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ORIGINAL PAPER

Efficient regeneration and antioxidative enzyme activities in *Brassica rapa* var. *turnip*

Bilal Haider Abbasi · Murad Khan · Bin Guo · Saleem Ahmed Bokhari · Mir Ajab Khan

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Abstract The regeneration potential and antioxidative enzyme activities of economically important *Brassica rapa* var. *turnip* were evaluated. Calli were induced from leaf explants of seed-derived plantlets on Murashige and Skoog (MS) medium incorporated with different concentrations of various plant growth regulators (PGRs). The highest leaf explant response (83%) was recorded for 2.0 mg l^{-1} benzyladenine (BA) and 1.0 mg l^{-1} α-naphthaleneacetic acid (NAA). Subsequent subculturing of callus after 3 weeks of culture, on medium with similar compositions of PGRs, induced shoot organogenesis. The highest shoot induction response (83%) was recorded for 5.0 mg l^{-1} BA after 5 weeks of transfer. However, 7.8 shoots/explant were recorded for 2.0 mg l^{-1} BA. The transferring of shoots to elongation medium resulted in 5.1-cm-long shoots on 10 mg l^{-1} of gibberellic acid (GA_3). Rooted plantlets were obtained on MS medium containing different concentrations of indole butyric acid (IBA). The determination of activities of antioxidative enzymes (superoxide dismutase [SOD], ascorbate peroxidase [APX], catalase [CAT], glutathione peroxidase [GPX], and peroxidase [POD]) revealed involvement of these enzymes in callus formation and differentiation. All of the activities were interlinked with each other and played significant roles in the scavenging of toxic free radicals. This study will help in the advancement of a regeneration protocol for *B. rapa* var. *turnip* and the understanding of the functions of antioxidative enzymes in plant differentiation.

Keywords *Brassica* · Regeneration · Plant growth regulators · Antioxidative enzymes · SOD · Brassicaceae

Abbreviations

BA	6-Benzyladenine
IBA	Indole butyric acid
GA_3	Gibberellic acid
MS	Murashige and Skoog medium
MS0	MS medium without plant growth regulators
NAA	α-Naphthaleneacetic acid
PGRs	Plant growth regulators
SOD	Superoxide dismutase
CAT	Catalase
APX	Ascorbate peroxidase
GPX	Glutathione peroxidase
POD	Peroxidase

B. H. Abbasi (✉) · M. Khan · M. A. Khan
Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan
e-mail: bhabbasi@qau.edu.pk

B. Guo
College of Life Sciences, Northwest University, North Taibai Road, 710069 Xi'an, People's Republic of China

S. A. Bokhari
Department of Chemistry, GC University, Lahore 54000, Pakistan

Introduction

The *Brassicaceae* family comprises ~350 genera and 3,000 species. Among the economically important *Brassica* species are *B. rapa*, *B. napus*, *B. oleracea*, etc. (Cogbill et al. 2010; Musgrave 2000). *B. rapa* is an oil-yielding plant, generally grown as farm crop, truck crop, or home-garden crop. Its roots are eaten in crude form or cooked as a vegetable, and its tops as potherb. Roots are also grown to

feed livestock. It is also an important source of dietary fiber and vitamin C (Choi et al. 2007). The powdered seed is used as a folk remedy for cancer. The roots, when boiled with lard, are used to treat breast tumors (Duke 1979). The stems and leaves are used against cancer, while a salve derived from the flowers help skin cancer (Hartwell 1971). It was cultivated in Europe for over 4,000 years, probably native to central and southern Europe, now spread throughout world, including most parts of the tropics. Terrell (1977) divides *B. rapa* into the following groups: *chinensis*, pak choi; *pekinensis*, Chinese cabbage; *perviridis*, spinach mustard; *rapifera*, turnip; and *ruvo*, kale, and the number of chromosomes was represented as 2n = 20. Together, these crops contribute significantly towards world food and fodder production (Choi et al. 2007). The consumption of rapeseed oil is increasing in the developed world (Walker and Booth 2001). However, very little information is available on the cultivation methods for this species, and protocols for yield and productivity are limited (Kopsell et al. 2003). The conventional cultivation practices of these plant species are subjected to variations in genetic resources and responses to chemical, biological, and environmental stimuli (Abbasi et al. 2007; Sinniah et al. 1998).

