STANDARDIZATION OF IN VITRO INDIRECT SHOOT REGENERATION PROTOCOL FOR INDIAN MUSTARD, BRASSICA JUNCEA (L) USING CYTOKININ, THIODIAZURON AS AN INDUCER

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Abstract: High frequency callus mediated rapid shoot organogenesis was induced in 2d old coyledonary explants of *Brassica juncea* cv Pusa bold, inoculated onto the MS media supplemented with very low concentration of thidiazuron (N-phenyl-N'-1, 2, 3-thidiazol-5-yl) urea) TDZ (0.1-1 µM). Highest number of Shoots (14-15) regenerated in vitro were obtained with 0.5 µM TDZ. This morphogenetic response of *B.juncea* is genotype dependent, amongst the other cultivars tested e.g. pusa bold,pusa bahar,9304,luxmi,T-59,B9,RH-30 and PJK,Pusa bold found to be most responsive both in regeneration frequency and in number of shoots/explants. Of the four explants e.g. hypocotyls, shoot tip, cotyledon and cotyledonary node, cotyledon produced maximum shoots/explants. The calli produced by 2 day old cotyledons of *B. juncea* on TDZ were subcultured twice on the same media, which produced about 8-9 and 5-6 shoots in 1st and 2nd subculture respectively. The regenerated shoots were rooted with 100% frequency on MS media supplemented with Indole-3-butyric acid (IBA; 1 µM) and mature fertile plants were developed in pots which showed a normal phenotype. Our experiments indicate that TDZ is a very potential factor to induce a rapid, high frequency shoot regeneration from young cotyledonary explants via a callus mediated phase in pusa bold ,which is a high yielding variety of Indian mustard widely grown in India.

Keywords: Agricultural, *Brassica*, Cotyledons, Regeneration, Shoots, phenotype.

Introduction

India is the third largest edible oil economy in the world. The value of agricultural economy is 17% of the country, whereas, oil crops account for almost 5% of the gross national product. Rapeseed-mustard (*Brassica* sp.) is the second largest indigenous oilseed crop after groundnut. The *B. juncea* contributes 32% of the total oilseed production in India (Pandey 2013). In India, Rapeseed-mustard is grown over an area of 57.62 lakh hectare with the average productivity 68.22 lakh tons (NMOOP 2017). Haryana is one of the highest Rapeseed-mustard growing state in India, having an area of 5.05 million hectare with the corresponding production of 8.05 million ton and an average yield of 1594 kg/ha. The important Rapeseed-mustard growing districts in Haryana state are Bhiwani, Mahendergarh, Rewari, Gurgoan, Hissar, Sirsa and Fatehabad (NMOOP 2017).

Though, India has the second largest acreage of oil seed *Brassica*, the productivity is low (Shivanna and Singh 2000). The low productivity is attributed to the poor agronomic practices and various biotic and abiotic stresses. In view of the acute shortage of edible oil and low productivity of oil seeds, tissue culture has attracted recent attention in order to increase the productivity of Rapeseed-mustard. A number of attempts are being made to improve the quality as well as the quantity of *B .juncea* using conventional breeding programs and/or tissue culture. However, the average yield is still far below the world average (Michael 2009).

TDZ is among the most active cytokinins. It was first synthesized by German Schering Corporation for defoliation of cotton, *Gossypium hirsutum* (Arndt et al. 1976). Due to its efficient role in tissue culture, it has gained a considerable attention. TDZ has shown cytokinin effects in multiple shoot regeneration. The frequencies are low in indirect differentiation of shoots from callus or suspension callus. Leaf segment (Guoet al. 2005), cotyledonary explant (Narasimhulu and Chopra, 1988), floral intermodal segment and ovary and callus phase by vegetative has helped in shoot organogenesis. It was shown that TDZ fulfilled the requirements of various regenerative responses of many different plant species (Guo et al. 2011).

Therefore in the present study, attempts were made to standardize a protocol for shoot regeneration system from explants by TDZ as an inducer. The efficient and reliable indirect shoot regeneration system was standardized in both cotyledons and hypocotyl explants.

