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ORIGINAL ARTICLE

Effect of potent cytokinin thidiazuron on in vitro morphogenic potential of broccoli (*Brassica oleracea* L. var. *italica*), an important vegetable crop

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Abstract Broccoli (*Brassica oleracea* var. *italica*) is an important nutritionally rich vegetable cole crop. Environmental stress, pests and diseases cause enormous yield losses because of a limited gene pool. Genetic manipulation is becoming an important method for broccoli improvement. The objective of the present study was to evaluate the potency of thidiazuron (TDZ) as a plant growth regulator in evoking morphogenic responses in hypocotyl and cotyledon explants of broccoli. Hypocotyl and cotyledon explants were used from 10 to 12 days old in vitro grown seedlings of broccoli and cultured on MS medium supplemented with wide range of TDZ concentrations for efficient in vitro shoot regeneration, viz., TDZ alone, TDZ with adenine, TDZ with naphthalene acetic acid (NAA) and TDZ with indole acetic acid (IAA). Among the 36 combinations of growth regulators used, the maximum percentage of shoot regeneration was observed in the hypocotyl explants (95.92 %) on MS medium supplemented with 2.0 µM TDZ and 0.5 µM IAA. The multiple shoot regeneration response of cotyledon explants producing shoot (88.88 %) were observed on MS medium supplemented with 2.0 µM TDZ and 0.59 mM adenine. Shoot multiplication and elongation were observed on the same medium. High frequency (93.99 %) of root regeneration was observed in in vitro regenerated shoots on MS medium supplemented with 1.08 µM NAA. The regenerated plantlets with well-developed shoots and root system were transferred to the pots containing a mixture of sand and soil and acclimatized. We recommend 2.0 µM TDZ and 0.5 µM IAA, and 2.0 µM TDZ and 0.59 mM adenine combinations for adventitious shoot regeneration from hypocotyl and cotyledon explants in broccoli cv. Solan green head respectively. This is the first report on high frequency organogenesis in broccoli cv. Solan green head using TDZ from India.

Keywords In vitro regeneration · Plant growth regulators · Shoot induction · Acclimatization · Genetic improvement

Introduction

Establishment of reproducible and highly efficient plant regeneration protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. Broccoli (*Brassica oleracea* var. *italica*) is an important vegetable crop belonging to *Brassica* species, which is nutritionally rich, high in vitamin A and C, soluble fiber, selenium, and anticancerous property attributable to sulforaphane, resulting from glucoraphanin (Kirsh et al. 2007). However, various biotic and abiotic stresses cause enormous crop yield losses during commercial cultivation of broccoli (Cao and Earle 2003; Yang et al. 2004). Broccoli cv. "Solan green head" was used in the present experiment because of its good yield potential and early maturity. However, this cultivar is severely affected by insect pests, such as diamondback moth (*Plutella xylostella*), cabbage looper (*Trichoplusia* sp.), beetles (*Phyllotreta cruciferae*) and aphids (*Brevicoryne brassicae*). Being an economically important crop, application of plant tissue culture and genetic engineering can be used to add target characteristics to broccoli cultivars, thus providing

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an opportunity to improve broccoli in many respects. For genetic improvement of broccoli via genetic transformation, an efficient and reproducible in vitro regeneration protocols required.

In vitro plant regeneration studies in broccoli using several types of explants have been reported by various workers, viz., peduncle (Christey and Earle 1991), anther (Chang et al. 1996), hypocotyl (Zhong and Li 1993; Puddephat et al. 2001; Kim and Botella 2002; Ravanfar et al. 2009; Huang et al. 2011), leaf tissue (Robertson and Earle 1986; Cao and Earle 2003; Kumar and Srivastava 2015) and cotyledon (Qin et al. 2006; Ravanfar et al. 2011). Thidiazuron, (*N*′-phenyl-*N*′-1,2,3-thidiazol-5-ylurea, TDZ) is a phenyl urea that has gained importance as a potent plant growth regulator (PGR) for in vitro regeneration systems of various crops (Murthy et al. 1995; Guo et al. 2005; Ravanfar et al. 2014). Despite the high efficacy of TDZ in inducing morphogenic responses in vitro, there is only one report (Ravanfar et al. 2014), where TDZ in an appropriate ratio with NAA has been used for efficient shoot regeneration from cotyledon explants of broccoli cv. Green Dragon King. To our knowledge, there are no reports available on morphogenic response of hypocotyl and cotyledon explants of broccoli (*B. oleracea* L. var. *italica*) using this potent cytokinin.