In vitro regeneration provides useful tools for the large-scale production and conservation of germplasm of elite plant species (Abbasi et al. 2007, 2010a, b; Ahmad et al. 2010). To date, no reports on the successful in vitro regeneration of *B. rapa* var. *turnip* from leaf explants of in vitro-grown plantlets are available. However, several protocols for regeneration and embryogenesis from isolated microspore, cotyledon, hypocotyls, peduncle, and petiole explants are reported for this elite species (Takasaki et al. 1997; Guo et al. 2000; Teo et al. 1997; Ferrie et al. 1995; Burnett et al. 1994; Khan et al. 2009; Tang et al. 2003;Cao et al. 1994; Burnett et al. 1992; Cogbill et al. 2010).

In vitro regeneration is a complicated phenomenon requiring a multi-disciplinary approach in its investigation (Meratan et al. 2009). The progression of the metabolic prototype that occurs during organogenesis involves the activation of specific enzymes at the appropriate times and regulation of their activity. The final result of this process is organogenesis (Abbasi et al. 2007). However, there is a growing interest in the functional role of reactive oxygen species (ROS) and corresponding scavenging enzymic and nonenzymic systems in plant growth (Dučić et al. 2003; Benson 2000). The appearance of ROS in the plant cells is generally linked with the free radical processes involved in the plant developmental processes, as well as its interaction with the environment. Furthermore, these free radicals have an important role in the metabolism and development of aerobic organisms; however, their uncontrolled production leads to oxidative stress. Benson (2000) reported the possibility that free radical-mediated stress has a role in tissue culture recalcitrance. To overcome this issue, antioxidative enzymes perform the check and balance of these free radicals. Recently, part of ROS and antioxidative enzymes with organogenesis in some species has been demonstrated (Tian et al. 2003; Meratan et al. 2009), but *B. rapa* remained yet to be studied.

Materials and methods

Seeds were collected from field-grown plants of *B. rapa* var. *turnip*. These seeds were surface-sterilized by the method of Abbasi et al. (2010a, b). Briefly, seeds were sterilized by immersion in 70% ethyl alcohol for ~3 min, 0.5% mercuric chloride solution for ~1 min, and washed three times with sterile distilled water. These seeds were incubated on Murashige and Skoog (MS; 1962) basal medium (MS0) for germination. Leaves were collected from ~4-week-old plantlets for regeneration purposes. To prevent the endogenous contamination of leaf explant, a similar protocol for decontamination was exploited.

For indirect shoot organogenesis, leaves from ~4-week-old seed-derived plantlets were cut into ~1.5-cm^2 pieces and placed onto MS medium containing different concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 mg l^{-1}) of either 6-benzyladenine (BA), kinetin (Kn), or gibberellic acid (GA_3) with or without 1.0 mg l^{-1} α-naphthaleneacetic acid (NAA). MS0 was used as a control for callus induction and shoot regeneration. About five leaf explants were incubated in a 100-ml Erlenmeyer flask containing 25 ml of solidified medium. After ~3 weeks of culture, the number of responding explants was recorded. Off-white friable callus was refreshed to MS medium with similar composition/concentrations of plant growth regulators (PGRs) for further growth and shoot organogenesis. Data on shoot induction response, number of shoots/explant, and the mean shoot length was recorded after ~5 weeks of callus subculturing. Elongated shoots were transferred to rooting medium containing different concentrations (1.0, 2.0, 3.0, and 4.0 mg l^{-1}) of indole butyric acid (IBA), and after ~5 weeks, rooted plantlets were removed, washed with sterile distilled water, and transferred to soil for further growth.

