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In vitro direct regeneration of Dalle Khursani (*Capsicum annum*) from salicylic acid treated explants

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Abstract
A study was conducted with cotyledon, shoot tip and hypocotyl explants derived from Dalle Khursani; an important chilli cultivar of Sikkim to estimate the *in vitro* regeneration efficiency of salicylic acid treated explants. Explants were cultured on MS media supplemented with different combination of plant growth regulators along with 500 µM salicylic acid. All the three explants type showed response to the growth media, however the response was best observed on cotyledon explants with an average of 2.75 shoots per explant. MS medium supplemented with 4 mg/l TDZ (Thidiazuron) and 500 µM salicylic acid was found to be best for *in vitro* direct regeneration from all the three types of explants with an average of 2.93 shoots per explant. MS medium containing 2 mg/l GA_3 (Giberrellic Acid 3) + 0.5 mg/l IAA (Indole Acetic Acid) was found to be suitable for both shoot elongation and rooting with mean shoot length of 3.10 ± 0.33 cm and 6.35 ± 0.98 roots per explant respectively.

Keywords: dalle khursani, regeneration, salicylic acid, capsaicin

Introduction
Chillies are the plant belonging to the genus *Capsicum* of the solanaceae family. *Capsicum* has its centre of origin in American tropics. *Capsicum* is derived from the Greek word 'kapsimo', meaning 'to bite'. There are thought to be 25-30 species of *Capsicum* of which 5 species; *C. annum* L, *C. frutescens* mill, *C. chinense*, *C. baccatum* L, and *C. pubescens* have been domesticated and cultivated (Kothari *et al.*, 2010) [13]. *Capsicum* is a self-pollinated dicot plant. However, there is an occurrence of cross pollination which leads to the formation of variants within the species. Hotness or pungency of the chilli pepper is due to the presence of capsaicin. It is an active component of chili peppers. Capsaicin is the main capsaicinoid in chilli peppers, followed by dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin (Reyes-Escogido *et al.*, 2011) [19]. Capsaicin present in chilli pepper is used as a counter irritant in Lumbago, Neuralgia, Rheumatic disorders and non-allergic Rhinitis; it has a tonic and carminative action. In combination with Cinchona, it is used for the treatment of intermittent and lethargic affliction, tympanitis and paralysis (Kothari *et al.*, 2010) [13]. The plants have also been used as folk remedies for dropsy, colic, diarrhea, asthma, arthritis, muscle cramp and toothache (Ravishankar *et al.*, 2003) [18].
Synthesis of capsaicin is influenced by the genotype and environment interaction (Harvell and Bosland, 1997) [9], therefore the capsaicin content of chilli can be manipulated by manipulating the culture conditions. It has been reported that the treatment of *Capsicum* tissues/cell with elicitor like salicylic acid and other environmental factors induces the capsacinoid synthesis including capsaicin (Nunez-Palenius and Ochoa-Alejo, 2005; Gutierrez-Carbajal *et al.*, 2010; Kehie *et al.*, 2012; Ancona-Escalante *et al.*, 2013) [16, 8, 12, 2]. However, there is no standardized protocol to obtain plant from those cell or tissue which has been treated with elicitor. Thus, standardization of protocol for regenerating chilli plants from explants which are treated with an elicitor like SA may help to produce chiili plants with higher capsaicin content.

Materials and Methods
Dalle Khursani (*Capsicum annuum)* Chromosome no: 2n=2x=24 (2n=2x=48 by Dafadar *et al*., 2012) [6] was used as material for the source of explants; it is one of the important genotype of chilli, which is extensively grown in the Indian state of Sikkim. It is grown in almost all part of the state and in its surrounding regions. It is grown for its pungent fruits which are used in pickles and sauces while the red dried fruits or their powder is used as hot spices. It is one of the hottest chilli pepper with a scoville rating of 100,000 to 350,000 SHU (Scovile Heat Unit) (For comparison King chilli has scoville rating of 330,000-1,000,000 SHU, Tabasco red