In this paper, we report a high-frequency shoot-regeneration ability of hypocotyl and cotyledon explants of broccoli (*B. oleracea* L. var. *italica* cv. Solan green head) using TDZ.

Materials and methods

Plant material and culture medium

The certified seeds of broccoli (*B. oleracea* L. var. *italica*) cv. Solan green head were obtained from the Department of Vegetable Science, Dr Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). The uniform and healthy seeds were selected, and used for aseptic germination. The seeds were thoroughly washed with teepol and then under running tap water and finally soaked in water for half an hour. After soaking the seeds were surface sterilized for 1 min in 0.1 % $HgCl_2$ and then rinsed 3–4 times with sterilized distilled water to remove traces of $HgCl_2$. The seeds were inoculated on half strength MS basal medium (Murashige and Skoog 1962) containing 0.5 % sucrose for germination. Aseptically grown 10–12 days old seedlings of broccoli were used as a source of explants (cotyledon and hypocotyl) for plant regeneration studies. The explants were cut into 0.5–1.0 cm size and cultured on MS medium supplemented with different concentration of growth regulators for shoot regeneration experiment. The pH of the medium was adjusted to 5.8 before adding agar–agar to the medium. The medium was poured in culture vessels and sterilized at 15 pounds per square inch for 15 min in an autoclave. All the aseptic manipulations were carried out under laminar air flow chamber. After the inoculation all the cultures were kept in the culture room at 26 ± 2 °C and 70–80 % humidity under 16/8 h light/dark photoperiod, with light intensity of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Plant regeneration from hypocotyl and cotyledon explants

Hypocotyl and cotyledon (0.5–1.0 cm) explants were excised and inoculated on the MS medium containing different concentrations of TDZ, TDZ and Adenine, TDZ and NAA and TDZ and IAA to optimize the culture medium for high frequency shoot regeneration (Tables 1, 2, 3, 4). The explants were evaluated for percent shoot regeneration and average number of shoots per explant. Shoots were sub-cultured on fresh medium every 4 weeks. The regenerated shoots (2–3 cm) obtained from both the explants were excised and individual shoot was transferred to the MS medium supplemented with three different concentrations of NAA (0.27, 0.54, 1.08 μM) for the root regeneration. The transferred shoots were then evaluated for the percent root regeneration after 25 days of inoculation.

Hardening of regenerated plantlets

The regenerated plantlets with well developed shoot and root system were carefully taken out and washed gently under running tap water in order to remove the traces of adhering medium. After removal of the medium, the plantlets were kept in running tap water for a few minutes so that they do not wilt after transfer to soil. Pots were filled half with sterilized soil and sand mixture in equal proportion and half with sand. The plantlets with washed roots were transferred to the pots. The plants were watered and covered with polythene bags to maintain high relative humidity. Water was sprayed twice a day to maintain high relative humidity. After a week when plants showed initial signs of establishment in pots with appearance of new leaves, polythene bags were temporarily removed daily for few hours. The plants were finally transferred to earthen pots and were acclimatized further in glass house. The percentage survivals of the hardened plants were recorded after 4–5 weeks of transfer to the pots.

