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IN VITRO PLANT REGENERATION IN *SINAPIS ALBA* AND EVALUATION OF ITS RADICAL SCAVENGING ACTIVITY

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Abstract

Feasible regeneration protocols and the antioxidant activity of regenerated tissues of an economically important plant, *Sinapis alba*, were evaluated and compared with seed-derived plantlets. Shoot regeneration was achieved from four-week-old seed-derived leaf explants, cultured on Murashige and Skoog (MS) medium incorporated with several plant growth regulators (PGRs). Optimum (89%) callogenic response was observed for 1.0 mg/l 6-benzyladenine (BA) followed by other PGRs. After three weeks of culture, subsequent sub-culturing of callus into MS medium with similar concentrations of PGRs induced shoot regeneration. Highest shoot regeneration frequency (85%) was recorded for 5.0 mg/l BA after six weeks of sub-culture. Highest (6.3) shoots/explant were recorded for 2.0 mg/l BA. Incorporation of 1.0 mg/l NAA into MS medium containing 5.0 mg/l BA produced shoots 5.8 cm long. In this study, BA was found to be the optimal PGR for induction of callus and shoot regeneration in *Sinapis alba*. Rooted plantlets from elongated shoots were transferred into MS medium containing different concentrations of indole butyric acid (IBA). Furthermore, the antioxidant potential of regenerated tissues was evaluated by using DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical. Regenerated shoots showed significantly higher radical scavenging activity than other tissues tested. This study contributes to a better understanding of the different mechanisms involved in morphogenesis and production of biologically active components in *Sinapis alba*.

Introduction

Sinapis alba, also known as *Brassica alba,* is an economically important plant of Brassicaceae, a large family of 350 genera and 3000 species (Musgrave 2000; Brown *et al.,* 2005; Cogbill *et al.,* 2010). Commonly known as yellow or white mustard, and growing well in hot and dry environments, *S. alba* is an annual spring crop and has a chromosome number of 2*n*=24 (Hemmingway 1976; Brown *et al.,* 2005). *S. alba* is grown for its seeds, which have bio-active compounds (Gareau *et al.,* 1990). The most significant one is a glucosinolate, p-hydroxybenzyl glucosinolate, also called sinalbin, that imparts a bitter taste to the seeds (Brader *et al.,* 2006). Sinalbin is biologically active upon hydrolysis and its hydrolyzed product has shown some bactericidal, antifungal and anticarcinogenic activities (Fahey *et al.,* 2001; Holst and Williamson, 2004; Brader *et al.,* 2006; Fimognari and Hrelia, 2007). Additionally, with the presence of higher amounts of important phytochemicals like erucic acid, oleic acid and a low content of monosaturated omega-9-fatty acid in seeds, *S. alba* is valuable for condiment use (Downey and Rakow, 1995). Its leaves are rich in vitamins, minerals and fiber and are used as a flavoring agent in mixed salads and also as a potherb (Steinmetz and Potter, 1996). This plant can tolerate drought and high-temperature conditions and has resistance to flea beetles and other pests (Downey *et al.,* 1975; Putnam, 1977; Lamb, 1980; Brown *et al.,* 2005).

Conventional cultivation procedures are compromising the crop quality and consumption of mustard species, which is increasing in the developed world with the passage of time (Walker and Booth, 2001; Ahmad *et al.,* 2011; Hussain *et al.,* 2011). Therefore, methods of *in vitro* regeneration are an attractive and promising option for the conservation, crop improvement, and large scale production of such economically important plant species (Abbasi *et al.,* 2007; Abbasi *et al.,* 2010a; Ahmad *et al.,* 2010). There have been no reports on successful regeneration of *S. alba* from leaf explants of *in vitro* seed-derived plantlets until now. However, explants from anthers, cotyledons, pistils, stamens and stigmas for the micropropagation of this medicinally important plant species have been reported (Klimaszewska *et al.,* 1983; Jain *et al.,* 1989; Zarychta *et al.,* 2007; Zarychta and Zenkteler, 2010). *Agrobacterium*-mediated transformation by infection of stem explants of *S. alba* and regeneration of transgenic plants have also been reported (Hadif and Batschauer, 1994).