Materials and Methods

Plant material

Certified seeds of eight cultivars viz., Pusa Bold, T-59, RH- 30, PJK, PusaBahar (PB), Luxmi 8812, 9304 and B9 of Indian Mustard, *Brassica juncea* L. Czern & Coss were obtained from Pulse Research laboratory, Division of Genetics, Indian Agriculture Research Institute, New Delhi and Oil Seed Section, Haryana Agriculture University, Hissar, Haryana.

Seed sterilization, germination and preparation of explants.

Healthy and mature seeds of *B. juncea* were given a quick rinse in 70% ethanol and then sterilized in 0.1% mercuric chloride for 6 min. After rinsing three times in sterile distilled water, seeds were aseptically germinated on filter paper under dark conditions. The seed coat was removed and the immature cotyledons with petiole and hypocotyl were excised from 2, 4 and 6 day old seedlings.

Culture medium and culture conditions

Cotyledon, hypocotyls explants were cultured on Murashige and Skoog's basal medium (Murashige and Skoog 1962) containing 3% sucrose and 0.8% agar agar along with the different concentrations of TDZ. The pH of the medium was adjusted to 5.8 using 0.1N NaOH and 0.1 N HCl prior to autoclaving. The explants were allowed to regenerate in 16:8 hour light: dark period cycle with cool white fluorescent light of 80 µmol m^{-2} s^{-1} at 25±2°C. For each treatment, 30 explants were cultured and each experiment was repeated at least four times. The multiple shoots developed from the explants were counted after 3-4 weeks.

Effect of pre-treatment of Thidiazuron (TDZ)

In this experiment, explants were soaked in liquid MS media containing 0.5µm TDZ for different intervals i.e., ½, 1, 2, 4, 8, 12 and 24 hrs and were transferred on PGRs-free MS basal media.

Rooting and transplantation

The 17 days old in vitro regenerated shoots were excised from proliferating explants and then transferred to MS media supplemented with different concentration of IBA (1-2 µM) or IAA (1-2 µM). After transplantation adventitious roots arise from basal portion of the shoots on two weeks. The plantlets with well-developed roots were removed from the culture tubes and then the roots were carefully washed under the running tap water.

Plants were grown in pots containing moist sand: soil: manure (1:1:1). Each pot was covered with a polythene bag to ensure humidity for first few days and subsequently, humidity was gradually reduced by making holes in the polythene bags. After 7 days it was completely removed and the plants were transferred to soil. Plants were survived in soil with no failure and produced phenotypically normal fertile plants with flowers and fruits.

Results

Callus mediated shoot organogenesis from cotyledon explant

Cotyledon explants of 2-6 day old *B. juncea* c.v. Pusa Bold seedlings were assessed for multiple shoot induction on MS medium containing different concentrations of TDZ. Upon supplementation of MS basal medium by TDZ, the cotyledon explants developed roots directly at the base in 100% of the cultures.

Addition of different concentrations (0.1-1µM) of TDZ to the basal medium induced multiple shoots in the petiolar cut region of the explants. A maximum of 14.33 $\pm$ 0.49 shoots per callus were obtained in all the cultures of 2-day old cotyledon explants, when 0.5 µM TDZ was supplemented in the MS Media (Figure 1). Further increase in the TDZ concentration did not improve regeneration, albeit lesser number of shoots developed. The calli produced by 2-day-old cotyledons of *B. juncea* on TDZ were sub-cultivated twice on the same medium, which produced about 8 to 9 shoots during the first time and 5 to 6 shoots the second time.

