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Optimization of *in vitro* conditions favourable for effective regeneration in Pusa Meghna Indian cauliflower

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ABSTRACT

Establishment of an efficient *in vitro* regeneration system is a pre-requisite for genetic modification of plants. The present study reports a high efficiency regeneration protocol for cauliflower cv. Pusa Meghna using cotyledon and hypocotyl explants cultured on different growth media with varying combinations of plant growth regulators. Maximum shoot regeneration response from hypocotyl (95.76%) and cotyledon (83.01%) was obtained on MS medium containing 1mg/L BAP and 1 mg/L BAP+0.1 mg/L NAA+0.5 mg/L $AgNO_3$+0.1 mg/L GA_3, respectively. Further optimization of rooting media for development of full grown plants showed maximum root regeneration of 92.20% and 94.86 % from hypocotyl and cotyledon explants grown on MS medium containing 0.5 mg/L IBA and 0.2 mg/L IBA, respectively. Utilization of the present established regeneration protocol would serve as a valuable tool for various crop improvement studies in cauliflower.

Key words: *Brassica oleracea* var. *botrytis*, cole crop, *in vitro* plant regeneration, hypocotyl, cotyledon.

INTRODUCTION

Brassicaceae family comprises of economically and nutritionally important highly diverse group of vegetable crops such as cauliflower, cabbage, broccoli, brussels sprouts, collards, savoy cabbage, kohlrabi, rutabaga, kale and turnip (Bhalla *et al*., 1, Singh *et al*., 22). Cauliflower is a cool season crop cultivated globally for its edible inflorescence known as curd. It exhibits anticancer activity due to the presence of glucosinates (Zhang *et al*., 26). India comes next after China in terms of production, accounting for annual production of 18.8 MT from an area of 452100 hectares (NHB, 16).

Pusa Meghna is an early maturing Indian cauliflower variety developed by the ICAR-Indian Agricultural Research Institute, New Delhi with an average yield of 12.5 t/ha. It is attacked by a large number of insect pests such as diamondback moth (*Plutella xylostella*), cabbage aphid (*Brevicoryne brassicae*), cabbage semilooper (*Helicoverpa armigera*) cabbage butterfly (*Pieris brassicae*), cabbage looper (*Tricho plusiani*), which negatively effects the yield of the crop (Gaur *et al*., 7). Development of genetic resistance through traditional breeding has been hindered due to the unavailability of resistance genes in the crossable germplasms. Also according to certain reports cauliflower is recalcitrant to genetic transformation (Puddephat *et al*., 20; Passelegue *et al*., 18).

Genetic modifications of crops *via Agrobacterium* mediated transformation rely on efficient plant regeneration system which in turn is skill dependent and is influenced by a large number of uncharacterized physiological factors such as type of explants, nutrients, hormones, gelling agent and other additives (Bhalla *et al*., 2; Siong *et al*., 24; Gerszberg *et al*., 9; Gambhir *et al*., 6). Different researchers have attempted *in vitro* regeneration in cauliflower under the influence of various parameters such as explants (cotyledon, hypocotyl, floral tissues, protoplasts) and media composition (Narasimhulu *et al*., 15; Bhalla *et al*., 2; Chikkala *et al*., 5; Pavlovic *et al*., 14; Yu *et al*., 25; Siong *et al*., 24; Gambhir *et al*., 8; Gaur *et al*., 7). Among these, hypocotyls and/or cotyledons received considerable attention due to their high potential for shoot organogenesis, somatic embryogenesis and protoplast culture (Cardoza *et al*., 3). Also, 6-benzylaminopurine (BA) and thidiazuron (TDZ) have been shown to be much effective in shoot induction (Bhalla *et al*., 1; Qin *et al*., 21; Yu *et al*., 25; Gaur *et al*., 7). TDZ is reported to aid in the rapid shoot regeneration in a number of plant species (Yu *et al*., 25; Gambhir *et al*., 6 and Gaur *et al*., 7). Further, additives like silver nitrate ($AgNO_3$) are known to inhibit ethylene and wound stress, thus, promoting regeneration efficiency followed by shooting (Chi *et al*., 4; Palmer 17). Thereby the present study aims to establish an effective *in vitro* regeneration system in Pusa Meghna by optimizing the factors governing regeneration which included the explant, media and plant growth regulators such as 6-benzylaminopurine