In vitro plant regeneration is a complex phenomenon involving different biochemical mechanisms for its progression (Meratan *et al.,* 2009). It is the process of the activation and regulation of certain enzymes at specific times for organogenesis (Abbasi *et al.,* 2007). Reactive oxygen species (ROS) have a functional role in the developmental processes of plants. ROS scavenging via enzymatic and nonenzymatic systems and the plasticity of ROS production and scavenging in response to different environmental stimuli have significant roles in the plant development (Benson 2000; Ducic *et al.,* 2003). Some *Brassica* species have been previously evaluated for radical scavenging activities (Tian *et al.,* 2003; Meratan *et al.,* 2009), but *S. alba* has not been studied yet.

Conventional approaches have proven inadequate for identifying the genes responsible for abiotic stress tolerance and insect resistance. Yield losses due to environmental stress could be reduced if these genes can be isolated for transforming other crops to improve these traits. Modern *in vitro* regeneration techniques have the potential to fulfill modern agronomy needs (Khan *et al.,* 2009). The main objective of the present study was to optimize the *in vitro* conditions for regeneration of *S. alba* from *in vitro* seed-derived leaf explants and to investigate regenerated tissues for antioxidant potentials.

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Materials and Methods

Seeds of *S. alba* were kindly provided by Dr. Mir Ajab Khan, Professor, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. These seeds were then decontaminated by surface sterilization through the protocol developed by Abbasi *et al.*, (2011). Seeds were immersed in 70% ethanol for three minutes, then soaked in 0.1% mercuric chloride solution for one minute and finally washed and rinsed four times with autoclaved sterile distilled water. Seeds were then allowed to germinate on MS (Murashige and Skoog, 1962) basal medium supplemented with 3% sucrose and 0.8% agar, under aseptic lab conditions (pH = 5.8, photoperiod of 16 h, light irradiance ~45µmol/m^2/sec and temperature = 25 ± 2°C). Explants from leaves of 4-week-old plantlets were excised and sterilized with the seed sterilization protocol to avoid endogenous contamination. Leaf explants were then transferred to organogenesis medium (MS basal medium) supplemented with different concentrations of 6-benzyladenine (BA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and gibberellic acid (GA_3), individually or in combination, under a laminar flow hood. For callus production and shoot organogenesis, leaf explants (1.0-1.5 cm^2) were placed onto MS medium supplemented with either BA (0.5, 1.0, 2.0, 5.0, 10.0 mg/l), 2,4-D (0.5, 1.0, 2.0, 5.0, 10.0 mg/l), GA_3 (0.5, 1.0, 2.0, 5.0, 10.0 mg/l) either alone or in combination with 1.0 mg/l NAA. Basal MS (MS0; without any hormone) was used as the control. About five to six leaf-explants were placed onto 30 ml of solidified MS medium in a 100 ml Erlenmeyer flask. After four weeks of culture time, callus induction (percent responding explants) was recorded. Yellowish-green callus was refreshed into MS basal medium of the same composition/concentrations of PGRs for further growth and development. Data on shoot organogenesis, including % shooting, number of shoots/explant and average shoot length were collected after six weeks of callus sub-culturing. Callus induction, percent shooting and rooting were recorded by visual inspections.

Elongated shoots were then transferred to establish satisfactory roots in media containing different concentrations (1.0, 2.0, 3.0, 4.0 mg/l) of indole butyric acid (IBA), and after 6 weeks rooted plantlets were removed, washed with sterile distilled water and transferred to soil for further growth.

Regenerated tissues like callus, shoots, seed-derived plantlets and *in vitro* regenerated plantlets were used to determine 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity by the procedure of Amarowicz *et al*., (2004). Briefly, 8.0 mg of plant extract was dissolved in 4 ml of pure methanol and then added to methanolic DPPH solution (1 mM, 0.5 ml). The resulting mixture was vortexed for 15 sec and left to stand for 30 min at room temperature. The absorbance of the resulting solution was measured by a spectrophotometer (Agilent 8453, CA, USA) at 517 nm. A methanolic solution of DPPH that had decayed and hence no longer exhibited purple color (2 mg of butylated hydroxyanisole (BHA) dissolved in 4 ml of methanol with 0.5 ml of DPPH solution added) was used for background correction instead of pure methanol. Finally, the radical scavenging activity was calculated as percentage of DPPH discoloration using the equation:

$$\% \text{ scavenging DPPH free radical} = 100 \times (1-A_E/A_D)$$

where A_E is absorbance of the solution when an extract was added at a particular concentration and A_D is the absorbance of the DPPH solution with nothing added. For statistical analysis, five culture flasks were used and data were collected from duplicates. ANOVA (Analyses of variance) and DMRT (Duncan multiple range test) were used for comparisons of means of different treatments. SPSS (Windows version 7.5.1, SPSS Inc., Chicago) was used to determine the significance at $p<0.05$.