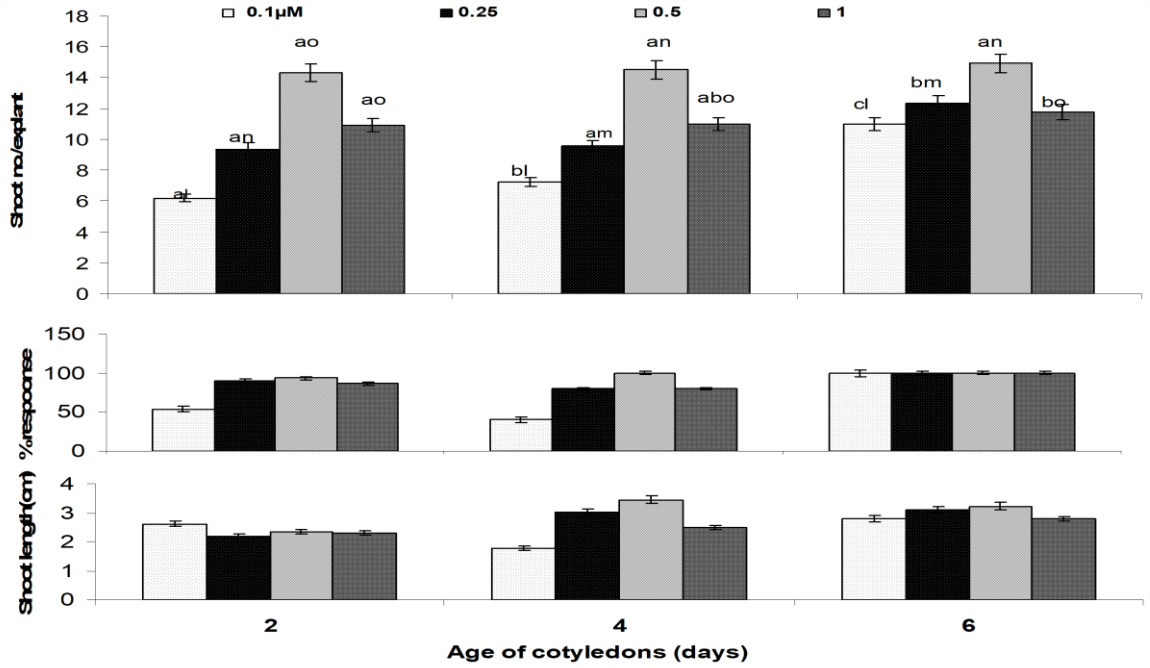

Fig 1 Effect of age of cotyledon explant of *Brassica juncea* cv. Pusa Bold on TDZ induced indirect shoot regeneration. Values are mean ± S.E. Data based on 30 explants per treatment and scored after 4 weeks of culture. Details are as per described in materials and methods.The data analysed by two way Anova, same letters don't differ at DMRT < 0.05.

Effect of TDZ on hypocotyl explant

The 2-day-old hypocotyl explants of *Brassica juncea* cv. Pusa Bold were assessed for multiple shoot induction on medium containing different concentration of TDZ. Addition of various concentrations in the range of 0.1 to 50µM of TDZ to the basal medium induced multiple shoots at either side of the explants. A maximum of 7.2 ±0.54 shoots per explant were obtained in 80% of the culture, when 10 µM TDZ was provided. As was observed in the case of cotyledon explants further increase in the TDZ concentration beyond 10 µM caused decreased regeneration of shoots (Table 1).The length of shoots was independent of the TDZ concentration (Table 3).

Effect of age

The 2-to-6-day old Cotyledonary explants produced multiple shoots from the petiolar cut region *in vitro* in response to the TDZ as early as 17-day. Maximum percentage response and number of normal regenerated shoots were obtained in 2-day old explants cultured on MS media supplemented with 0.5 µM TDZ. However in the older seedlings, 20 percent of the shoots were deformed at 0.5 µM TDZ.

Table 1 Morphogenic response of hypocotyls of *Brassica juncea* cv. Pusa Bold cultured on different concentration of TDZ

TDZ (μM)	% Regenerating explants	Number of shoots/explant*	Shoot length (cm)
0.1	46.6	$4.28^{c} \pm 0.7$	1.54 ± 0.07
0.5	66.6	$4.6^{c} \pm 0.5$	1.36 ± 0.09
1	66.6	$5.9^{ab} \pm 0.3$	1.2 ± 0.08
5	100	$6.7^{a} \pm 0.4$	1.63 ± 0.13
10	80	$7.2^{a} \pm 0.54$	1 ± 0.06
20	20	$2.4^{d} \pm 0.23$	1.1 ± 0.09

Values are mean ±S.E. Data are based on 24 explants per treatment and scored after 4 weeks of culture.Data have been analysed by one-way ANOVA and mean values followed by same letters don't differ significantly at DMRT<0.05.

Effect of genotype

2-day-old cotyledon explants of T-59, B9, PJK, Luxmi (8812), Pusabahar, Pusa Bold, 9304, and RH-30 genotypes were induced by various concentrations of TDZ in MS Medium. The shoot regeneration was highest (14 – 15 shoots) in case of PusaBold, followed by 9304 (13 – 14 shoots), PB and luxmi (8 – 9 shoots) and T-59, RH-30, PJK and B9 (4 – 5 shoots) (Table 2).

