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High frequency regeneration of plants from cotyledon and hypocotyl cultures in *Brassica oleracea* cv. Pride of India

Geetika Gambhir, Pankaj Kumar*, D.K. Srivastava

Department of Biotechnology, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan 173230, Himachal Pradesh, India

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ABSTRACT

Morphogenic potential of cabbage cv. Pride of India, for multiple shoot induction was tested under *in vitro* conditions using cotyledon and hypocotyl explants. Aseptically grown seven to nine days old seedlings of cabbage were used as source of explants for reproducible plant regeneration studies. Forty different concentrations and combinations of TDZ (alone), TDZ with adenine, TDZ with NAA and TDZ with IAA were tried. Maximum shoot regeneration response from cotyledon explants (91.11%) and hypocotyl (94.40%) was obtained on MS medium containing 0.330 mg/l TDZ + 79.70 mg/l Adenine and 0.220 mg/l TDZ + 0.088 mg/l IAA, respectively. Rooting was achieved within two to three weeks on all the rooting media, but MS medium containing 0.10 mg/l NAA produced the maximum number of strong and healthy roots (100%). The regenerated complete plantlets with healthy roots and shoot system were transferred to pots containing sterilized cocopeat and successfully acclimatized and no phenotypic variations were observed among regenerated plants. Highly efficient, reproducible plant regeneration protocol has been standardized in cabbage cv. Pride of India, which would be valuable for *Agrobacterium*-mediated gene transfer studies in cabbage.

1. Introduction

Brassica vegetables are an economically important and highly diversified group of crops such as cabbage, broccoli, Brussels sprouts, cauliflower, collards, Savoy cabbage, kohlrabi, rutabaga, and turnip [1,2,3], belonging to the family *Brassicaceae*. Cabbage "nutrient rich" economically important vegetable crop with higher amount of vitamins C, K, A and folic acid, fiber, flavonoids, proteins, minerals and connected with secondary metabolites called glucosinolates contributed to anticarcinogenic properties [4,5]. Cabbage cv. "Pride of India" is recommended for cultivation in the Himachal Pradesh by Department of Vegetable Science, Dr Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India and is used in crop breeding program because of its high yield potential and early maturity. In, India especially in Himachal Pradesh, it is widely grown as off-season vegetable crop and for seed production. Various stress (biotic and abiotic) results in quantitative and qualitative yield losses [5,6,7]. Generally, this crop is damaged by cabbage butterfly worm or fungal diseases caused by *Botrytis cinerea*, *Alternaria brassicola*, *Plasmodiophora brassicae*, or *Pythium* spp. To improve the yield of *B. oleracea*, considerable research has been conducted to optimize tissue culture and transformation protocols [5,7]. For genetic improvement of cabbage using genetic engineering, an efficient and reproducible *in vitro* regeneration protocols required. *In vitro* plant regeneration studies have been carried out in cabbage using different explants such as cotyledons [8–15], and hypocotyl [8,9,16,17,11,18,19,15,20,7]. Thidiazuron (TDZ), a substituted phenylurea (*N*-phenyl *N*'-1,2,3-thiadiazol-5-ylurea) has high efficacy in induction of morphogenic response *in vitro,* initially used as a cotton defoliant [21], and aids in rapid shoot regeneration of a number of plant species [22–26,20]. In this paper, we report high frequency shoot regeneration potential of cotyledon and hypocotyl explants in cabbage cv. Pride of India using cytokinin TDZ.

2. Material and methods

2.1. Plant material and culture medium

The certified seeds of cabbage cv. Pride of India were obtained from Vegetable Sciences department of Dr Y.S. Parmar University of Horticulture and Forestry, Solan. The seeds were surface sterilized and inoculated on the MS half strength basal medium [27])

Abbreviations: MS, Murashige and Skoog; IAA, Indole-3-acetic acid; NAA, Naphthalene acetic acid; TDZ, Thidiazuron.

* Corresponding author.
E-mail addresses: geetikabt@gmail.com (G. Gambhir), pksharmabiotech@gmail.com (P. Kumar), dksuhf89@gmail.com (D.K. Srivastava).