Results and Discussion

The goals of the current study were to optimize the *in vitro* growth conditions, develop a competent reproducible protocol for regeneration of *S. alba* from seed-derived leaf explants and investigate the antioxidant activity of regenerated tissues. Seeds collected were viable, were not recalcitrant to *in vitro* germination and showed a feasible frequency of germination of ~80% (data not shown). Contamination levels were decreased by ~75% (data not shown) over untreated seeds. Many protocols for the decontamination of leaf explants have shown inhibitory effects on micropropagation (Makunga *et al.,* 2003; Eeswara *et al.,* 1999; Atak and Celik, 2009; Debnath, 2009). We did not observe any inhibitory effect of the sterilization protocol on regeneration (data not shown).

The response, activity and effects of different plant growth regulators such as BA, GA_3, 2,4-D singly, or BA and GA_3 in combination with 1 mg/l NAA, on indirect organogenesis were investigated (Figs. 1-5). Various PGRs like BA, GA_3, 2,4-D alone or BA and GA_3 in combination with 1 mg/l NAA were tested for regeneration purposes.

Leaf explants of *S. alba* responded to all PGRs used in the present work (Fig. 2). After three weeks callus induction was initiated. After four weeks callus proliferation became more rapid. The callus produced was fast-growing and yellowish-green in color. The highest success of callus induction (89% of explants) was recorded for 1.0 mg/l BA (Fig. 2). GA_3 alone (2.0 mg/l) showed a moderate response while callus induction recorded for 2,4-D (22%) was significantly lower than all the PGRs applied in the experiment (Fig. 2). We observed no callus formation on MS medium without added PGRs. These observations agree with Khan *et al*., (2009), who found a high percentage of callus induction (91.43%) in *Brassica rapa* genotype Tori-7 on media supplemented with 2 mg/l BA + 0.1 mg/l NAA + 2.0 mg/l $AgNO_3$.

In the current study the incorporation of NAA into MS medium containing BA or GA_3 did not enhance explant response in comparison to BA and GA_3 used alone. However, in a previous report, 1.0 mg/l BA and 1.0 mg/l NAA produced a 75.3% callus response from cotyledonary explants of *S. alba* (Jain *et al.,* 1989). Our data showed the same callogenic response for BA 2.0 mg/l and GA_3 2.0 mg/l. However, lower callus induction was observed for 2,4-D. Khan *et al*., (2002) reported similar results, with no callus formation on 2,4-D. These observations contradict the findings of Ali *et al*., (2007), who found that callus induction ability in *B. napus* cultivars was enhanced by using 2,4-D in combination with BA and $AgNO_3$.

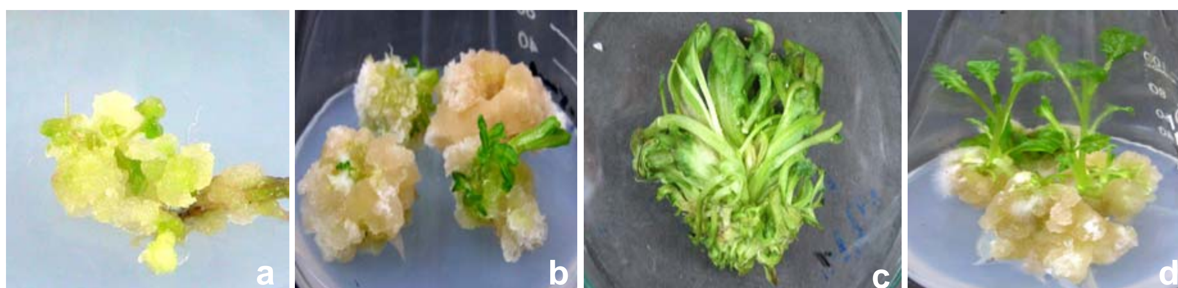

Fig. 1. Plant regeneration in *Sinapis alba* (a) Callus (b) Shoot regeneration (c) Shoot elongation and (d) Shoot proliferation.

BA showed an efficient response for callus induction in our study and has been reported as an optimal PGR in callus induction of various medicinally important plants (Ahmad *et al.,* 2010; Abbasi *et al.,* 2010b). Callus cultures were refreshed to MS medium of the same composition and concentration of PGRs. Subsequently shoots started appearing and data were recorded after 6 weeks of culture time. BA alone (5.0 mg/l) showed the best shooting response (82%) for a single PGR; however, NAA in combination with BA or GA_3 produced the optimal shooting response. These observations agree with the findings of Abbasi *et al*., (2010a).