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(BA), thidiazuron (TDZ), α-naphthalene acetic acid (NAA), 2,4-dichloro phenoxy acetic acid (2,4-D), gibberellic acid (GA_3), silver nitrate ($AgNO_3$).

This highly efficient and reproducible regeneration protocol is now successfully being used for the transformation of Pusa Meghna with *Agrobacterium tumefaciens* harbouring *cry1b/cry1c* to confer protection against a devastating insect pest diamondback moth (*Plutella xylostella*).

MATERIALS AND METHODS

Pusa Meghna seeds were procured from the Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi. First, the seeds were treated with Tween-20 to remove surface contaminants. Then the explants were treated with 70% ethanol for 2-3 min followed by washing with sterile water for 5-6 times. Thereafter, the seeds were sterilized with 0.1% $HgCl_2$ for 3 min and washed thoroughly with sterile water for 5-6 times under aseptic conditions. The sterilized seeds were then blotted on sterile filter paper and allowed to dry. After drying the seeds were inoculated on MS and B5 medium containing 3% sucrose and 0.8% agar for seed germination (Murashige and Skoog 14; Gamborg *et al*., 8). To maintain the aseptic conditions, the medium was autoclaved at 15 lbs pressure for 15 min followed by conductance of experiment in laminar air flow chamber. The cultures were grown at 25±2°C temperature and 70-80 % humidity under 16/8 h light/dark photoperiod in the culture room with light intensity of 40µmol $m^{-2}s^{-1}$ using cool fluorescent lamps.

For efficient regeneration, expanded green and turgid cotyledons and hypocotyls (0.5-1.0 cm) developed from 5-6 day old seedlings were used (Fig. 1a, 1b) and cultured on MS basal and B5 medium supplemented with varying concentrations of BAP, TDZ, IBA alone or in combinations of BAP+2,4-D, BAP+NAA, BAP+GA_3, BAP+$AgNO_3$, BAP+NAA+GA_3+$AgNO_3$, respectively. For every treatment, 5 replicates each with 20-25 explants were set up and further each experiment was repeated thrice. The shoot regeneration was monitored at every 7 day time interval. Explants were evaluated for percent shoot regeneration based on the mean number of shoots/ explant. The elongated regenerated shoots (2-3 cm) developed from explants were isolated and individual shoots were cultured on root induction MS and B5 medium containing varying concentrations of IBA (0.1-0.5 mg/L). Transferred shoots were evaluated for percent root induction post 4 weeks of culturing.

The plantlets thus, developed were washed with luke warm water specifically roots to remove traces of adhered agar and then were transferred into a test tube containing water and kept in plant tissue culture room for 3-5 days. These plantlets were further transferred into soilrite pots and covered with perforated polythene bags in order to maintain humidity and gaseous exchange. The pots were then transferred to phytotron and kept for hardening for 10-12 days. The perforated bags were removed and the plants were kept for one week under same conditions. The plants were then transferred into pots containing potting mixture consisting of soil, sand and FYM (2:1:1), which were then ready to transfer under natural environmental conditions (Fig. 1g, 1h).

The experiment was conducted in completely rendomized design. The statistical analysis based on mean values per treatment was carried out for the analysis of variance (ANOVA) with Tukey test using SPSS 23 (IBM, Chicago, USA).