Effects of different explants

The 2-day old cotyledon, hypocotyl, cotyledonary node and shoot tip explants responded positively to 0.5 μM TDZ. The maximum shoots were regenerated in the cotyledon explants (14 – 15 shoots) followed by the cotyledonry node (12 shoots), shoot tip (10 – 11 shoots) and hypocotyl (4 – 5 shoots) explants(Figure 2).

Figure 2. Indirect *in vitro* shoot organogenesis in *B.juncea* cv. Pusa Bold using cotyledon and hypocotyl explants

a) Cotyledon explant 10 days) b) Multiple shoots in TDZ c) Multiple shoots (After 17 days) d) Sub-cultured callus with multiple shoots e) Hypocotyl explant f) Induction of shoot from both cut end of hypocotyl g) Multiple shoots h) Rooting i) *In vitro* flowering j) Mature plant in soil

Table 2 Morphogenic response of 2-day old cotyledons derived from different genotype of *Brassica juncea* using TDZ as induction factor

Cultivar	% Regenerating explants	Number of shoots/explant*	Shoot length (cm)
Pusa Bold	100	$14.33^{a} \pm 0.49$	
9304	80	$13.25^{b} \pm 0.05$	2.47 ± 0.22
PB	80	$9.5^{c} \pm 0.30$	1.75 ± 0.26
Luxmi(8812)	60	$8.62^{c} \pm 0.43$	1.71 ± 0.12
T-59	70	$5.85^{d} \pm 0.37$	2.78 ± 0.22
B9	70	$4.71^{d} \pm 0.38$	1.02 ± 0.14
RH-30	70	$4.33^{d} \pm 0.35$	2.34 ± 0.11
PJK	80	$4.25^{d} \pm 0.12$	2.21 ± 0.276

Values are mean ±S.E. Data are based on 24 explants per treatment and scored after 4 weeks of culture. Data have been analysed by one-way ANOVA and mean values followed by same letters don't differ significantly at DMRT<0.05.

Table 3 Morphogenic response of different explants derived from *Brassica juncea* cv Pusa Bold using 0.5µM TDZ as induction factor

Explant used	% Regenerating explants	Number of shoots/explants*	Shoot length (cm)
Large cotyledon	100	$14.33^{a} \pm 0.49$	2.34 ± 0.05
Small cotyledon	100	$14^{a} \pm 0.37$	2.68 ± 0.15
Shoot tips	100	$10.4^{c} \pm 0.4$	2.59 ± 0.146
Cotyledonary node	100	$12^{b} \pm 0.42$	2.56 ± 0.11
Hypocotyl	66.6	$4.6^{d} \pm 0.5$	1.36 ± 0.09

Values are mean ±S.E.Data are based on 24 explants per treatment and scored after 4 weeks of culture. Data have been analysed by one-way ANOVA and mean values followed by same letters don't differ significantly at DMRT<0.05

Table 4 Effect of the preculture of 2 days old cotyledon derived from *Brassica juncea* in liquid medium containing TDZ (0.5 μM) and cultured on MS media.

Time (Hours)	% Regenerating explants	Number of shoots/explant	Shoot length (cm)
1/2	60	1.4 ± 0.23	1.1 ± 0.012
1	80	3.6 ± 0.45	1.2 ± 0.02
2	80	4.8 ± 0.33	1.4 ± 0.02
3	100	5.6 ± 0.38	2.1 ± 0.01
4	100	7.8 ± 0.43	2.2 ± 0.2
5	100	7.4 ± 0.32	2.3 ± 0.19
7	40	7.1 ± 0.43	2.0 ± 0.11
12	0	0	0
24	0	0	0

Values are mean ±S.E.Data are based on 24 explants per treatment and scored after 4 weeks of culture. Data have been analyzed by one-way ANOVA and mean values followed by same letters do not differ significantly at DMRT<0.05

Effect of pre-soaking

The 2-day old cotyledons were soaked in 0.5μM TDZ for a period ranging from 1/2-24 hours and cultured on MS basal medium. Maximum numbers of shoots (7-8 shoots) were obtained when cotyledon was soaked for 4 to 5 hours. The cotyledons were not regenerated at all in the case of pre-soaking for more than 7 hrs.