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containing 0.5 percent sucrose for seed germination. *In vitro* grown seven-nine days old seedlings were used as a source of hypocotyl and cotyledon explants for plant morphogenesis (Fig. 1a). The age of explant is related with the age of *in vitro* grown aseptic seedlings from where the explants (hypocotyl and cotyledon) were excised. Completely green fully expanded cotyledons and hypocotyl which were greenish in colour and turgid nature from seven to nine days old aseptically grown seedlings were used for efficient shoot regeneration studies. Different concentrations and combinations of thidiazuron along with auxins i.e. TDZ alone, TDZ + Adenine, TDZ + NAA, TDZ + IAA were used in the MS basal medium for efficient plant regeneration response. Stock solution of Thidiazuron (TDZ) was prepared by dissolving 10 mg of TDZ in minimum amount of DMSO and the final volume was made to 10 ml by using double distilled water. The pH of the culture medium was adjusted to 5.8 before agar–agar addition to the medium. Then medium was

Fig. 1. Efficient plant regeneration from cotyledon and hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).
a. Aseptically germinated 7–9 days old seedlings of cabbage cv. Pride of India.
b. Callus formation and shoot initiation from cotyledon explants on medium supplemented with (MS+ 0.33 mg/l TDZ + 79.9 mg/l Adenine).
c. Shoot regeneration from cotyledon explants on medium supplemented with (MS+ 0.33 mg/l TDZ + 79.9 mg/l Adenine).
d. Callus formation and shoot initiation from hypocotyl explants on medium supplemented with (MS+ 0.22 mg/l TDZ + 79.9 mg/l Adenine).
e. Shoot regeneration from hypocotyl explants on medium supplemented with (MS+ 0.22 mg/l TDZ + 79.9 mg/l Adenine).
f. Root regeneration from the *in vitro* developed shoot regeneration from explants on the medium supplemented with (MS + 0.10 mg/l NAA).
g. Fully developed plantlets of cabbage taken out of medium (after proper washing) after 15 days of culturing showing well developed root system. (Later on transferred to pots containing pre-sterilized cocopeat for acclimatization).
h. Regenerated plantlets transferred to plastic pots containing mixture of pre-sterilized cocopeat covered with polythene bags for hardening.
i. Plantlets transferred to potting mixture (containing sand + soil + FYM).

poured in different culture vessels and sterilized at 15 pounds per square inch for 15 min in an autoclave. Aseptic manipulations were carried out in laminar air flow chamber. All the inoculated cultures were grown at 26 ± 2 °C temperature and 70–80% humidity under 16/8 h light/dark photoperiod in the culture room with light intensity of 40 μmol m^{-2} s^{-1} using cool white fluorescent lamps.

2.2. Plant regeneration from cotyledon and hypocotyl explants

For obtaining high efficiency shoot induction, cotyledon and hypocotyl (0.5–1 cm) explants were excised and cultured on MS medium with various combination and concentrations of TDZ (mg/l) alone, TDZ with adenine (mg/l), TDZ with NAA (mg/l) and TDZ with IAA (mg/l). For every combination, 5 flasks with 5 explants each were inoculated and each experiment was repeated thrice. Observations were taken at the interval of 7 days till shoot regeneration. Explants were evaluated for percent shoot regeneration with mean number of shoots per explants. After every four weeks, regenerated elongated shoots were subcultured. The regenerated shoots (2–3 cm), obtained from both the explants were separated and individual shoot was transferred to the root induction MS medium containing different auxins concentrations of IAA, NAA and IBA to get a complete plantlet. Transferred shoots were evaluated for percent root regeneration after 4 weeks of culturing.

2.3. Hardening of regenerated plantlets

After proper *in vitro* roots and shoot development, the plantlets were carefully taken out so that no damage should occur to their delicate root system. The roots were lightly washed in running tap water to get rid of adhering medium. *In vitro* raised plantlets after washing were hardened using sterilized cocopeat mixture and sheltered with polythene bags to maintain relative humidity. The plantlets were finally transferred to pots containing sterilized potting mixture (consisting of sand + soil + FYM). The potted plantlets were kept under varying conditions of humidity and light intensity and observed for growth/survival.