RESULTS AND DISCUSSION

Initially, to check the effect of BAP on shoot regeneration on cotyledon and hypocotyl explants, two different medium i.e. MS and B5 were supplemented with varying concentrations of BAP. Direct organogenesis was observed after 22-25 days for hypocotyl explants and 30-32 days for cotyledon explants. From the data, it is quite evident that BAP at 1 mg/L was found to induce effective multiple shoot regeneration with 95.76 percent shoot regeneration, and 8.33 average number of shoots/ hypocotyl in MS medium, while the corresponding values were found to be 80.39 and 6.33 in B5 medium, respectively. Likewise, under similar concentrations of BAP, percent shoot regeneration, and average number of shoots/explant from cotyledon were found to be 72.19 and 6.00 in MS medium while the corresponding values were found to be 56.12 and 4.00 in B5 medium, respectively (Table, Fig. 1d). As evident from our studies, higher concentration of BAP (1 mg/L) had a significant effect on shoot regeneration and number of shoots/explant produced from cotyledons or hypocotyls cultured on either MS or B5 medium (Table 1). However, the response of hypocotyls was much better both in terms of shoot regeneration and number of shoots/explant as compared to cotyledons (Table 1). Bhalla *et al*. (2) reported that there is significant role of BAP in shoot differentiation in cauliflower and we also had similar findings. Earlier, it was observed that BAP either alone or in combination with hormones has been shown to have a potential role for shoot regeneration and multiplication in other crucifers (Metz *et al*., 12; Jin *et al*., 10; Munshi *et al*., 13; Maheshwari *et al*., 11).

As noted earlier, since BAP at 1 mg/L was much effective in shoot regeneration, the effects of different

Table 1: Effect of BAP on shoot regeneration from cotyledon and hypocotyl explants of Pusa Meghna cauliflower on MS and B5 medium.

Treatment	Cotyledon		Hypocotyl	
	Average percent shoot regeneration	Average no. of shoots/explant	Average percent shoot regeneration	Average no. of shoots/explant
MS+0.2mg/L BAP	19.19^{ef} ±0.51	0.00^{e}±0.00	55.73^{c} ± 0.42	3.33^{cd}±0.33
MS+0.4mg/L BAP	24.97^{e} ±0.28	1.67^{cd}±0.33	52.72^{d} ± 0.50	3.00^{d}±0.00
MS+0.6mg/L BAP	23.05^{ef}±0.40	0.67^{de}±0.33	55.92^{c} ± 0.43	4.00^{cd}±0.33
MS+0.8mg/L BAP	39.02^{c} ±0.30	2.67^{bc}±0.33	66.12^{b} ± 0.82	4.67^{c}±0.33
MS+1.0mg/L BAP	72.19^{a} ±0.19	6.00^{a}±0.58	95.76^{a} ± 0.88	8.33^{a}±0.33
B5+0.2mg/L BAP	18.02^{f} ±1.16	1.67^{cd}±0.33	42.29^{f} ± 0.64	2.67^{d}±0.00
B5+0.4mg/L BAP	18.14^{f} ±0.90	2.00^{cd}±0.00	46.42^{e} ± 0.55	3.00^{d}±0.33
B5+0.6mg/L BAP	18.17^{f}±0.81	2.00^{cd}±0.00	49.99^{d} ± 0.47	3.67^{cd}±0.33
B5+0.8mg/L BAP	31.89^{d} ±0.71	2.67^{bc}±0.33	51.84^{d} ± 0.51	4.67^{c}±0.33
B5+1.0mg/L BAP	56.12^{b} ±0.59	4.00^{b}±0.00	80.39^{a} ± 0.22	6.33^{b}±0.33

*Different letters for mean per cent shoot regeneration show significant differences after ANOVA and Tukey's standartized range (hsd) test ($p<0.05$). sd=standard deviation