For antioxidative enzyme activities, the extract was prepared from plant tissue by modifications to the method of Meratan et al. (2009); briefly, the homogenate of seed-derived plantlet (control), calli, shoots, and regenerated plantlet was extracted with ice-cold 0.5 M Tris–HCl (pH 6.8) buffer. The extracts were centrifuged at 10,000 rpm for 20 min at 4°C and the resulting supernatant was kept in the freezer and used for enzyme assays. A UV–visible spectrophotometer (Agilent 8453, CA, USA)

was used to determine the absorption of extracts. Superoxide dismutase (SOD; EC 1.15.1.1) was determined by the method of Giannopolitis and Ries (1977), catalase (CAT; EC 1.11.1.6) was determined by the method of Arrigoni et al. (1992), peroxidase (POD; EC 1.11.1.7) was determined according to Abeles and Biles (1991), and ascorbate peroxidase (APX; EC 1.11.1.11) was determined by the method of Miyake et al. (2006).

For statistical analyses, each treatment consisted of four Erlenmeyer flasks and all experiments were repeated once. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used for comparisons among means. However, for antioxidative enzyme activities, data were collected from three replicates and represented as the mean ± standard deviation (SD).

Results and discussion

Seeds collected from wild-grown plants were viable and showed a germination frequency of about 80% (data not shown). However, the decontamination protocol did not show any inhibitory action on seed germination. This protocol was previously established to decontaminate explants, but the results produced here are supporting its application to surface-sterilize the seeds of *B. rapa* var. *turnip* (Abbasi et al. 2010a, b; Ahmad et al. 2010). Clorox has been reported to surface-sterilize the seeds of rapid-cycling *B. rapa* (Teo et al. 1997). However, Burnett et al. (1994) found that the presence of BA and NAA in the germination medium enhanced the seed germination rate of *B. rapa* spp. *oleifera*.

A feasible regeneration protocol for *B. rapa* var. *turnip* from leaf explant was successfully established (Fig. 1). The effects of various PGRs such as benzyladenine (BA), kinetin (Kn), gibberellic acid (GA_3), and α-naphthaleneacetic acid (NAA) on the leaf-explant response of *B. rapa* were evaluated. Callus response was observed within 3 weeks following culture (Fig. 2). However, the maximum callus response (83%) was recorded for 2.0 mg l^{-1} BA and 1 mg l^{-1} NAA. Similar findings were previously made for other explants of *B. rapa* also (Khan et al. 2009; Tang et al. 2003). Furthermore, thidiazuron (TDZ) with NAA induced adventitious shoot regeneration in rapid-cycling *B. rapa* (Cogbill et al. 2010). The callus was initially white in color and became yellow after about 18 days. We noted in the current report that the addition of 1 mg l^{-1} NAA enhanced callus production in *B. rapa* var. *turnip*. On the contrary, the addition of NAA has shown inhibitory effects in callus induction in some species (Abbasi et al. 2010a, b). Notwithstanding, 2.0 mg l^{-1} BA and 2.0 mg l^{-1} GA_3 produced similar results for callus induction. Minimum callus response was recorded for 10 mg l^{-1} Kn. Teo et al. (1997) observed direct shoot regeneration from cotyledon-explants of rapid-cycling *B. rapa* incubated on MS medium incorporated with BA and NAA. 2,4-Dichlorophenoxyacetic acid (2,4-D) has also been shown to yield the best results for callus induction in *B. rapa* (Guo et al. 2000). These differences showed that regeneration in *Brassica* is highly genotype-dependant (Cardoza and Stewart 2004). BA has been shown to give an efficient response in callus induction in several elite plant species (Ahmad et al. 2010; Abbasi et al. 2010a, b), which was also observed in the present report. Callus formation is important for studies of indirect morphogenesis like somatic embryogenesis and for the metabolic pathways of biologically active molecules (Meratan et al. 2009).