2.4. Statistical analysis

Data recorded for the different parameters used in the plant regeneration experiments were subjected to completely randomized design (CRD). The statistical analysis based on mean values per treatment was made using analysis of variance for completely randomized design [28].

3. Results

3.1. Multiple shoot induction from cotyledon and hypocotyl explants

Cotyledon and hypocotyl explants were excised from seven to nine days old aseptically grown seedlings and cut into small pieces of 0.5–1.0 cm in size, which were cultured on MS medium supplemented with various combinations and concentrations of TDZ (mg/l), TDZ + Adenine (mg/l), TDZ + NAA (mg/l) and TDZ + IAA (mg/l). The colour of explants did not change. No change in colour of media was observed and Shoot elongation and multiplication was achieved on the same shoot regeneration medium.

3.1.1. Effect of TDZ (in MS medium) on shoot regeneration

Out of ten different concentrations of TDZ for shoot regeneration tried. Direct organogenesis was observed after 70–75 and 33–35 days of culturing for cotyledon and hypocotyl explants, respectively. Maximum per cent shoot regeneration (48.88%) and (90.73%) was obtained from cotyledon and hypocotyl explants on MS basal medium supplemented with 0.220 mg/l TDZ (Table 1).

3.1.2. Effect of TDZ and adenine (in MS medium) on adventitious shoot bud regeneration

Ten different combinations and concentrations of TDZ and Adenine were used for shoot regeneration. During early days of cultures, the explants began to expand and swelled. Slight callusing was observed around cut surfaces of cotyledon explants after 28–30 days. In hypocotyl explants callus initiation was observed after 15–18 days in all media. Adventitious shoot bud formation/shoot regeneration from cotyledon and hypocotyl explants started after 55–60 and 32–35 days of culturing respectively. Maximum per cent shoot regeneration (91.11%) with maximum number of shoots per explant (2.997) was obtained from the cotyledon explants on MS basal medium containing 0.330 mg/l TDZ with 79.70 mg/l adenine. Whereas, in case of cultured hypocotyl explants, maximum shoot regeneration response (92.59%) with highest number of shoots per explants (2.94) were observed in MS basal medium supplemented with 0.220 mg/l TDZ + 79.7 mg/l adenine. Direct shoot regeneration was observed after 35–40 days of culturing in cotyledon explants (Table 2, Fig. 1b–e).

3.1.3. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration

MS medium supplemented with ten different combinations and concentrations of TDZ and NAA were used for shoot regeneration.

Table 1
Effect of various concentrations of TDZ alone (in MS medium) on shoot regeneration from cotyledon and hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).

Medium composition (MS basal medium)	Average number of shoots regenerated per explants	Percent shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
	Cotyledon		Hypocotyl	
0.110 mg/l TDZ	0.506	26.66(30.97)	1.347	55.55(48.20)
0.220 mg/l TDZ	1.063	48.88(44.36)	2.423	90.73(72.47)
0.330 mg/l TDZ	1.197	46.66(43.09)	2.627	87.03(68.98)
0.440 mg/l TDZ	0.840	35.55(36.59)	1.070	47.03(43.30)
0.550 mg/l TDZ	0.486	26.66(30.97)	2.440	74.07(59.42)
0.660 mg/l TDZ	0.796	35.55(36.59)	2.160	75.92(60.65)
0.770 mg/l TDZ	0.463	22.22(28.07)	2.477	77.77(61.87)
0.880 mg/l TDZ	0.573	26.66(31.09)	2.810	88.89(70.93)
0.990 mg/l TDZ	0.510	24.44(29.47)	1.700	57.40(49.31)
1.100 mg/l TDZ	0.730	33.33(35.19)	1.810	61.11(51.45)

*The values in parenthesis are arc sine transformed values.
$CD_{0.05}$ 0.1965 8.547(5.435) 0.9637 7.869(5.470).
SE ± 0.0942 4.097(2.605) 0.0941 3.772(2.622).

Table 2
Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from cotyledon and hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).