Table 1 Effect of various concentrations of TDZ (in MS basal medium) on shoot regeneration from hypocotyls and cotyledon explants of broccoli (*Brassica oleracea* L.var. *italica*)

Medium composition [MS basal + TDZ (μM)]	Hypocotyl		Cotyledon	
	Average no. of shoots explants^{-1}	Shoot regeneration (%)	Average no. of shoots explants^{-1}	Shoot regeneration (%)
0.25	2.96	74.81 (59.86)[a]	1.08	43.33 (41.14)[a]
0.5	3.73	79.26 (62.89)	**1.51**	**53.33 (46.90)**
0.75	2.11	76.66 (61.10)	1.16	34.44 (35.91)
1.0	4.31	77.78 (61.90)	1.06	29.25 (32.70)
1.25	2.44	76.66 (61.10)	1.10	40.44 (39.43)
1.5	**4.22**	**81.11 (64.23)**	1.06	35.55 (36.57)
1.75	3.23	72.22 (58.17)	0.95	29.25 (32.72)
2.0	3.18	62.96 (52.50)	0.82	22.00 (27.91)
2.5	3.11	62.22 (52.10)	1.26	35.55 (36.57)
LSD ($p \leq 0.05$)	1.020	5.866 (3.644)	0.331	7.297 (4.351)
SE±	0.341	1.959 (1.217)	0.111	2.437 (1.453)

Bolded values represents highest mean number of shoots/explant with highest percent shoot regeneration

[a] The values in the parenthesis are arc sine transformed values

Table 2 Effect of various concentrations and combinations of TDZ and adenine (in MS basal medium) on shoot regeneration from hypocotyl and cotyledon explants of broccoli (*Brassica oleracea* L. var. *italica*)

Medium composition (MS basal+)		Hypocotyl		Cotyledon	
TDZ (μM)	Adenine (mM)	Average no. of shoots explants^{-1}	Shoot regeneration (%)	Average no. of shoots explants^{-1}	Shoot regeneration (%)
0.25	0.59	3.17	82.96 (65.66)[a]	2.14	68.51 (55.84)[a]
0.50	0.59	**4.15**	**91.10 (72.85)**	3.02	66.66 (54.78)
0.75	0.59	3.16	81.11 (64.21)	2.06	54.81 (47.74)
1.0	0.59	3.37	91.10 (72.85)	1.62	42.22 (40.50)
1.25	0.59	3.04	71.48 (57.70)	1.93	73.33 (58.91)
1.5	0.59	2.63	60.37 (50.96)	3.17	81.11 (64.23)
1.75	0.59	1.92	67.77 (55.38)	2.78	82.59 (65.34)
2.0	0.59	3.95	82.22 (65.12)	**3.46**	**88.88 (70.70)**
2.5	0.59	3.66	75.55 (60.39)	2.09	51.10 (45.61)
LSD ($p \leq 0.05$)		0.553	5.041 (4.184)	0.468	6.173 (4.154)
SE±		0.185	1.684 (1.397)	0.156	2.062 (1.387)

Bolded values represents highest mean number of shoots/explant with highest percent shoot regeneration

[a] The values in the parenthesis are arc sine transformed values

Statistical analysis

Each treatment consisted of at least 30 explants and each experiment was repeated thrice. The data recorded for the different parameters were subjected to "completely randomized design". The statistical analysis based on mean values per treatment was made using analysis of variance of CRD (Gomez and Gomez 1984).

Results and discussion

Multiple shoot induction from hypocotyl and cotyledon explants

Hypocotyl explants showed swelling at margins after 4–6, where as cotyledon explants, showed initial expansion after 1 week of culturing on different combinations and

Table 3 Effect of various concentrations and combinations of TDZ and NAA (in MS basal medium) on shoot regeneration from hypocotyl and cotyledon explants of broccoli (*Brassica oleracea* L.var. *italica*)