pepper sauces has rating of 2500-5000 SHU, and pure capsaicin has scoville rating of 16,000,000 SHU). The seeds were collected from Temi village, South Sikkim, India. The seeds were taken inside the laminar flow chamber and were surface sterilized with 70% ethanol for 10-15 sec, followed by sterilization with 2-4 % Sodium hypochlorite (NaClO) for 15-20 minutes, and after that seeds were washed thoroughly with sterile distilled water atleast 4-5 times to remove the traces of NaClO. Sterilized seeds were then inoculated in culture bottles containing MS basal media without any growth regulators for germination. After 15 to 20 days of inoculation, the explants namely, the cotyledon, shoot tip and hypocotyl were excised from the germinated seedlings.

Murashige and Skoog (MS) medium supplemented with different plant growth regulators which were previously identified in our lab for efficient regeneration of Dalle khursani (Bhutia *et al.,* 2016) [4] were used to estimate the regeneration efficiency of different explants of Dalle Khursani under Salicylic acid treatment. Explants excised from *in vitro* germinated seedlings were cultured on MS medium supplemented with various concentrations and combinations of growth hormones for direct regeneration along with 500 µM of salicylic acid (Table 1). Explants were cultured on salicylic acid containing media for 3-4 weeks. The cultures were maintained in the culture room at 25°C ± 0.5°C temperature, photoperiod regime of 16 hrs light and 8 hrs dark and with a relative humidity (RH) of 60%.

Table 1: Shoot induction media containing salicylic acid for direct regeneration

MS Media	Plant growth regulators (mg/l)					Salicylic acid (µM)
	TDZ	BAP	KIN	IAA	GA_3	
M_1	4.0	-	-	-	-	500
M_2	4.0	-	2.0	-	-	500
M_3	-	8.0	2.0	-	-	500
M_4	4.0	-	-	0.5	0.5	500
M_5	6.0	-	2.0	-	-	500

(TDZ = Thidiazuron; BAP = Benzyl Amino Purine; IAA = Indole Acetic Acid; KIN = Kinetin; NAA = Naphthalene Acetic Acid, GA3 = Giberrellic Acid 3)

Regenerated shoots were sub-cultured on MS media supplemented with various concentrations and combinations of growth regulators or on MS media without any growth regulators which were earlier found to be efficient (Bhutia *et al.,* 2016) [4] for shoot elongation and rooting (Table 2).

Table 2: Different concentration and combinations of growth regulators for shoot elongation and rooting

MS Media	Plant growth regulators (mg/l)			
	KIN	GA_3	NAA	IAA
ER_1	0.0	0.0	0.0	0.0
ER_2	0.0	2.0	0.5	0.0
ER_3	0.0	0.0	2.0	0.0
ER_4	0.0	2.0	0.0	0.5
ER_5	0.0	0.5	0.0	2.0

The observations that were recorded from the *In vitro* culture included the explants response percentage (percentage of explants responding to *In vitro* culture), number of shoots per explants, shoot length, number of roots and the root length. After 8 to 9 weeks of culture, the elongated and rooted seedlings were transferred into the mixture of artificial soil and autoclaved soil for hardening. Artificial soil was prepared by mixing Perlite, Vermiculite and Peat (1:1:1 ratio) and was further mixed with autoclaved soil at 1:1 ratio. Fully developed elongated shoots with roots were taken out from the culture tubes and the base portion was washed thoroughly with running water to remove the nutrient medium attached on it. Regenerated seedlings were transplanted on mixture of artificial soil and sterilized soil and then transferred to greenhouse for hardening. For each treatment, 10 replications were used and the experiment was repeated three times. Completely Randomized Design (CRD) was used as the experimental design and for statistical analysis statistical software SPSS version 17.0 was used.

Results and Discussion

Effect of explant

All the three explants types showed response to the growth media, however the response was best observed on cotyledon explants with an average of 2.75 shoots per explants, followed by hypocotyl explants with an average of 2.24 shoots per explant and shoot tip explants with an average of 1.57 shoots per explant (Table 3). However, the highest average response of 77.36 % was observed in shoot tip explants followed by hypocotyl explants with 64 % response and cotyledon explants with 58.87 % response. Analysis of variance showed significant differences among the means of the explants at 1% level of significance (Table 4).