Medium composition (MS basal medium)	Average number of shoots regenerated per explants	Percent shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
	Cotyledon		Hypocotyl	
0.110 mg/l TDZ + 79.7 mg/l adenine	2.930	88.88(70.73)	2.347	87.03(68.98)
0.220 mg/l TDZ + 79.7 mg/l adenine	0.773	26.66(31.09)	2.947	92.59(74.42)
0.330 mg/l TDZ + 79.7 mg/l adenine	2.997	91.11(72.88)	2.070	75.92(60.56)
0.440 mg/l TDZ + 79.7 mg/l adenine	1.373	48.51(44.13)	1.070	35.18(36.37)
0.550 mg/l TDZ + 79.7 mg/l adenine	1.530	46.66(43.03)	1.610	62.96(52.52)
0.660 mg/l TDZ + 79.7 mg/l adenine	0.773	35.00(36.26)	1.980	64.81(53.68)
0.770 mg/l TDZ + 79.7 mg/l adenine	1.910	66.66(54.80)	1.883	64.81(53.63)
0.880 mg/l TDZ + 79.7 mg/l adenine	0.530	22.22(28.07)	1.607	59.26(50.39)
0.990 mg/l TDZ + 79.7 mg/l adenine	1.350	44.44(41.80)	1.477	57.41(49.28)
1.10 mg/l TDZ + 79.7 mg/l adenine	1.197	42.22(40.52)	1.790	61.09(51.48)

*The values in parenthesis are arc sine transformed values.
$CD_{0.05}$ 0.2040 9.779(6.197) 0.1762 9.453(5.873).
SE ± 0.0978 4.668(2.971) 0.0844 4.532(2.815).

Adventitious shoot bud formation from cotyledon and hypocotyl explants started after 30–35 and 27–35 days of culture. Maximum per cent shoot regeneration (84.44%) with mean number of shoots per explant (2.57) were obtained from the cotyledon explants on MS basal medium containing 0.990 mg/l TDZ + 0.02 mg/l NAA in cotyledon explants. Whereas, in cultured hypocotyl explants, maximum shoot regeneration (81.48%) with average number of shoots per explants (2.05) were observed on MS basal medium supplemented with 0.880 mg/l TDZ and 0.02 mg/l NAA (Table 3).

3.1.4. Effect of TDZ and IAA (in MS medium) on shoot bud regeneration

Out of ten different combinations and concentrations of TDZ and IAA used for multiple shoot regeneration response. Shoot regeneration from both explants started after 27–35 days of culturing. Maximum per cent shoot regeneration (66.66%) with mean number of shoots per explant (1.91) were obtained from the cotyledon explants on MS basal medium containing 0.990 mg/l TDZ with 0.088 mg/l IAA and in hypocotyl, maximum shoot regeneration (94.44%) with maximum average number of shoots per explants (2.99) were observed on MS basal medium supplemented with 0.220 mg/l TDZ + 0.088 mg/l IAA (Table 4).

3.2. Root induction and hardening of regenerated plantlets

In vitro developed shoots were excised and cultured on MS medium containing different concentrations of IAA, NAA and IBA. Root regeneration was observed after two weeks. Maximum percent root regeneration was observed on NAA (100%), IAA (97.20%) and IBA (80%), respectively. After 18–25 days, healthy and vigorous root system has been developed. Little callusing was observed only on medium supplemented with IAA (Table 5). After the well developed root and shoot system, cabbage plantlets were taken out of the culture tubes, taking various precautions to avoid any damage to its delicate root system. The root portion of the

Table 3
Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from cotyledon and hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).