Medium composition (MS basal+)		Hypocotyl		Cotyledon	
TDZ (μM)	NAA (μM)	Average no. of shoots explants^{-1}	Shoot regeneration (%)	Average no. of shoots explants^{-1}	Shoot regeneration (%)
0.25	0.107	0.84	28.88 (32.49)[a]	1.08	57.40 (49.24)[a]
0.5	0.107	0.99	34.99 (36.24)	0.99	51.10 (45.61)
0.75	0.107	1.02	41.11 (39.86)	1.33	64.44 (53.37)
1.0	0.107	1.64	44.44 (41.78)	1.64	72.96 (58.64)
1.25	0.107	1.86	49.99 (44.98)	1.83	82.22 (65.04)
1.5	0.107	**1.94**	**51.10 (45.61)**	**2.86**	**83.33 (65.94)**
1.75	0.107	1.12	34.81 (36.13)	2.44	76.29 (60.88)
2.0	0.107	0.58	25.55 (30.32)	1.57	48.88 (44.34)
2.5	0.107	0.95	46.84 (43.15)	1.15	37.22 (37.56)
LSD ($p \leq 0.05$)		0.213	6.502 (3.805)	0.368	5.386 (3.380)
SE±		0.071	2.171 (1.271)	0.123	1.799 (1.129)

Bolded values represents highest mean number of shoots/explant with highest percent shoot regeneration

[a] The values in the parenthesis are arc sine transformed values

Table 4 Effect of various concentrations and combinations of TDZ and IAA (in MS basal medium) on shoot regeneration from hypocotyl and cotyledon explants of broccoli (*Brassica oleracea* L.var. *italica*)

Medium composition (MS basal+)		Hypocotyl		Cotyledon	
TDZ (μM)	IAA (μM)	Average no. of shoots explants^{-1}	Shoot regeneration (%)	Average no. of shoots explants^{-1}	Shoot regeneration (%)
0.25	0.5	2.87	82.22 (65.04)[a]	–[b]	–[b]
0.5	0.5	3.66	88.88 (70.70)	–[b]	–[b]
0.75	0.5	3.14	82.59 (65.32)	–[b]	–[b]
1.0	0.5	3.52	87.03 (69.43)	–[b]	–[b]
1.25	0.5	3.05	88.51 (70.19)	–[b]	–[b]
1.5	0.5	4.11	91.47 (73.29)	–[b]	–[b]
1.75	0.5	3.26	87.03 (68.97)	–[b]	–[b]
2.0	0.5	**4.28**	**95.92 (80.44)**	–[b]	–[b]
2.5	0.5	4.06	93.70 (75.44)	–[b]	–[b]
LSD ($p \leq 0.05$)		0.370	6.219 (7.195)		
SE±		0.124	2.077 (2.403)	–[b]	–[b]

Bolded values represents highest mean number of shoots/explant with highest percent shoot regeneration

[a] The values in the parenthesis are arc sine transformed values

[b] In case of cotyledon explants, only callus formation takes place, there was no shoot or root differentiation from callus even after eight weeks of culturing

concentrations of growth regulators used. The colour of the explants turned purple green from green, while no change in the colour of the media was observed. Callus initiation at the cut ends was observed after 13–15 days in hypocotyl explants, whereas in cotyledon explants callus initiation was observed between 18 and 20 days. Shoot initiation was observed between 27 and 30 days in hypocotyl explants and between 40 and 42 days in case of cotyledon explants (Fig. 1A–D). After 7–8 weeks, significant differences were observed among the treatments for percentage of explants forming shoots. Highest shoot induction response was observed in case of hypocotyl explants, highest percentage of shoot formation (95.92 %) and mean number of shoots (4.28) resulted from the treatment containing 2.0 μM TDZ and 0.5 μM IAA. Meanwhile, for cotyledon explants, highest percent shoot regeneration (88.88 %), with highest mean number of shoots (3.46) per explants were observed on MS medium supplemented with 2.0 μM TDZ and