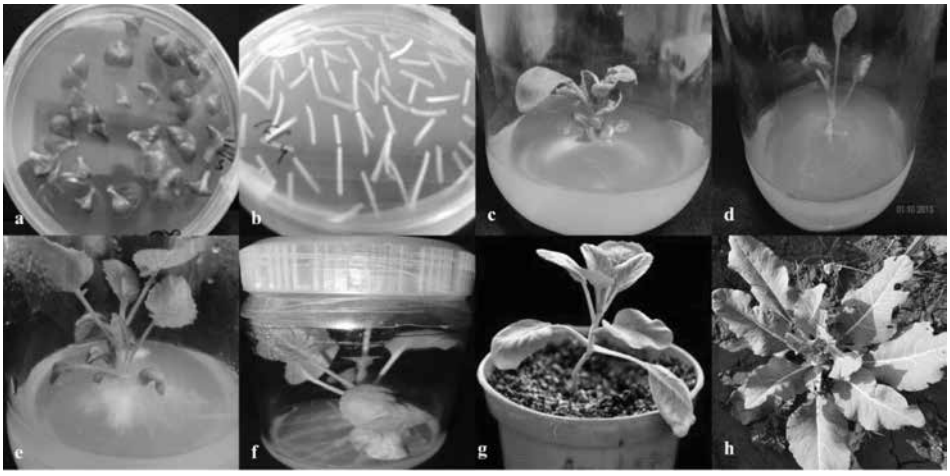

Fig. 1. Efficient plant regeneration from cotyledon and hypocotyl explants of Pusa Meghna cauliflower (*Brassica oleracea* L. var. *botrytis*) **a)** Aseptically inoculated cotyledon explants on MS basal medium **b)** Aseptically inoculated hypocotyl explants on MS basal medium **c)** Shoot regeneration from cotyledon explants on medium supplemented with (MS+1mg/L BAP+0.1mg/LNAA+0.1mg/LGA3+0.5mg/L$AgNO_3$) **d)** Shoot regeneration from hypocotyl explants on medium supplemented with (MS+1mg/IBAP) **e)** Root regeneration from the *in vitro* developed shoot regeneration from hypocotyl explant on the medium supplemented with MS+0.5mg/IIBA **f)** Root regeneration from the *In vitro* developed shoot regeneration from cotyledon explant on the medium supplemented with (MS+0.2mg/LIBA) **g)** Fully developed plantlets of cauliflower transferred into pot **h)** Acclimatized plants transferred to the field.

components i.e. 2,4-D, NAA, GA_3, $AgNO_3$ on shoot regeneration from cotyledon and hypocotyl explants was studied by supplementing 1 mg/L of BAP in MS and B5 medium.

Among the different combinations of hormones in MS and B5 medium containing 1 mg/L BAP, the highest shoot regeneration and no. of shoots/explant from cotyledon in MS+1 mg/L BAP+0.1 mg/L NAA+0.1 mg/L GA_3+0.5mg/L $AgNO_3$ was found to be 83.01 and 3.33 whereas the corresponding value was found to be 38.32 and 1.67 in B5 medium, respectively (Table 2, Fig. 1c). In hypocotyl, shoot regeneration and no. of shoots/explant cultured in MS+1 mg/L BAP+0.1 mg/L 2,4-D was found to be 66.92 and 3.00 whereas in B5 medium it was 45.18 and 3.67, respectively (Table 2). Using different permutation

and combination of hormones, media and explants, it was deciphered that shoot regeneration and no. of shoots/ explant was higher in MS medium compared to B5 medium. Also, shoot regeneration was observed higher when cotyledon was used as an explant rather than hypocotyl and *vice versa* was true for shoots/explants. This might be due to the synergistic effects of the hormones supplemented in the regeneration medium. Bhalla *et al*. (2) also reported that enhanced shoot regeneration required at least two growth regulators, and in our experiment this criterion was found to be true in cotyledon explant. Radke *et al*. (22) also observed that the long-term effect of $AgNO_3$ leads to the vitrification of shoots from the hypocotyl of *Brassica rapa*. In our study, $AgNO_3$ alone or in combination with NAA and GA_3 had a positive effect on percent shoot regeneration and number of shoots/explant of cotyledons in MS medium containing 1 mg/L BAP (Table 2). On the contrary, $AgNO_3$ impacted shoot regeneration from hypocotyls but the number of shoots/ explant were enhanced (Table 2).