GA_3 induced shoot organogenesis response at significantly higher levels than other PGRs tested (Fig. 3). Zarychta *et al*., (2007) studied organogenesis of *Sinapis alba* from pistil and stamen explants and observed adventitious shoots in MS basal medium incorporated with BA and 2,4-D. In another report, BA in combination with 2,4-D induced optimal shooting response for the stigma explants of *S. alba* (Zarychta & Zenkteler, 2010). In the present study we observed the minimal shooting response with 2,4-D.

Medium containing 2.0 mg/l BA resulted in the maximum number of shoots (6.3 shoots/explant) in *S. alba* (Fig. 4). By increasing the concentration of BA to 2.0 mg/l, the number of shoots also increased. The experiments with a combination of BA and NAA showed a decline in the shooting response (Fig. 4). On the other hand, with an increase in the concentration of BA from 0.5 to 2.0 mg/l we observed an increase in shoot production as reported earlier for *Brassica carinata* (Jaiswal *et al.,* 1987), *B. napus* (Moloney *et al.,* 1989) and *B. juncea* (Sharma *et al.,* 1990).

However, it is assumed that a synergistic combination of BA with NAA facilitates multiple shooting responses as reported previously in *B. rapa* (Guo *et al.,* 2000). In another report this combination of BA with NAA was not satisfactory for a shooting response in rapid-cycling *B. rapa* on MS medium having BA and NAA in combination or thidiazuron alone (Abbasi *et al.*, 2011; Guo *et al.*, 2011). In our work GA_3 induced multiple shooting at 10 mg/l.

Our results contradict observations that BA and NAA in synergistic combination enhance shoot regeneration frequency as previously reported by Abbasi *et al*., (2010). Khan *et al*., (2009) found a combination of BA, NAA and $AgNO_3$ optimal to induce a maximum number of shoots/explant in *Brassica* spp. Abbasi *et al*., (2010) found that incorporation of NAA with GA_3 or BA increased the number of shoots as compared to BA or GA_3 used alone for *Silybum* spp. Magyar-Tabori *et al*., (2010) concluded that the type and concentration of cytokinins is largely dependent on genotype. In contrast, Abbasi *et al*., (2011), found that the addition of NAA with either BA or GA_3 significantly inhibited number of shoots/explants in *B. rapa*. The differences in data may be due to the use of different explants sources. Fig. 5 shows the longest shoot (5.8 cm) was recorded from the medium containing 5.0 mg/l BA and 1.0 mg/l NAA. Makunga *et al*., (2003) also observed similar findings for *Thapsia* spp. However, Lucchesini *et al*., (2009) found that BA alone promoted good shoot length in *Echinacea angustifolia*.

Furthermore, incorporation of NAA into MS basal medium containing GA_3 showed moderate shoot length (4cm) in our study (Fig. 5). Ahmad *et al*., (2010) found longer shoots in *Piper* spp. on BA in combination with GA_3 than other PGRs. However, GA_3 did not produce optimal lengths for shoots in *Silybum marianum* (Abbasi *et al.,* 2010). Surprisingly, shooting response was observed for all of the PGRs tested in the present study but BA showed the strongest shooting results (Figs. 2-5). In our study, 2,4-D did not produce good results for callogenesis or shoot organogenesis (Figs. 2 & 5).

For root organogenesis the regenerated shoots were refreshed to MS basal medium supplemented with different concentrations of IBA (Table 1). IBA at concentrations of 1.0-3.0 mg/l induced good root formation. The best rooting responses (percentage of shoots rooting, 74%), maximum number of roots per shoot (6.6) and greatest length of root (9.8 mm) were found for 3.0 mg/l IBA. In our study IBA at a higher concentration (4.0 mg/l) inhibited rooting in *S. alba*. IBA at 3.0 mg/l was found to be optimal for rooting response by Abbasi *et al*., (2011) in *B. rapa*. Ahmad *et al*., (2010) found a direct relationship between rooting and the concentration of IBA in *Piper*. However, in different *Brassica* spp., NAA has been reported to be most effective for rooting (Teo *et al*., 1997; Khan *et al.,* 2009; Cogbill *et al.,* 2010). It is therefore concluded that *Brassica* spp. are not recalcitrant to commonly used auxins for rooting.

