THIDIAZURON INDUCED PLANT REGENERATION IN BRASSICA RAPA VAR. TURNIP VIA SEED DERIVED CALLI INDUCTION AND RADICAL SCAVENGING ACTIVITY

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ABSTRACT

The present study is first report on in vitro regeneration and antioxidant potentials of economically important Brassica rapa var. turnip from seed derived calli and using Thidiazuron (TDZ) (1-phenyl-3- (1, 2, 3- Thiadiazol-5-yl) as primary growth regulator. Results exhibited that seed germination medium supplemented with TDZ (2mgL⁻¹) and NAA (1mgL⁻¹) yielded highest frequency of callus induction (71.3%) within 7 days of culture. Shoot differentiation was achieved within five weeks of callus transfer to MS medium with similar concentrations of growth regulators. Medium containing TDZ (1mgL⁻¹) and BA (1mgL⁻¹) provided optimum conditions for in-direct shoot organogenesis (28 shoots per callus). Furthermore, the addition of TDZ (0.5mgL⁻¹) to MS medium containing BA (1mgL⁻¹) produced as long as 2.8cm shoot. TDZ improved antioxidant potential of regenerated plantlets, roots and shoots as compared to control plants and their parts. Highest frequency of radical scavenging activity was observed in seed derived calli (80.1%). All in vitro regenerated plants were successfully rooted and acclimatized to in vivo conditions. This study contributes to better understanding of mechanism of TDZ as potential growth regulator for regeneration via seed derived callus and increased radical free scavenging activity in Brassica rapa var. turnip.

Key words: Brassica rapa var. turnip, seed derived callus, TDZ, regeneration, radical scavenging activity

INTRODUCTION

Brassica rapa var. rapa L. (turnip) is an important vegetable and medicinal plant, that is highly appreciated and consumed around the world (Fernandes et al., 2007). The roots of this plant are eaten in crude form or cooked as a vegetable, while tops are fed to livestock. Turnip greens are a good source of lutein, vitamin A, folate, vitamin C, vitamin K and calcium (Duke, 1979; Haytowitz et al., 2008). It is demonstrated that a high ingestion of Brassica vegetables lessens the threat of age-related chronic diseases (Kris-Etherton et al., 2002) and reduces the risk of several types of cancer (Kristal and Lampe, 2002; Wang et al., 2004).

Conventional cultivation methods applied in developing countries are inadequate to maintain crop quality of Brassica species (Sajid and Aftab, 2009). Therefore plant tissue culture / in vitro regeneration has arisen as an alternative tool for the conservation and rapid reproduction of these medicinal species (Castillo and Jordan, 1997; Saxena et al., 1997; Sanyal et al., 1998). In vitro plant regeneration involves different mechanisms for activation and regulation of certain enzymes at particular growth stage (Meratan et al., 2009; Abbasi et al., 2011a). Induction of organogenic callus from explant culture is the key step in in vitro plant propagation. Furthermore, scavenging of Reactive Oxygen Species (ROS) through enzymatic and non-enzymatic systems in response to different environmental stimuli has significant roles in the plant development (Benson, 2000; Ducic et al., 2003). The Radical scavenging potential of Brassica species has been evaluated at numerous levels (Tian et al., 2003; Meratan et al., 2009), but, not in B. rapa var. turnip.

In history, regeneration has been achieved in many species of genus Brassica, by using microspore, leaf, cotyledon, hypocotyls, peduncle, and petioles as explants source (Guo et al., 2005; Khan et al., 2009; Abbasi et al., 2010 a, b; Cogbill et al., 2010). Despite the fact, that regeneration via seed derived callus induction may reduce the duration and expense of these practices, our survey of literature found that few reports are available on plantlet regeneration using seeds as explants (Al-Khayri et al., 1992; Mii et al., 1994; Griffin and Dibble 1995; Prakash et al., 1999; Chengalrayan et al., 2005).
Therefore, the present manuscript is the first report on successful regeneration of *B. rapa* var. *turnip* via seed derived callus induction and determination of its radical scavenging activity.