Data on shoot regeneration was recorded after 5 weeks of subculture (Fig. 3). The highest shooting (83%) was recorded for leaf explants cultured on medium containing 5 mg l^{-1} BA. Moderate concentrations of BA have shown the highest shooting response, but a higher concentration (10 mg l^{-1}) has shown inhibitory action. In the current study, the addition of NAA in medium already containing BA significantly inhibited the percent shoot induction. These findings were similar to the observations of Ahmad et al. (2010) and in contrast to Abbasi et al. (2010a, b) for other plant species. Nonetheless, BA was more effective than other PGRs used in current report. However, Guo et al. (2000) concluded from their work on *B. rapa* that the combination of auxin and cytokinin is effective in multiple shoot induction response. Cogbill et al. (2010) found no shoot regeneration in rapid-cycling *B. rapa* on MS medium incorporated with BA and NAA or TDZ alone.

The highest number (7.8) of shoots/explant was recorded for 5.0 mg l^{-1} of BA and the lowest number (1.6) of shoots/explant was recorded for 0.5 mg l^{-1} BA and 1.0 mg l^{-1} NAA (Fig. 4). It was observed that the addition of NAA in medium incorporated with either BA or Kn significantly inhibited the number of shoots/explant. Similar findings were previously reported for other plant species (Ahmad et al. 2010). Furthermore, the addition of NAA in medium containing GA_3 did not show any significant change (Fig. 4). Our results did not support the observations that the synergistic combination of auxin with cytokinin promoted shoot regeneration frequency (Abbasi et al. 2010a, b). 16.7 shoots/explant were obtained on medium containing 3.0 mg l^{-1} TDZ and 1.0 mg l^{-1} NAA in rapid-cycling *B. rapa* (Cogbill et al. 2010). The addition of $AgNO_3$ in shoot regeneration medium significantly enhanced the number of shoots/explant in different *Brassica* spp. (Khan et al. 2009; Cogbill et al. 2010). Previously, the presence of NAA along with GA_3 and BA was found to increase the number of shoots as compared with BA or GA_3 alone for *Silybum* (Abbasi et al. 2010a, b). The study of Teo et al. (1997) showed that the addition of

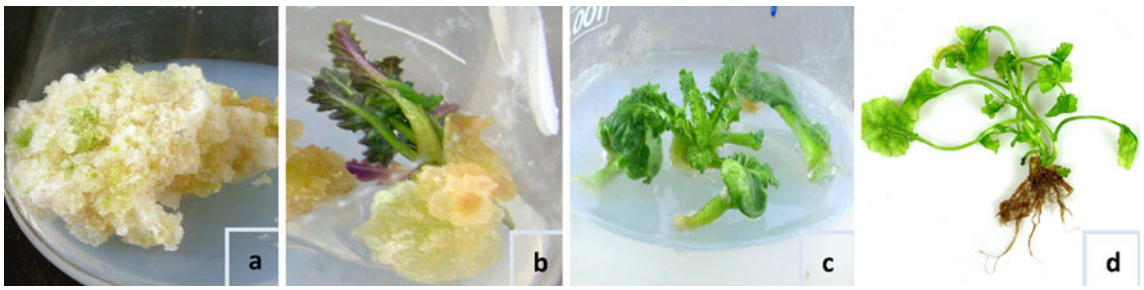

Fig. 1a–d Plant regeneration in *Brassica rapa*. **a** Callus. **b** Shoot regeneration. **c** Shoot elongation. **d** Regenerated plantlets