Accumulation of secondary metabolites in regenerated plantlets is similar in nature to those found in mother plants (Shilpa *et al.,* 2010). Antioxidant potential of regenerated tissues of *S. alba* was determined by using DPPH free radical (Fig. 6). We found regenerated shoots had significantly higher antioxidant activity than other regenerated tissues. Regenerated shoots had high antioxidant activity (59%) in comparison to regenerated plantlets (40%), seed-derived plantlets (42%) and callus (33%). Callus cultures were found to have lower antioxidant activity. Ahmad *et al*., (2010) had similar observations for black pepper. Abbasi *et al*., (2010a) found higher antioxidant activity in callus cultures of *Silybum*. This variation in data suggests the accumulation of different components during different phases of growth. The regeneration protocol established here has shown feasible amounts of active antioxidants from seed-derived plantlets. This protocol could be scaled up to a bioreactor level to produce chemically consistent *S. alba* plantlets.

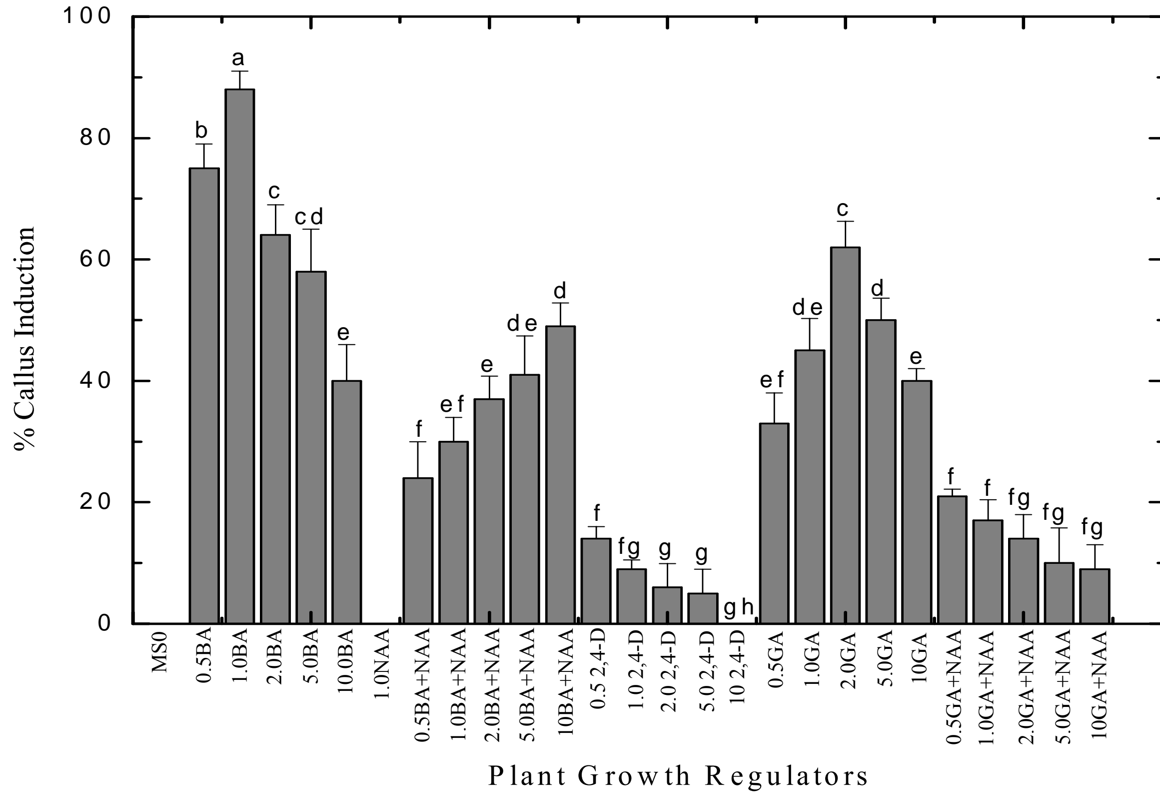

Fig. 2. Effects of various concentrations of BA, 2,4-D, GA_3 with or without 1 mg/l NAA on percent callus induction of *S. alba.* Data were collected after 4 weeks of culture. Values are means of 5 replicates. Columns within same grid lines are not significantly different at $p<0.05$.