**MATERIALS AND METHODS**

*Plant Material and Decontamination*

The seeds of *B. rapa* var. *turnip* were collected from NARC (National Agricultural Research Centre), Islamabad, Pakistan. These seeds were then surface sterilized according to the modified protocol of the Abassi *et al.* (2010 a, b). Briefly, seeds were immersed in 70% ethanol for ~3 min followed by 0.1% mercuric chloride for ~1 min and finally washed three times in sterile distilled water.

*Seed Germination and Callus Induction*

This assay was conducted to obtain the seed germination at optimum level and to assess the comparative effects of exogenous growth regulators on seed derived calli formation. For this purpose, MS basal medium (Murashige and Skoog, 1962) containing 30% sucrose, solidified with 0.8% (w/v) agar was modified with different concentrations of PGRs including TDZ (0.5, 1, 2, 4, 8 mgL⁻¹) alone or in combination with BA (1.0 mgL⁻¹), KN (1.0 mgL⁻¹) and NAA (1.0 mgL⁻¹). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min. Five seeds were placed per 100 ml flask containing 30 ml medium. The culture was incubated in dark for 48 h, subsequently transferred to light conditions and maintained at 25 °C with 16-h photoperiod using cool white fluorescent tubes and an irradiance of 40 mol m⁻² s⁻¹. Frequency for seed germination was recorded after 2 days of transfer to light conditions; while the rate of seed-derived callus induction was determined after 10 days as percentage of seed forming calli.

*Shoot Proliferation and Acclimatization*

Seed derived calli were subsequently used for regeneration purpose by subculturing and maintaining on medium with similar compositions and conditions as mentioned. Data on shoot numbers and mean shoot lengths were recorded after 3 weeks of inoculation. For this purpose, the plants were harvested and shoot length was recorded with the help of a suitable scale from the top of the medium to the tip of shoot minus 1.0 cm (size of the original explant). Elongated shoots were transferred to ½ strength MS medium, ½ strength MS medium supplemented with NAA (0.5 mgL⁻¹) and ½ strength MS medium with IBA (0.5 mgL⁻¹) for root induction. Autoclaved soil sand and cow dung in ratio of 1:2:1 was used for acclimatization of rooted plants to in vivo conditions.

*DPPH (1,1-diphenyl-2-picrylhydrazyl) free Radical Scavenging Activity*

The procedure described by Amarowicz *et al.* (2004) was used to assess the potential of prepared extracts to scavenge free radical DPPH with some alterations. Briefly, 2.0 mg of plant tissue extracts dissolved in methanol were added to methanolic solution of DPPH⁺ (1 mM, 0.5 ml). The resultant mix was vortexed for 15 sec and then allowed to stand for 30 min at 30 °C and examined on spectrophotometer at 517 nm. A decomposed methanolic solution of DPPH⁺ without purple color (achieved by dissolving 2 mg of butylated hydroxyanisole (BHA) in 4 ml methyl alcohol with 0.5 ml of DPPH⁺ solution added) was selected for background alteration, other than pure methanol. Finally following formula was used to determine the radical scavenging activity as percentage of DPPH⁺ discoloration;

\[
\text{Scavenging DPPH⁺ free radical (\%)} = 100 \times (1 - \frac{AE}{AD})
\]

Where AE is absorbance of the solution, when extract has been added at a particular level and AD is the absorbance of the DPPH⁺ solution with nothing added.

**Statistical Analysis**

MS medium without any plant growth regulator was used as control in this experiment. All experiments were repeated three times. Randomized complete block design (RCBD) was used for treatments. Each treatment consisted of five Erlenmeyer flasks with five explants/calli, and ‘standard error of the mean’ was used for comparisons among means.