Effect of media

The results showed that MS medium supplemented with 4 mg/l TDZ and 500 µM salicylic acid was best for *in vitro* direct regeneration from all the three types of explants with an average of 2.93 shoots per explant and response percentage of 73.14. Lowest response of 57.77 % was observed on MS medium supplemented with 6 mg/l TDZ + 2 mg/l KIN and 500 µM salicylic acid and lowest number of shoots per explant was observed on MS medium containing 8 mg/l BAP + 2 mg/l KIN and 500 µM salicylic acid (Table 3)

Effect of Media x Explant (Interaction)

Analysis of variance showed no significant differences among the interaction between explant x media (interaction) at 1% and 5% level of significance (Table 4). Irrespective of explants, MS medium containing 4 mg/l TDZ showed better result in terms of number of shoots regenerated per explant with 2.93 shoots per explants and irrespective of media, cotyledon explants showed best results in terms of number of shoots with an average of 2.75 shoots per explants (Table 3).

Table 3: Effect of growth regulators and salicylic acid on direct regeneration of shoots from explants of *Capsicum annuum* (Dalle khursani) after 4 weeks of culture

Media	Number of shoots/explants (Mean ± SE_M)			Media Mean
	cotyledon	shoot	Hypocotyls	
M1	3.33 ± 0.44	2.28 ± 0.24	3.19 ± 0.39	2.93
M2	2.69 ± 0.42	1.95 ± 0.19	2.38 ± 0.31	2.34
M3	2.20 ± 0.47	1.03 ± 0.12	1.33 ± 0.27	1.52
M4	3.06 ± 0.19	1.46 ± 0.19	2.33 ± 0.35	2.28
M5	2.50 ± 0.40	1.13 ± 0.17	1.96 ± 0.31	1.86
Explant Mean	2.75	1.57	2.24	
Critical Difference		0.05		0.01
CD (Explant)		0.76		1.24
CD (Media)		0.62		0.93

Table 4: Analysis of variance (ANOVA) showing significant differences among means of different explants and different combination of growth regulators with salicylic acid and non- significant differences among interaction between explants and media.

Anova					$F_{tabular}$	
SOV	**Df**	**SS**	**MSS**	**$F_{calculated}$**	**5%**	**1%**
Replication	2	0.170	0.085	1.094		
Treatment	14	21.939	1.567	20.066		
Explant (E)	2	10.608	5.304	67.923	3.2	5.1
Media (M)	4	10.235	2.558	32.766	2.22	3.05
(E x M)	8	1.094	0.136	1.752	1.91	2.5
Error	28	2.186	0.078			
Total	44	24.296				

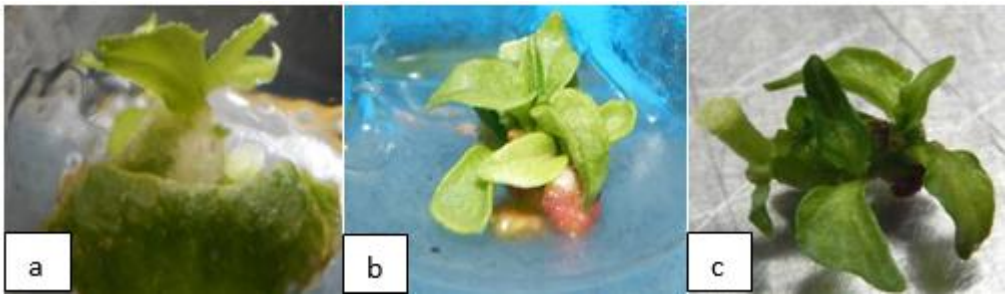

Fig 1: Direct regeneration on shoot induction media containing salicylic acid from (a) Cotyledon explant. (b) Shoot tip explant. (c) Hypocotyl explant.

Shoot elongation and rooting

Different combinations of growth regulators showed different effects on shoot elongation and rooting on sub cultured regenerated shoots. Some combination of growth regulators showed better result for shoot elongation and other combinations showed better result for root induction and root elongation.