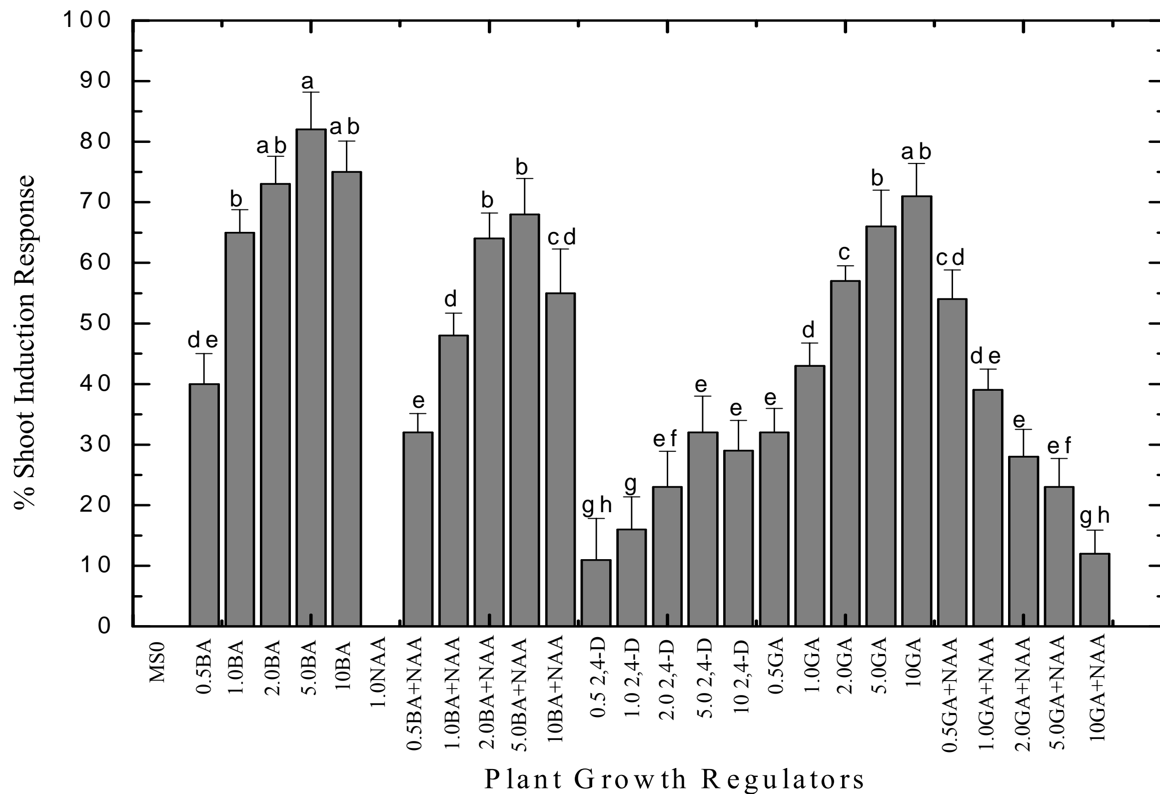

Fig. 3. Effects of various concentrations of BA, 2,4-D, GA_3 with or without 1 mg/l NAA on percent shooting in *S. alba*. Data were collected after 6 weeks of sub-culture on MS media with similar composition of plant growth regulators. Values are means of 5 replicates. Columns within same grid lines are not significantly different at $p<0.05$.