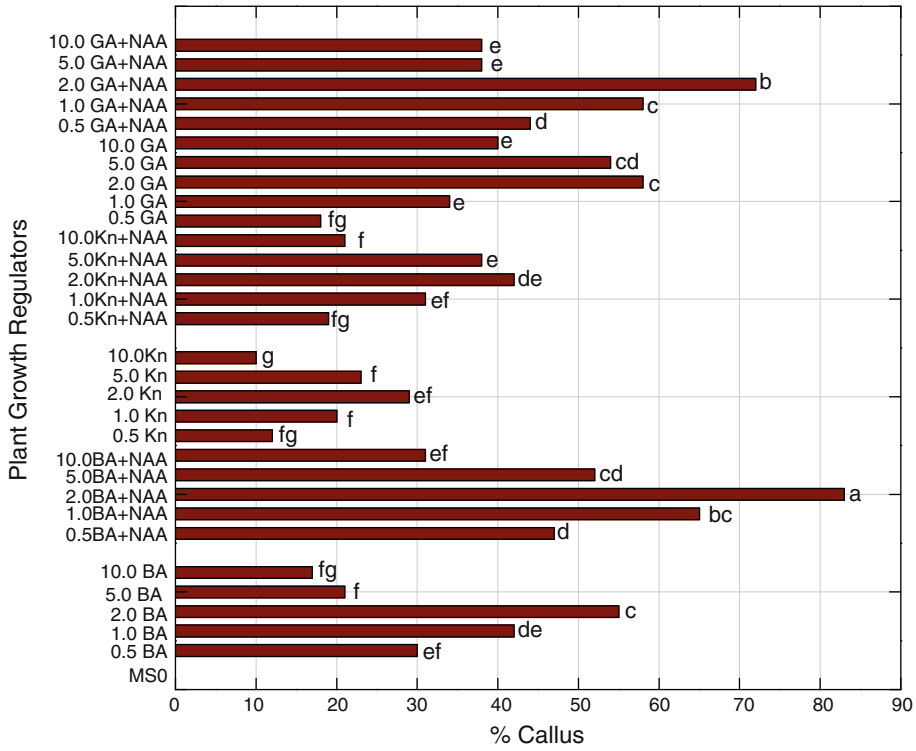

Fig. 2 Effects of various concentrations of 6-benzyladenine (BA), kinetin (Kn), or gibberellic acid (GA_3) with or without 1 mg l^{-1} α-naphthaleneacetic acid (NAA) on the percent callus induction of *B. rapa* var. *turnip*. The data were collected after 3 weeks of culture. The values are the means of four replicates. Means with the same letter are not significantly different at $P < 0.05$

aminoethoxyvinylglycine increased the number of shoots formed per explant. The addition of silver ions had shown positive effects for *Brassica* spp. (Tang et al. 2003). However, the actual mechanism of silver ions on in vitro shoot regeneration has not yet been elucidated (Abbasi et al. 2007, 2010a, b). Surprisingly, shoot induction was recorded for all of the PGRs tested in the present study (Figs. 3, 4, and 5).

The highest mean shoot length (5.1 cm) was observed in the presence of 10 mg l^{-1} GA_3 (Fig. 5). In a previous study, it was found that a combination of BA and GA_3 promoted longer shoots than other PGRs (Ahmad et al. 2010). Contrarily, GA_3 could not produce the highest shoot length response in some plant species (Abbasi et al. 2010a, b). Chikkala et al. (2009) concluded from their study that mature leaf explants produced fewer number of shoots than younger leaves of rapid-cycling *B. oleracea* and broccoli, but the number of shoots and regeneration frequency was higher in cauliflower. Kuginuki et al. (1997) studied varietal differences in embryogenic and regenerative ability in the microspore culture of Chinese cabbage (*B. rapa* spp. *pekinensis*). However, our results showed that GA_3 did not produce the highest percent shoot induction or number of shoots/explant (Figs. 3, 4, and 5). Unfortunately, no report is available regarding shoot length in *Brassica*.