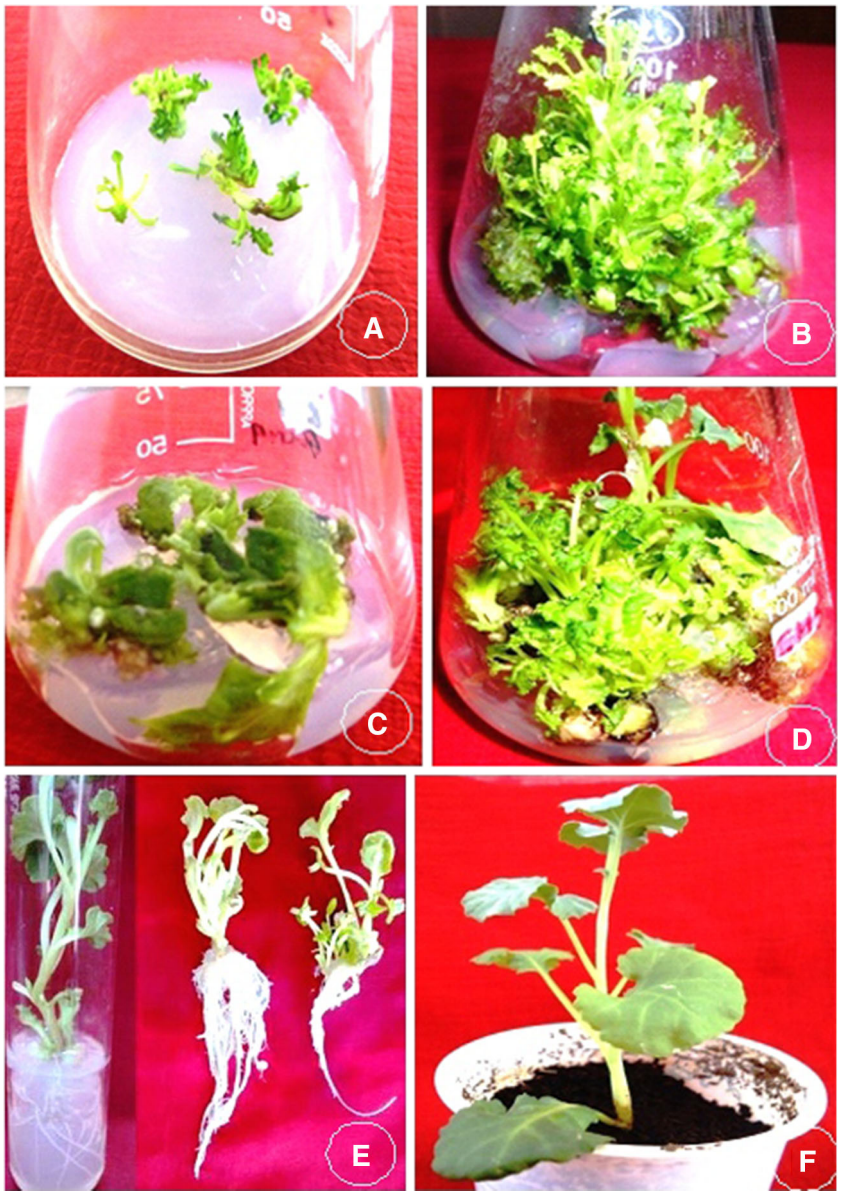

Fig. 1 Plant regeneration in broccoli (*Brassica oleracea* L. *italic*) cv. Solan green head: shoot regeneration from hypocotyl explants on MS medium supplemented with 2.0 µM TDZ and 0.5 µM IAA (**A**), multiple shoot regeneration from hypocotyl explants on MS medium supplemented with 2.0 µM TDZ and 0.5 µM IAA (**B**), shoot regeneration from cotyledon explants on MS medium supplemented with, and 2.0 µM TDZ and 0.59 mM adenine (**C**), multiple shoot induction from cotyledon explants on MS medium supplemented with 2.0 µM TDZ and 0.59 mM adenine (**D**), root regeneration in in vitro regenerated shoots on MS medium supplemented with 1.08 µM NAA and development of in vitro plantlet (**E**) and successful acclimatization of in vitro developed plantlet in the pot after 30 days (**F**) (colour figure online)

0.59 mM adenine. Ravanfar et al. (2014) reported that TDZ in an appropriate ratio with NAA increased shoot formation from cotyledon explants of broccoli cv. Green Dragon King. Varied responses could be due to genotypic differences of the cultivar or plants reacting differently during in vitro organogenesis.

