अलग कर कैलेसिंग एवं आगे की पुनर्जनन के लिए विभिन्न उपचार दिए गये। संवर्धन की शुरुआत के लिए पूर्ण सान्द्र मुराशीग और स्कूग (एमएस) माध्यम, अर्ध सान्द्र एमएस माध्यम (1/2 एमएस) और बी–5 माध्यम से उत्तम पाया गया। कैलस प्रवृत दोनों बीजपत्र (98.11 प्रतिशत) एवं बीजपत्राधार (97.97 प्रतिशत) में अधिक पाया गया जब पूर्ण सान्द्र मुराशीग और स्कूग (एमएस) माध्यम में 5.0 मिग्रा प्रति ली. 2,4 डाई क्लोरो फीनोक्सी एसिटिक एसिड (2,4–डी) एवं 1.0 मिग्रा. प्रति ली. काएनेटीन (केएन) मिला था। कैलस बहुगुणन के लिए एमएस + 5.0 मिग्रा. प्रति ली. 2,4–डी + 1.0 मिग्रा. प्रति ली. काएनेटीन (केएन) उपयुक्त पाया गया गया। बीजपत्राधार व्युत्पन्न कैलस पर प्ररोह कलिका तब उत्पन हुआ जब एमएस माध्यम में थाईडायाजुरोन (टीडीजेड) (1.5 मिग्रा. प्रति ली.) का उपयोग साइटोकाइनिन के रूप में किया गया।

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