To check the effect of TDZ on multiple shoot regeneration from hypocotyl and cotyledon explants, varying concentrations of TDZ was supplemented in MS and B5 medium. The highest per cent shoot regeneration and number of shoots/explant from hypocotyl cultured on MS medium with 0.2 mg/L TDZ was found to be 79.99 and 2.67, while the corresponding values from cotyledons cultured on MS medium with 0.4 mg/L was found to be 71.69 and 2.0, respectively (Table 3). Our findings are not in accordance with Gaur *et al*., 7, where they observed positive effect of TDZ on shoot regeneration from hypocotyls, whereas cotyledons showed a very poor response and that too at higher concentrations. This might be attributed to the varied response of the genotype under study. As noted in our study, higher concentrations of TDZ had a negative impact on shoot regeneration. Similar observations as well as shoot deformation were also noted by Yu *et al*., (25).

To examine the effect of IBA on root induction from shoots developed from hypocotyl and cotyledon explants. *in vitro* developed shoots were excised and cultured on MS and B5 medium supplemented with varying concentrations of IBA. Root induction was noted after 10-12 days. IBA at lower concentrations had positive effect on root induction from cotyledon derived shoots whereas higher concentrations favoured root induction from shoots developed from hypocotyls cultured either on MS or B5 medium (Table 4). Maximum root induction was observed from cotyledons (94.86) at 0.2 mg/L, whereas for hypocotyl it was 92.20 at 0.5 mg/L on MS medium (Table 4, Fig. 1e, 1f). Gaur *et al*., 7 observed maximum root induction on MS medium containing 0.05 % IBA. Under all experimental conditions, the MS medium was found to be optimal for efficient shoot regeneration as compared to B5 medium. However, the cotyledon and hypocotyl explants showed a varied response in *in vitro* regeneration according to the supplement of various growth regulators either alone or in combination.

Table 2: Effect of BAP, NAA, $AGNO_3$, GA_3 (in MS and B5) on the shoot regeneration from cotyledon and hypocotyl explants of Pusa Meghna indian cauliflower.

Treatment	Cotyledon		Hypocotyl	
	Average percent shoot regeneration	Average no. of shoots/ explant	Average percent shoot regeneration	Average no. of shoots/ explant
MS+1mg/LBAP+0.1mg/L2,4-D	44.44^{e}±1.61	1.33^{d}±0	66.92^{a}±3.81	3.00^{b}±0.33
MS+1mg/LBAP+0.1mg/LNAA	49.44^{d}±0.60	1.67^{c}±0.33	56.16^{b}±3.38	3.67^{ab}±0.33
MS+1mg/LBAP+0.5mg/L$AgNO_3$	61.81^{c}±0.79	2.00^{abc}±0.33	44.87^{de}±2.22	3.00^{b}±0.33
MS+1mg/LBAP+0.1mg/LGA_3	72.22^{b}±1.92	2.67^{ab}±0.33	51.44^{c}±4.12	4.67^{a}±0.33
MS+1mg/LBAP+0.1mg/LNAA+0.5mg/L$AgNO_3$+0.1mg/LGA_3	83.01^{a}±2.19	3.33^{a}±0.57	40.99^{ef}±1.53	4.67^{a}±0.33
B5+1mg/LBAP+0.1mg/L2,4-D	16.68^{h}±1.42	0.00^{d}±0.33	45.18^{d}±2.39	3.67^{ab}±0.58
B5+1mg/LBAP+0.1mg/LNAA	16.97^{h}±0.84	0.67^{cd}±0	51.42^{c}±4.36	4.33^{ab}±0.33
B5+1mg/LBAP+0.5mg/L$AgNO_3$	26.91^{g}±1.63	1.00^{cd}±0	38.69^{fg}±0.61	3.67^{ab}±0.33
B5+1mg/LBAP+0.1mg/LGA_3	29.81^{g}±0.84	1.33^{bcd}±0.33	38.76^{fg}±1.44	3.67^{ab}±0.33
B5+1mg/LBAP+0.1mg/LNAA+0.5mg/L$AgNO_3$+0.1mg/LGA_3	38.32^{f}±0.25	1.67^{bc}±0	36.35^{g}±0.46	3.67^{ab}±0.00

*Different letters for mean per cent shoot regeneration show significant differences after ANOVA and Tukey's standardised range (hsd) test ($p<0.05$). sd= standard deviation.