Medium composition (MS basal medium)	Average number of shoots regenerated per explants	Percent shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
	Cotyledon		Hypocotyl	
0.110 mg/l TDZ + 0.02 mg/l NAA	1.220	46.66(43.08)	1.680	51.85(46.06)
0.220 mg/l TDZ + 0.02 mg/l NAA	0.910	53.33(47.69)	1.810	64.81(53.71)
0.330 mg/l TDZ + 0.02 mg/l NAA	2.287	73.33(59.64)	1.957	75.89(60.62)
0.440 mg/l TDZ + 0.02 mg/l NAA	1.817	57.78(49.48)	1.363	51.85(46.06)
0.550 mg/l TDZ + 0.02 mg/l NAA	1.550	55.55(48.20)	1.570	59.26(50.34)
0.660 mg/l TDZ + 0.02 mg/l NAA	1.597	60.00(50.77)	1.163	35.18(36.37)
0.770 mg/l TDZ + 0.02 mg/l NAA	2.197	66.66(54.80)	1.477	61.11(51.42)
0.880 mg/l TDZ + 0.02 mg/l NAA	2.530	80.00(63.44)	2.050	81.48(64.56)
0.990 mg/l TDZ + 0.02 mg/l NAA	2.573	84.44(66.87)	0.903	35.18(36.37)
1.10 mg/l TDZ + 0.02 mg/l NAA	1.350	44.44(41.80)	1.180	50.00(45.00)

*The values in parenthesis are arc sine transformed values.
$CD_{0.05}$ 0.1837 19.445(12.215) 0.1445 6.458(3.929).
SE ± 0.0880 9.321(5.855) 0.0693 3.096(1.883).

Table 4
Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from cotyledon and hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).

Medium composition (MS basal medium)	Average number of shoots regenerated per explants	Percent shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
	Cotyledon		Hypocotyl	
0.110 mg/l TDZ + 0.088 mg/l IAA	0.000	0.00(0.00)	2.253	81.46(64.55)
0.220 mg/l TDZ + 0.088 mg/l IAA	0.000	0.00(0.00)	2.997	94.44(76.36)
0.330 mg/l TDZ + 0.088 mg/l IAA	0.000	0.00(0.00)	2.107	74.07(59.42)
0.440 mg/l TDZ + 0.088 mg/l IAA	0.000	0.00(0.00)	2.810	90.73(72.47)
0.550 mg/l TDZ + 0.088 mg/l IAA	0.000	0.00(0.00)	2.457	83.51(66.05)
0.660 mg/l TDZ + 0.088 mg/l IAA	1.153	44.44(41.80)	1.497	59.40(50.43)
0.770 mg/l TDZ + 0.088 mg/l IAA	0.950	46.66(43.09)	1.423	53.70(47.13)
0.880 mg/l TDZ + 0.088 mg/l IAA	0.863	28.88(32.48)	1.980	59.26(50.34)
0.990 mg/l TDZ + 0.088 mg/l IAA	1.910	66.66(54.73)	0.997	33.33(35.26)
1.10 mg/l TDZ + 0.088 mg/l IAA	1.087	44.44(41.80)	1.627	51.85(46.06)

*The values in parenthesis are arc sine transformed values.
$CD_{0.05}$ 0.1250 3.588(2.134) 0.1854 4.606(3.207).
SE ± 0.0599 1.720(1.5 0.0890 2.208(1.537).

Table 5
Effect of different concentrations of various auxins on per cent root regeneration from *in vitro* developed shoots of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).

Medium Composition (MS basal medium)	IAA	NAA	IBA
0.05 mg/l	97.20(84.39)	77.78(61.97)	69.44(56.49)
0.10 mg/l	97.20(84.39)	100(90.00)	80.55(63.94)
0.20 mg/l	86.09(68.32)	94.40(78.77)	71.66(56.84)
$CD_{0.05}$ 7.796(10.628)			
SE ± 3.711(5.058)			

*The values in parenthesis are arc sine transformed values.

plantlets were then placed over the sterilized cocopeat and covered with sterilized cocopeat gently to full capacity of the cup. The plantlets were covered with polythene bags and watered daily, to maintain high humidity. After two to three weeks, plants showed emergence of new leaves, they were uncovered. Finally, transferred to the pots containing sterilized potting mixture (Sand: soil: FYM mixture). The plants were watered adequately daily. Maximum percent survival was 80% percent and showing morphological uniformity with no phenotypic variations among regenerated plants (Table 5; Fig. 1f–i).