Shoot elongation was observed better in MS medium containing 2 mg/l GA_3 + 0.5 mg/l IAA with mean shoot length of 3.10±0.33 cm, followed by MS medium without growth regulators with 3.00 ± 0.36 cm mean shoot (Figure 2). Similar combination was used by Kumar *et al*., 2012 [14], but the concentration of growth regulators was higher in the present case. There are various reports which suggested that the addition of GA_3 alone or in combination with either auxin or cytokinin in media was suitable for enhancing elongation of regenerated shoots (Kumar *et al*., 2005; Golegaonkar and Kantharajah, 2006; Joshi and Kothari, 2006; Channappagoudar, 2007; Verma *et al*., 2013; Pishbin *et al*., 2014) [15, 7, 11, 5, 22, 17]. MS media without any growth regulators was also found to be suitable for shoot elongation with an adequate shoot length of 3.00 ± 0.36 cm (Figure 2). There are various reports which also suggested the similar results (Hyde and Phillips, 1996; Ahmad *et al*., 2006; Siddique and Anis, 2006; Song *et al*., 2010) [10, 1, 20].

MS media containing 2 mg/l GA_3 + 0.5 mg/l IAA was best for root induction with an average of 6.35 ± 0.98 roots per explant (Figure 2) with 70 % response. Similar results were reported earlier where presence of IAA in media was suitable for root induction (Joshi and Kothari, 2006; Kehie *et al*., 2012, Dafadar *et al*., 2012) [11, 12, 6]. In previous reports MS media amended with NAA was best for root induction (Hyde and Philips, 1996; Dafadar *et al*., 2012) [10, 6]. In the present study, MS medium containing 2 mg/l NAA showed the second best results in terms of number of roots per shoot 5.55 ± 0.94 with a 55 % response. According to the present results, MS medium without any growth regulators was also best for root induction with an average of 4.60 ± 0.87 roots per shoot and 65 % response (Figure 2). This result was similar to those reported by Arous *et al*. (2001) [3] and Song *et al*. (2010) [21].

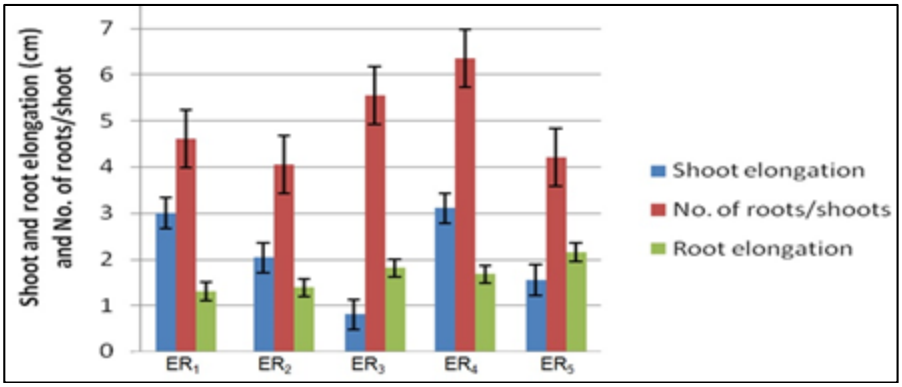

Fig 2: Graphical representation showing effects of different media composition on shoot elongation, root induction and root elongation on *In vitro* regenerated shoots

Hardening

In vitro regenerated, elongated and rooted seedlings of Dalle khursani (*Capsicum annuum*) were taken out from the culture tubes after 8-9 weeks of sub-culturing and the traces of *in vitro* growing media from the basal region or roots were removed by washing thoroughly with running tap water.

Plants were than transplanted into mixture of artificial soil and normal soil at 1:1 ratio. The cultures were kept inside the laboratory where temperature and lights were under controlled conditions for hardening of tender regenerated shoots for at least 15-20 days after which they were transferred to the green house and transplanted into bigger pots. In this experiment, more than 70 % of *in vitro* regenerated and rooted seedlings survived hardening.

Fig 4: (a) Shoot elongation (b) rooting and (c) hardening of *In vitro* regenerated seedlings

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