Regenerated shoots collected from shoot organogenesis medium were transferred to MS medium supplemented with different concentrations of IBA for root organogenesis (Table 1; Fig. 1d). The highest rooting (72%), maximum number (6.4) of roots/shoot, and highest root length (9.6 mm) was recorded for 3.0 mg l^{-1} IBA. However, a higher concentration than this (4.0 mg l^{-1}) was found to be inhibitory for rooting parameters in *B. rapa* var. *turnip*. Figure 1d shows healthy rooted plantlets, which were

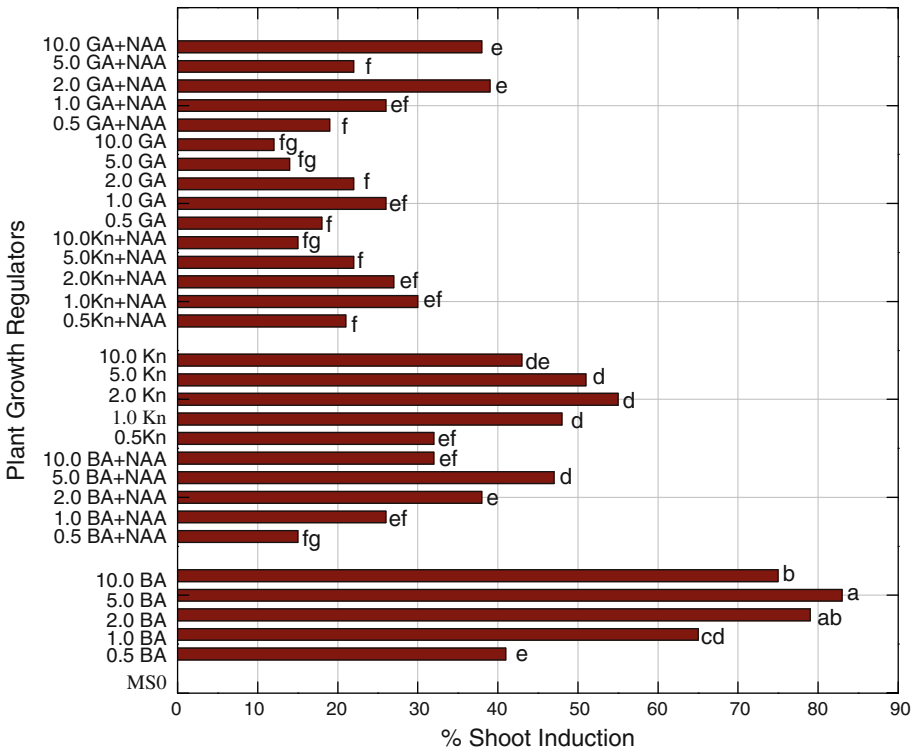

Fig. 3 Effects of various concentrations of BA, Kn, and GA_3 with or without 1 mg l^{-1} NAA on the percent shooting in *B. rapa* var. *turnip*. The data were collected after 5 weeks of subculture to MS media with a similar composition of plant growth regulators (PGRs). The values are the means of four replicates. Means with the same letter are not significantly different at $P < 0.05$