**RESULTS AND DISCUSSION**

Although the role of TDZ as an effective PGR is extensively studied in many other species of genus *Brassica*; however, during this particular investigation, TDZ alone and, in combination with other PGR was evaluated for its *in vitro* regeneration potential via seed derived callus induction in *B. rapa* var. *turnip*. All the applied treatments significantly improved the *in vitro* regeneration process (Fig. 1). The results are illustrated in Table. 1. During seed germination assay, none of the applied treatments (i.e. PGRs) could significantly enhance the frequency of seed germination as compared to untreated controls (83± 7.3 %). While, soft granular and purple callus initiation was
achieved at the root side of in vitro germinated seedlings after 7 days of seed culture on medium supplemented with TDZ alone (0.5-8 mgL⁻¹) and TDZ (0.5-8 mgL⁻¹) + (1 mgL⁻¹) NAA.

Table 1 Effect of various concentrations of TDZ alone, and in combination with regularly applied PGRs on frequency of seed germination and seed derived callus induction in B. rapa var. turnip.

<table>
<thead>
<tr>
<th>Exogenous Plant Growth Regulators (mgL⁻¹)</th>
<th>Frequency of seed germination ± SE</th>
<th>Percentage of calli formation ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDZ</td>
<td>BA ± SE</td>
<td>KN ± SE</td>
</tr>
<tr>
<td>0.5</td>
<td>- 64.8±2.5</td>
<td>43.3±2.1</td>
</tr>
<tr>
<td>1</td>
<td>- 56.7±2.1</td>
<td>47.5±2.3</td>
</tr>
<tr>
<td>2</td>
<td>- 59.2±4.0</td>
<td>63.5±2.0</td>
</tr>
<tr>
<td>4</td>
<td>- 61.6±3.3</td>
<td>37.7±3.1</td>
</tr>
<tr>
<td>8</td>
<td>- 52.1±2.5</td>
<td>29.1±2.7</td>
</tr>
<tr>
<td>0.5</td>
<td>1 55.5±5.1</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>1 60.5±0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>1 67.2±2.3</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>1 65.6±4.0</td>
<td>0.0</td>
</tr>
<tr>
<td>8</td>
<td>1 65±3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>1 70.7±6.5</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>1 58.3±11</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>1 66.5±3.2</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>1 62.3±4.2</td>
<td>0.0</td>
</tr>
<tr>
<td>8</td>
<td>1 60±3.7</td>
<td>0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>- 47.6±10.2</td>
<td>54.8±3.9</td>
</tr>
<tr>
<td>1</td>
<td>- 54.5±4.1</td>
<td>57.1±1.8</td>
</tr>
<tr>
<td>2</td>
<td>- 58±5.0</td>
<td>71.3±0.7</td>
</tr>
<tr>
<td>4</td>
<td>- 56.7±1.5</td>
<td>42.2±0.4</td>
</tr>
<tr>
<td>8</td>
<td>- 53.5±1.4</td>
<td>31.8±1.3</td>
</tr>
</tbody>
</table>

The seeds of B. rapa var. turnip failed to produce calli on MS⁰, TDZ + BA and TDZ + KN containing medium while producing seedlings only. The amount of TDZ in the medium and absence/presence of NAA influenced the seed derived callus induction rate (Table 1). The rate of callus induction in TDZ alone medium was far behind than TDZ + NAA supplemented medium where average frequencies noted were 44.22 ± 2.44 % while 51.44 ± 1.62% respectively. These results are in agreement to the findings of Taskin et al. (2003), where, 100% callus was obtained using TDZ alone or in combination with NAA in Arabis gunnisoniana. On other hand, Chengalrayan et al. (2005) who observed no callus induction from germinated seeds in TDZ containing medium, except on 2,4-D (2,4-Dichlorophenoxyacetic acid) and picloram-containing media in Sugarcane (Saccharum spp. hybrid cv. CP 84-1198). Highest frequency of seed derived callus formation (71.3±0.7%) was observed on TDZ (2 mgL⁻¹) with NAA (1 mgL⁻¹).