4. Discussion

Plant regeneration studies are genotypic specific and the cabbage cultivar i.e. Pride of India has not been worked out so far using thidiazuron; synthetic phenylurea cytokinin like compound that has been proven to be highly effective regulator of shoot morphogenesis in hypocotyl and cotyledon explants. Cabbage cultivar i.e. Pride of India is the most commercially successful cultivar grown in Himachal Pradesh and other part of India. In order to establish an efficient genetic transformation system in the cultivar, we considered it necessary to test the efficiency of plant regeneration from cotyledon and hypocotyl explants for experimental use.

In the present investigation, young tender, healthy seven to nine days' old aseptically grown seedlings were used as a source of explants (cotyledon and hypocotyl). TDZ is a synthetic phenylurea cytokinin has been confirmed to be highly effective regulator of shoot morphogenesis [29–33,20]. It is also effective for shoot regeneration in recalcitrant species [34–37]. The effect of various concentrations of thidiazuron alone and in combination with Adenine, NAA and IAA were studied for enhancing the shoot regeneration frequency from hypocotyl and cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India). Different explants responded with different intensity under *in vitro* conditions. Several published literature for *Brassica* species regeneration whether they include transformation step or not, are based on TDZ [38–44,26], reported that the TDZ based media found to be very efficient for enhancing the frequency of shoot regeneration in *Brassica* species.

In the present investigation, out of 40 combinations and concentration of thidiazuron (alone), Thidiazuron +Adenine, Thidiazuron +NAA and thidiazuron +IAA tried for both the explants. Hypocotyl explants showed maximum percent shoot regeneration 94.44% on TDZ and proved it better explant for its high efficacy for maximum shoot induction response. These results were in agreement with the published literature by Cheng et al. [40] and Ravanfar et al. [45]. Lu et al. [41] reported that TDZ was more efficient and rapid for shoot regeneration and regeneration frequency reached 98.8% on MS medium containing 0.25 mg/l TDZ + 0.5 mg/l NAA and 5.0 mg/l $AgNO_3$ in cotyledon explants. Fan et al. [14] compared the effect of BA and that TDZ and found TDZ was more effective in inducing shoot regeneration in cotyledon explants of Chinese cabbage. [46] also reported high efficiency shoot regeneration from hypocotyl explants using TD [20] reported thidiazuron was found to be superior over the various cytokinin in promoting shoot regeneration from leaf and petiole explants in cabbage. Only five medium (Thidiazuron + IAA) for cotyledon explants were ineffective, this might be due to synergistic effect of supplemented hormones for regeneration studies. Similar results were reported by Kumar and Srivastava Kumar and Srivastava (2015a,b) in *Brassica* cv. Solan Green Head.

Successful rooting of *in vitro*-derived shoots is an integral part of each plant regeneration protocol. Different auxins i.e. IAA, IBA and NAA were tested for root regeneration ability from *in vitro* developed shoots. 100% root induction response was observed using 0.10 mg/l NAA. These results are in agreement with the observations of [5] in different cabbage cultivars Amager', 'Kamienna głowa', 'Sława of Enkhuizen', and 'Brunświcka'. Similar results have been reported by Kumar and Srivastava [46,47] in broccoli cv. Solan green head. Chen et al. [48] used half strength MS medium containing 0.1 mg/l NAA for regeneration of roots. Memon et al. [49] also used different concentration of NAA for rooting and found 88% root regeneration on medium containing 0.3 mg/l NAA. High percentage (97.20%) of root regeneration was also observed on MS medium containing IAA, but MS media containing NAA was most effective and produced the maximum number of strong and healthy roots. The regenerated complete plantlets of cabbage were

successfully acclimatized having morphological uniformity. No apparent phenotypic variations were observed among the regenerated plants.

5. Conclusion

The goal of present investigation was to develop an efficient, reliable and high-frequency plant-regeneration protocol for the introduction of a desirable gene in cabbage, was successfully achieved. This study revealed that 0.22 mg/l TDZ + 0.088 mg/l IAA and 0.33 mg/l TDZ + 79.70 mg/l adenine were effectual in evoking morphogenic responses from hypocotyl and cotyledon explants of cabbage cultivar "Pride of India" with multiple shoot induction response and can be favorably exploited for *in vitro* genetic manipulation.

Conflict of interest

The authors have no conflict of interest to declare.

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