Table 3: Effect of various concentrations of TDZ alone (in MS and B5 medium) on shoot regeneration medium from cotyledon and hypocotyl explants of Pusa Meghna indian cauliflower.

Treatment	Cotyledon		Hypocotyl	
	Average percent shoot regeneration	Average no. of shoots/explant	Average percent shoot regeneration	Average no. of shoots/explants
MS+0.2mg/LTDZ	60.88^{b}±1.05	1.66^{ab}±0.33	79.99^{a}±0.23	2.67^{a}±0.33
MS+0.4mg/LTDZ	71.69^{a}±0.31	2.00^{a}±0.33	56.59^{c}±0.47	0.67^{e}±0.33
MS+0.6mg/LTDZ	57.13^{c}±0.44	1.00^{bc}±0.33	41.84^{f}±0.78	0.00^{d}±0
MS+0.8mg/LTDZ	52.82^{e}±0.51	0.66^{bcd}±0.33	38.76^{g}±0.72	0.00^{d}±0
MS+1.0mg/LTDZ	46.42^{f}±0.55	0.00^{d}±0	31.35^{i}±0.78	0.00^{d}±0
B5+0.2mg/LTDZ	54.54^{d}±0.48	1.00^{bc}±0.33	69.99^{b}±0.35	1.67^{b}±0.33
B5+0.4mg/LTDZ	36.72^{g}±0.75	0.00^{d}±0	45.27^{e}±0.59	0.00^{d}±0
B5+0.6mg/LTDZ	33.95^{h}±0.72	0.00^{d}±0	49.99^{d}±0.52	0.67^{c}±0.33
B5+0.8mg/LTDZ	29.81^{i}±0.71	0.00^{d}±0	33.32^{h}±0.71	0.00^{d}±0
B5+1.0mg/LTDZ	15.07^{j}±0.93	0.00^{d}±0	22.76^{j}±0.73	0.00^{d}±0

*Different letters for mean per cent shoot regeneration show significant differences after ANOVA and Tukey's standartized range (hsd) test ($p<0.05$). sd= standard deviation.

Table 4: Effect of various concentrations of IBA alone (on MS and B5 medium) on root regeneration from cotyledon and hypocotyl explants of Pusa Meghna indian cauliflower.

Treatment	Cotyledon	Hypocotyl
	Percent Root induction	Percent Root induction
MS+0.1 mg/L IBA	85.89±2.74	35.67±1.23
MS+0.2 mg/L IBA	94.86±0.57	39.57±0.73
MS+0.3 mg/L IBA	58.07±0.15	52.71±0.49
MS+0.4 mg/L IBA	46.44±0.37	62.95±0.39
MS+0.5 mg/L IBA	38.56±0.35	92.20±0.13
B5+0.1 mg/L IBA	90.10±0.72	40.85±1.24
B5+0.2 mg/L IBA	87.07±0.79	50.02±1.29
B5+0.3 mg/L IBA	44.46±1.5	39.60±0.66
B5+0.4 mg/L IBA	37.23±0.71	42.84±0.67
B5+0.5 mg/L IBA	33.32±0.68	83.60±0.16

*Different letters for mean per cent root regeneration show significant differences after ANOVA and Tukey's standardized range (hsd) test ($p<0.05$). sd= standard deviation.

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Conflicts of interest: The authors declare that they have no conflict of interest.

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