TDZ alone enhanced the process of callogenesis exponentially when used in lower concentrations (0.25 – 2 mgL⁻¹), yielding maximum rate of 63.5±2.0 % at 2 mgL⁻¹. Similarly lowest rate of seed derived callus induction (29.1±2.7 %) was recorded for TDZ (8mgL⁻¹) followed by 31.8±1.3 at TDZ (8 mgL⁻¹) combined with NAA. Medium supplemented with low concentrations of TDZ (0.5-2 mgL⁻¹) yielded moderate to high amounts of callus, whereas, higher concentrations of TDZ (< 2mgL⁻¹) resulted into decreased rate of callus induction. This result was in contrast to the Basalma et al. (2008) and Webster et al. (2006), where a lower concentration of TDZ suppressed callus formation and higher concentration was supportive to the process. This difference might be due to different explant types used for callus induction. The role of TDZ alone for optimal calli induction in is verified in other reports as well (Zhong and Tian, 2007; Taskin et al., 2003). Seed derived calli induced at seedlings, were subcultured above for shoot differentiation and proliferation, by using MS medium with similar compositions and concentrations of PGR as mentioned above.

Shoot organogenesis

Shoots started appearing from cultured calli and data was recorded after 5 weeks of culture time (Fig. 1b). MS⁰ medium alone was unable to induce any morphogenic response from seed derived calli, and did not promote further growth. Therefore, in vitro germinated seedlings on MS⁰ medium were maintained for further 5 weeks and used as positive control throughout the experiment. Each of the applied PGR treatment resulted into emergence of indirect shoots formation with obvious differences in yield frequencies among them. Average numbers of shoots observed were 11.5 per regenerated calli with mean shoots length of 1.64cm (Fig. 2; 3).
Fig. 1. a-d Regeneration and acclimatization of *B. rapa* var. *turnip* plants from *In vitro* seed derived callus by applying different concentrations of PGRs. a. *In vitro* seed derived callus induction at the base of germinated seedlings after 10 days, where purple color shows the presence of anthocyanin. b. Adventitious shoot induction from seed derived calli. c. Rooted plant. d. Acclimatization.

Fig. 2. Effects of various concentrations of TDZ alone, in combination with cytokinins (BA, KN) and auxin (NAA) on number of shoots per explant of *B. rapa* var. *turnip*. The values are the means of three replicates with standard error.
Fig. 3. Effects of various concentrations of TDZ alone and in combination with cytokinins (BA, KN) and auxin (NAA) on mean shoot length per explants of B. rapa var. turnip. The data were collected after 30 days of subculture on MS media with a similar composition of PGRs. The values are the means of three replicates with standard error.

MS media with exogenous addition of TDZ and BA efficiently enhanced shoot response with maximum number of shoots per callus/explant (28) at TDZ (1.0 mgL⁻¹) in combination with BA (1.0 mgL⁻¹). Similarly, maximum mean shoot length (2.8cm) was recorded at TDZ (0.5mgL⁻¹) with BA (1.0 mgL⁻¹). The effectiveness of TDZ for shoot induction in many woody species has already been reported (Huetteeman and Preece, 1993; Kim et al., 1997; Thimmappaiah et al., 2002; Ahmad and Anis, 2007). Dua and Pijut (2008) reported that medium supplemented with BA and TDZ worked well for shoot elongation and shoot proliferation in green ash (Fraxinus pennsylvanica). Furthermore, the consistency of our results is supported by the conclusion of Christey et al., (1999) where high efficiency of shoot regeneration was obtained in B. napus using TDZ and BA.

In our study, TDZ alone induced shoot organogenesis response at higher level than other PGRs (KN, NAA), by yielding second highest response regarding shoot numbers and shoot lengths after TDZ + BA (Fig. 2). Synergistic activity of TDZ and KN was average and produced moderate response of shoot number and mean lengths. Sango et al. (1996) achieved pea plant regeneration via organogenesis using TDZ that significantly influenced shoot formation. Moreover, the combination of KN with TDZ induced higher number of shoots per explant than obtained by Abbasi et al. (2011b) in B. rapa var. turnip incubated on KN alone and KN + NAA.

Moreover, NAA in combination with TDZ was less responsive for inducing indirect shoots and showed minimal response on shoot numbers and lengths when compared to TDZ alone and TDZ combined with cytokinins. Using cotyledonary explants of Rapid-cycling fast plants (Brassica rapa; RCBr), Cogbill et al. (2010) observed shoot induction on similar combination. These differences may be due to explant type or genotype.