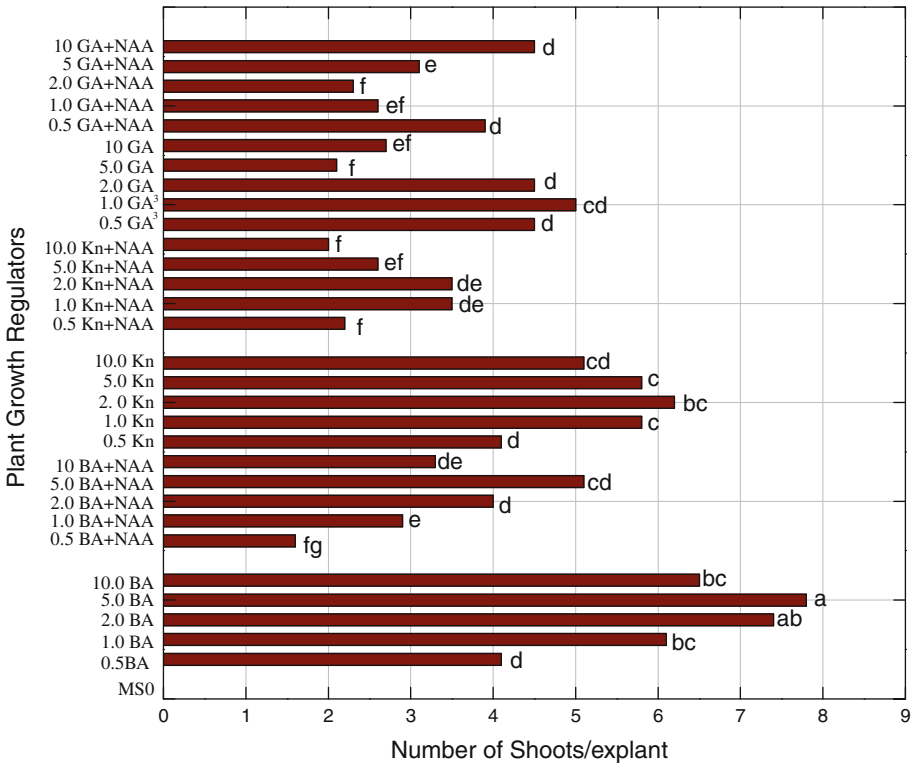

Fig. 4 Effects of various concentrations of BA, Kn, and GA_3 with or without 1 mg l^{-1} NAA on the number of shoots per explant in *B. rapa* var. *turnip*. The data were collected after 5 weeks of subculture to MS media with a similar composition of PGRs. The values are the means of four replicates. Means with the same letter are not significantly different at $P < 0.05$

transferred to potted soil for further growth. Initially, the roots were white in color and fragile, which subsequently became brown and thick. A direct relationship between root induction and IBA concentration was previously reported (Ahmad et al. 2010). Nonetheless, MS basal medium alone could also induce rooting in some members of other families (Abbasi et al. 2010a, b). Previously, the use of NAA exhibited optimal rooting in different *Brassica*

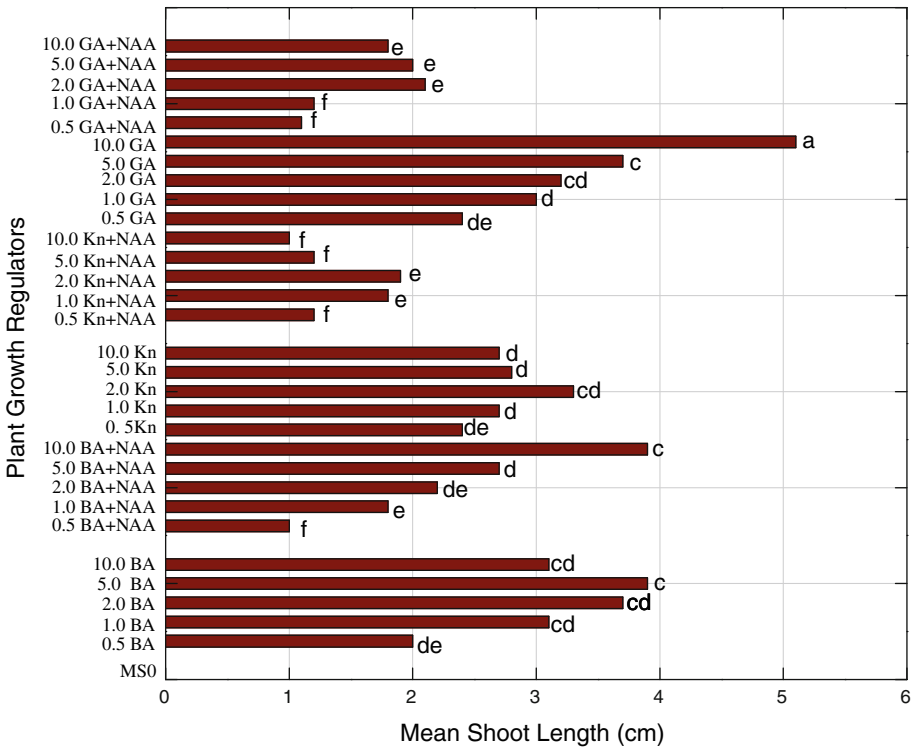

Fig. 5 Effects of various concentrations of BA, Kn, and GA_3 with or without 1 mg l^{-1} NAA on the mean shoot length in *B. rapa* var. *turnip*. The data were collected after 5 weeks of subculture to MS media with a similar composition of PGRs. The values are the means of four replicates. Means with the same letter are not significantly different at $P < 0.05$