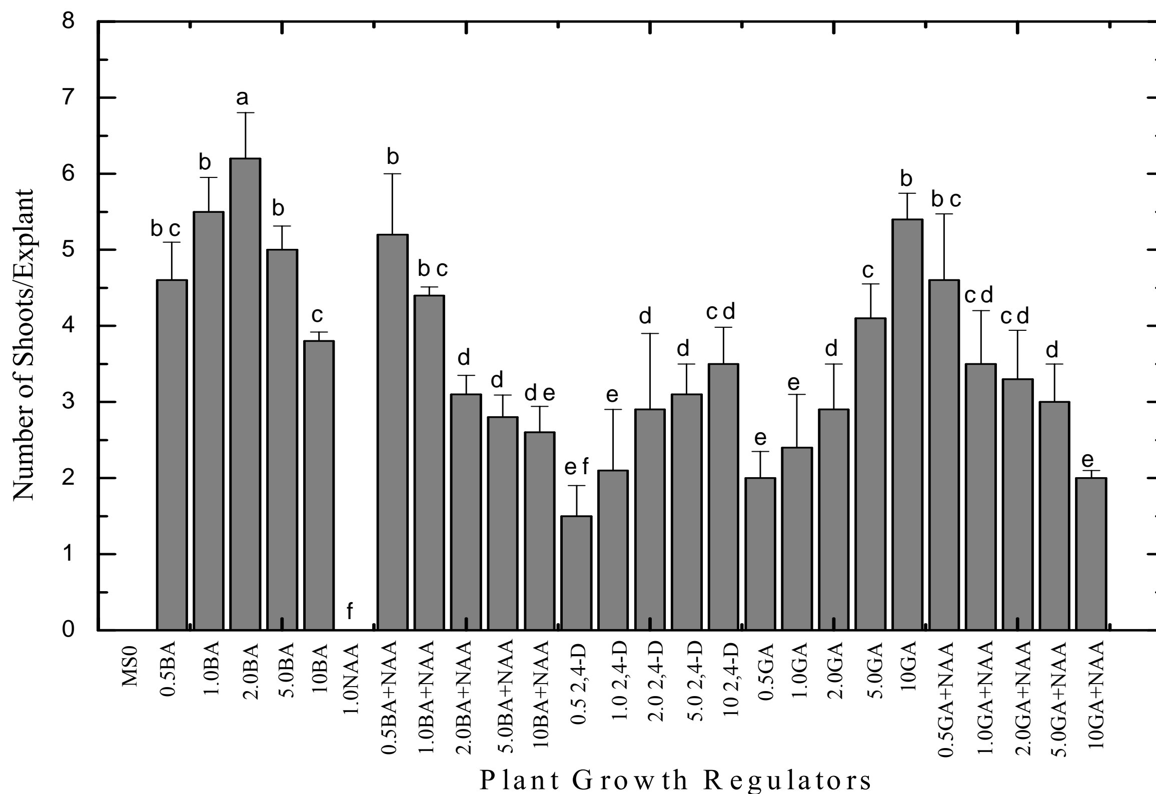

Fig. 4. Effects of various concentrations of BA, 2,4-D, GA_3 with or without 1 mg/l NAA on number of shoots per explant in *S. alba*. Data were collected after 6 weeks of sub-culture on MS media with similar composition of plant growth regulators. Values are means of 5 replicates. Columns within same grid lines are not significantly different at $p<0.05$.