Increasing the concentration of TDZ from lower (0.5mgL⁻¹) to moderate concentration (4mgL⁻¹) enhanced the in vitro regeneration of B. rapa var. turnip; while higher concentration (8.0 mgL⁻¹) was found inhibitory and drastically reduced the growth rate. These conclusions are in accordance with the findings of many authors (Murthy et al., 1998; Guo et al., 2005; Mallikarjuna and Rajendrudu, 2007). Similar concentrations of TDZ (0.1-4 mgL⁻¹) are found as optimum for shoot induction process which clearly supports our findings.

**Roots induction and establishment of in vitro regenerated Plantlets**

In our study highest rate of visible rooting (60 %) was obtained at ½ strength MS alone followed by 30% at ½strength medium supplemented with NAA (0.5 mgL⁻¹), while IBA failed to induce roots (Table 2).

The regenerated plantlets were successfully transplanted into plastic pots containing sterile soil, sand, and cow dung in a 1:2:1 ratio for acclimatization. Gradually the plantlets were adapted to the soil. Khan et al. (2009) and various other authors have reported high effectiveness of this medium in root induction. The rooted plantlets were transferred to plastic pots for hardening and were acclimatized successfully.
Table 2. Effects of different combinations of phytohormone in half strength MS medium on the root initiation of B. rapa var. turnip

<table>
<thead>
<tr>
<th>Supplements</th>
<th>No. of incubated</th>
<th>No. of shoots with roots</th>
<th>Root formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>½ MS</td>
<td>15</td>
<td>9</td>
<td>60.00</td>
</tr>
<tr>
<td>½ MS + 0.5 mg L⁻¹ NAA</td>
<td>15</td>
<td>5</td>
<td>30.00</td>
</tr>
<tr>
<td>½ MS + 0.5 mg L⁻¹ IBA</td>
<td>15</td>
<td>0</td>
<td>00.00</td>
</tr>
</tbody>
</table>

Antioxidant Activity (DPPH free Radical scavenging activity)

In the present study, the antioxidant activity of in vitro regenerated plantlets, calli and tissues was determined and compared with wildly grown plantlets and their tissues (controls). The results showed that in vitro derived complete plantlets had higher antioxidant activity (~80.1%) as compared to wild plants of brassica rapa var. turnip. Likewise in vitro derived shoots, roots, callus showed higher activity then those of shoots and roots harvested from wild plants. Callus showed significantly more activity as compared to re-differentiated plant tissues and roots had higher activity as compared to shoots in both cases (wild & in vitro). Among the individual plant tissues, the root part of the plant was found to be more active as compared to shoots (Fig. 4). Meratan et al. (2009) found the increased accumulation of antioxidant enzymes during callus differentiation in various species of Brassica, while Fernandes et al. (2007) revealed the DPPH assay for the dietary turnip (B. rapa var. rapa. L) and found that flower buds to be the most active part and exhibits a strongest antioxidant activity among all parts, followed by the leaves and stems, while Turnip roots showed a significantly lower antioxidant capacity; however, TDZ induced regenerated tissues showed higher activity then wild ones.

![Fig. 4. Comparison of DPPH free radical scavenging activity in TDZ induced regenerated plantlets, calli, shoots and roots of B. rapa var. turnip. Wildly grown plantlets and their parts served as positive controls.](image)

CONCLUSION AND RECOMMENDATIONS

The present report illustrates an efficient protocol for the regeneration and antioxidant potential in Brassica rapa var. turnip. TDZ in combination with NAA has been found to induce calli from seeds while highest level of antioxidative potential was found in TDZ induced regenerated shoots. These results explain the critical role of TDZ in Brassica regeneration and enhancing antioxidant activity. Further efforts are needed to optimize protocols by using other plant growth regulators in combination with TDZ to improve the regeneration efficiency and antioxidative secondary metabolites production in Brassica rapa var. turnip.

REFERENCES


Kaleem Ullah Kakar, et al. Thidiazuron induced plant regeneration in brassica rapa var. turnip… 240