In vitro rooting and hardening of regenerated plants

The 17 days old in vitro regenerated shoots were transferred to root inducing media, MS (1, l /2) alone or along with IBA (1-2μM) or IAA (1-2 μM), which produced (l00%) adventitious roots from the basal portion of the shoots in two weeks. The plantlets developed in vitro were transferred to pots containing moist sand: soil: manure (1:1:1).They survived in soil with no failure and produced phenotypically normal fertile plants with flowers and fruits after two weeks of the transfer to in vitro rooting media for acclimatization and hardening

Discussion

Thidiazuron (TDZ) is a potent synthetic plant growth regulator for organogenic, regeneration, and somatic embryogenesis (Dewir 2018). Chemically, TDZ is a substituted phenylurea (N-phenyl-N′-1,2,3-thiadiazol-5-ylurea) with high cytokinin activity (Murthy 1988). It induces callus mediated rapid, low cost shoot organogenesis. In medicinal and horticultural crops there is an increased use of TDZ for *in vitro* propagation of plants (Deepa *et al*.2018).

TDZ supplementation in MS medium at a concentration of 0.5μM induced shoot organogenesis (14-15shoots per explant) with high regeneration frequency (100%). However, the regeneration decreased with the increase in the TDZ concentration above 0.5 μM. The TDZ is known to induce callus formation (Lin 1993) and multiple shoot differentiation (Rizvi 2001). It is known to induce callus mediated rapid, low-cost shoot organogenesis from cotyledon and hypocotyl explants. Other workers also reported best axillary proliferation when low concentration of TDZ was supplied to various plants (summarised by Pai and Desai 2018). 0.2% of TDZ along with 0.1% of 6-benzyladenine induced 67 to 93% plant regeneration frequency with an average of 35 shoots/ explants of *Medicago sativa* (Kumar et al. 2013). Even low concentrations of 6-benzylaminopurine were found to promote direct shoot organogenesis in *Brassica juncea* (Dhania and Singh 2016).

In addition to the concentration of the TDZ, other confounding factors of the frequency of multiple shoot formation include the genotype of the plant, type of explants and their age, and pre-treatment by soaking in the TDZ. The 0.5μM TDZ was found to be active in all the cultivars used in the study, though with varying effect. The

concentration works best for the cultivar Pusa Bold, followed by 9304, PB, Luxmi, T-59, RH-30, PJK and B9. However, better responses of the other cultivars with other concentrations of TDZ cannot be ruled out. Since, the organogenesis of a plant vary from genotype to genotype (Rani et al.2013), the observation is not surprising.

Each cell of a plant is totipotent. However, the totipotency varies from cell to cell. Higher the meristematic activity, higher is the totipotency. For organogenesis, fresh and active cells, for instance stem tip are thus, taken as explants. The cotyledons retain the ability to transform into leaf primordia and have meristematic activity near its base (Chandler 2008). The comparison of various fresh tissues of *Brassica juncea* revealed that 2-day-old cotyledon explants are best suited for indirect shoot organogenesis using TDZ, followed by cotyledonary node, shoot tip and hypocotyl explants. It may be worth mentioning over here that the response to TDZ gradually decreased with aging of the cotyledon explants (Table 4).

Pretreatment with TDZ was also found to alter the shoot organogenesis. Different time intervals of pre-soaking was compared and it was observed that soaking for 4 hours works best for the indirect shoot regeneration in Pusa Bold. The pretreatment of plants with TDZ has been reported to increase the organogenesis in *Curculigo orchioides* (Thomas 2007), *Cicer arietinum* (Kumari et al. 2017), *Picrorhiza kurroa* (Patial et al. 2017) and *Brassica oleracea* (Pavlovi et al. 2010). Pretreatment by soaking in TDZ beyond 7 hours diminishes the ability of the shoot regeneration in the Pusa Bold of *B. juncea*.

In conclusion, the cotyledon explant of 2-day-old seedling supplied with 0.5µM of TDZ works best for the indirect shoot organogenesis of *Brassica juncea* cultivar Pusa Bold giving rise to 14-15 shoots per explants. Further, the in vitro regenerated shoots developed viable roots upon IAA or IBA treatment and produce phenotypically normal fertile plants with flowers and fruits when transferred to the pots.

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