A total of 36 combinations of plant growth regulators were used for shoot regeneration studies. In the case of hypocotyl explants, out of nine concentrations of TDZ used, highest percent shoot regeneration (81.11 %) with highest mean number of shoots (4.22) per explants was observed on MS medium containing 1.5 µM TDZ, whereas in the case of cotyledon explants, highest mean number of shoots per explant was 1.51 with 53.33 % shoot regeneration on MS medium supplemented with 0.5 µM TDZ. Out of nine different combinations of TDZ and adenine, the hypocotyl explants showed highest shoot regeneration response (91.10 %) with highest mean number of shoots (4.15) with 0.5 µM TDZ + 0.59 mM adenine treatment combination. However in the case of cotyledon explants, highest shoot regeneration was (88.88 %) with a mean number of shoots per explant of 3.46 on MS medium containing 2.0 µM TDZ and 0.59 mM adenine (Tables 1, 2). Out of nine different concentrations and combinations of TDZ and NAA, 0.5 µM TDZ + 0.107 µM NAA showed highest percent shoot regeneration of 51.10 % with 1.94 mean number of shoots per explant in case of hypocotyl explants and 83.33 % shoot regeneration and 2.86 average number of shoots per explant in case of cotyledon explants (Table 3).

Table 5 Effect of various concentrations of NAA (in MS basal medium) on per cent root regeneration from in vitro developed shoots of broccoli (*Brassica oleracea* L. var. *italica*)

Medium composition MS (half strength) basal medium + NAA (μM)	Percent root regeneration
0.27	78.15 (8.897)[a]
0.54	81.11 (9.061)
1.08	**93.99 (9.746)**
LSD ($p \leq 0.05$)	1.365
SE±	0.387

Bolded values represent highest percent root regeneration

[a] The values in the parenthesis are square root transformed values

Highest shoot regeneration response (95.92 %) and average number of shoots (4.28) were observed on MS medium supplemented with 2.0 μM TDZ + 0.5 μM IAA. In case of cotyledon explants callus initiation was observed after 12–14 days of culturing and callus proliferation after 25–28 days of culturing. There was no shoot or root differentiation from callus even after 8 weeks of culturing (Table 4).

Root induction and acclimatization of regenerated plantlets

In vitro developed elongated shoots (about 2–3 cm long) from hypocotyl and cotyledon were excised and cultured on MS medium supplemented with different concentration of NAA. Root regeneration started after 10–14 days of inoculation. The high percent root regeneration response (93.99 %) was observed on MS medium containing 1.08 μM NAA and well developed complete plantlets with shoot and root system were observed after 20–22 days of culturing. Lazzeri and Dunwell (1986) reported that in some cases high concentration of NAA was more effective for root regeneration as compared to high concentration of IAA or IBA. Ravanfar et al. (2009) reported that medium supplemented with 0.2 mg l^{-1} IBA was most suitable for root regeneration. Similarly Sharma et al. (2014) reported that the growth regulator IBA resulted in highest percentage of root regeneration. The regenerated complete plantlets of broccoli were successfully acclimatized with 75 % plantlets survival frequency, having morphological uniformity (Table 5; Fig. 1E, F).

Conclusion

The primary aim of our work to develop a reliable and high-frequency plant-regeneration protocol for the introduction of a desirable gene in broccoli, was successfully achieved. This study showed that 2.0 μM TDZ and 0.5 μM IAA, and 2.0 μM TDZ and 0.59 mM adenine were effective in evoking morphogenic responses from hypocotyl and cotyledon explants of broccoli cultivar "Solan green head" with highest shoot induction, and can be favorably exploited for genetic engineering purposes. To the best of our knowledge this is the first report on high frequency organogenesis in broccoli cv. Solan green head using thidiazuron from India.

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