Table 1 Effects of different concentrations of indole butyric acid (IBA) on percent rooting, number of roots per shoot, and approximate root length after ~5 weeks of culture on rooting media

IBA (mg l^{-1})	Rooting (%)	No. of roots/shoot	Root length (mm)
1.0	20d	1.8d	6.2c
2.0	58c	3.5b	7.9b
3.0	72a	6.4a	9.6a
4.0	65b	3.9b	8.8a

The values are the means of four replicates

Means with the same letter within each column are not significantly different at $P < 0.05$

spp. (Teo et al. 1997; Khan et al. 2009; Cogbill et al. 2010). Thus, it may be generalized that *Brassica* is not recalcitrant to commonly used auxins for rooting.

Activities of antioxidative enzymes (SOD, APX, CAT, GPX, and POD) were detected in all of the regenerated tissues and were compared with those of wild-grown plantlets (Table 2). SOD activity was found to be higher in regenerated plantlets followed by regenerated shoots, wild plantlets, and calli, respectively. APX, GPX, and POD activities were found to be higher in calli than the other tissues tested. Meratan et al. (2009) reported enhanced accumulation of antioxidant enzymes during callus differentiation. Higher levels of CAT activity were detected in regenerated shoots. This increase in the activity of CAT suggests its effective scavenging of H_2O_2 produced in regenerated shoots and its high activity could be correlated to the process of differentiation that occurred during shoot or root induction. Previously, similar observations were reported for other plant species (Gupta and Datta 2003; Meratan et al. 2009). The involvement of antioxidative

Table 2 Activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and peroxidase (POD) (U/mg protein) in wild-grown plantlets, calli, regenerated shoots, and regenerated plantlets of *Brassica rapa* var. *turnip*

Tissue	SOD	APX	CAT	GPX	POD
Wild plant	12.5 ± 1.2bc	9.3 ± 1.1e	25.3 ± 2.1bc	14.7 ± 1.0b	32.1 ± 1.2b
Callus	9.75 ± 0.9c	31.7 ± 2.3a	1.21 ± 1.1f	20.5 ± 0.4a	44.8 ± 0.7a
Regenerated shoots	16.28 ± 1.3b	26.4 ± 0.8b	35.9 ± 1.5a	10.6 ± 1.3c	28.91 ± 1.3bc
Regenerated plantlets	20.41 ± 1.2a	14.53 ± 1.3d	24.8 ± 0.9bc	15.94 ± 0.9b	7.83 ± 0.8f

The values are the means of three replicates

Means with the same letter within each column are not significantly different at $P < 0.05$

enzymes in differentiation has already been reported for *Acanthophyllum* and strawberry (Meratan et al. 2009; Tian et al. 2003).

Our data is showing that the activity of SOD increased during differentiation, and the activities of APX, GPX, and POD decreased during differentiation (Table 2). Nevertheless, CAT showed independent behavior. Previously, a positive relationship of CAT and POD was observed with growth and differentiation (Gupta and Datta 2003). It was also observed that cells which had undergone extreme levels of oxidative stress became necrotic and eventually died. Incipient oxidative damage could be detrimental, as this has the potential to generate genetic errors which could potentially lead to genetic instability (Benson 2000). Oxidative stress, therefore, has far-reaching consequences for in vitro plant systems (Fantel et al. 1998). We conclude from our study that all of the antioxidative enzymes contribute, to some extent, to growth and differentiation in *B. rapa* var. *turnip*, but their involvement at the molecular level has yet to be elucidated.

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