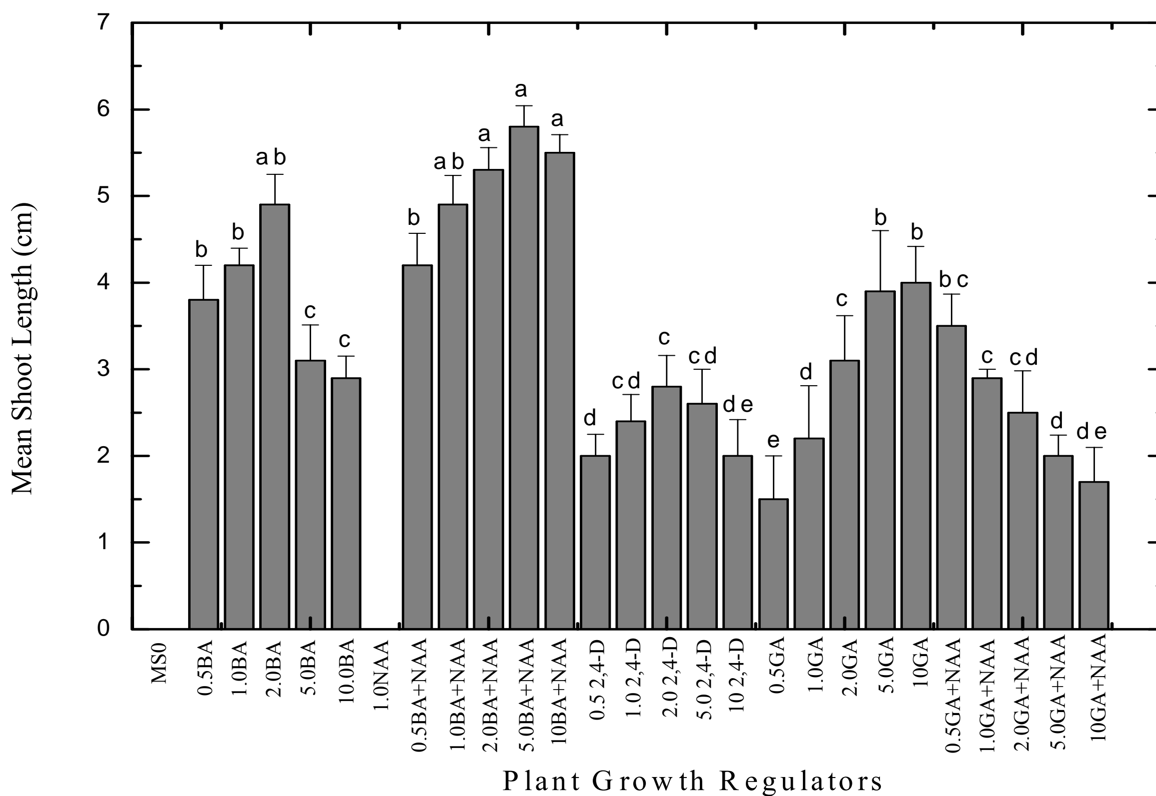

Fig. 5. Effects of various concentrations of BA, 2,4-D, GA_3 with or without 1 mg/l NAA on mean shoot length in *S. alba*. Data were collected after 6 weeks of sub-culture on MS media with similar composition of plant growth regulators. Values are means of 5 replicates. Columns within same grid lines are not significantly different at $p<0.05$.

Table 1. Effects of different concentrations of indole butyric acid (IBA) on percentage rooting, number of roots per shoot and approximate root length after ~6 weeks of culture on rooting media. Values are means of 5 replicates. Means with common letters are not significantly different at p<0.05.

IBA (mg/l)	Rooting (%)	No. of roots/shoot	Root length (mm)
1.0	22 ± 2.4^{d}	2.1 ± 0.3^{d}	6.4 ± 0.6^{b}
2.0	60 ± 3.1^{b}	3.7 ± 0.4^{c}	8.2 ± 0.3^{a}
3.0	74 ± 2.9^{a}	6.6 ± 0.1^{a}	9.8 ± 0.4^{a}
4.0	67 ± 1.8^{b}	4.1 ± 0.3^{bc}	9.1 ± 0.3^{a}

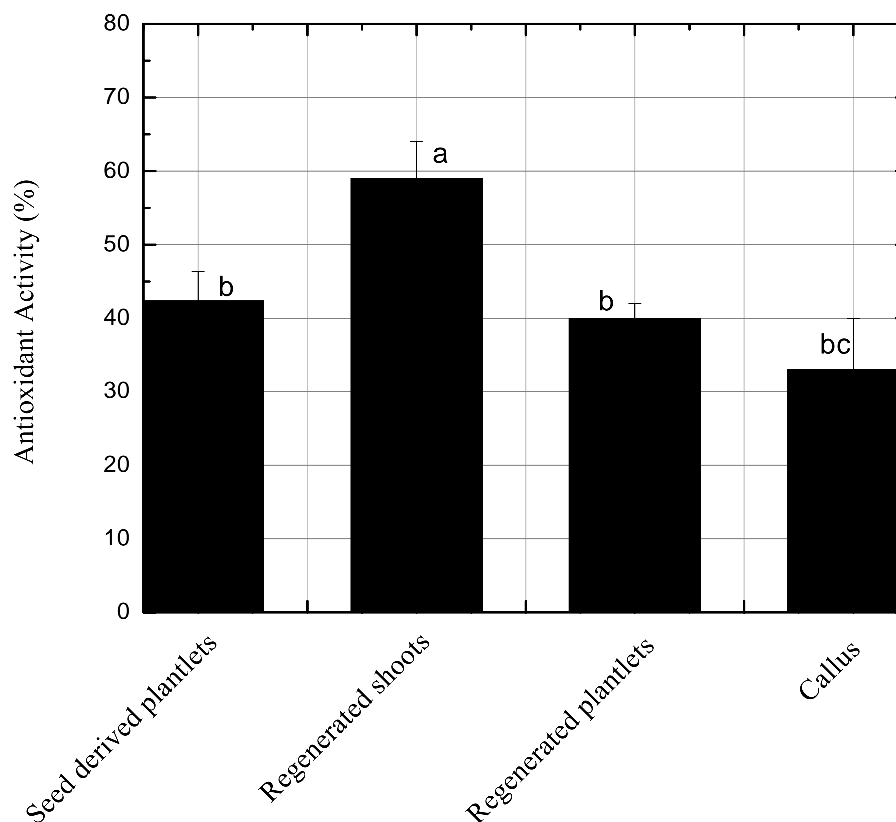

Fig. 6. Antioxidant activity of regenerated tissues of *S. alba*. The activity was determined by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) as free radical. Values are means of triplicates with standard deviation. Means with common letters are not significantly different at p<0.05.

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