Assessment of Changes in Physiological and Biochemical Behaviors in Grey-haired Acacia Tree (Acacia gerrardii)– an Important Plant of Arid Region

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Abstract High frequency plantlet regeneration was achieved in cotyledonary nodes of *Acacia gerrardii* from 15 days old *in vitro* grown seedlings, cultured on Murashige and Skoog (MS) medium supplemented with benzyladenine (BA), kinetin (Kn) and α- naphthalene acetic acid (NAA) either alone or in combinations. The highest regenerative response was observed on a medium containing 5.0 μM BA and 0.5 μM NAA where 90% of the cultures responded with an average shoot number (9.40 ± 0.70) per explant in 8 wk time. *In vitro* shootlets were transferred to root induction medium consisting of MS medium supplemented with auxins NAA or indole-3-butyric acid (IBA). Rooting was best in a medium supplemented with 2.0 μM IBA. Rooted plantlets were acclimatized and transferred to the field with 70% survival rate. Increase in chlorophyll (Chl) a, b, carotenoid levels, MDA and proline contents were observed in plantlets acclimatized for 28 days. As the acclimatization continues, an upregulation of the superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) enzyme activities were also observed. The results indicate that micropropagated plantlets develop an antioxidant enzyme system after *ex vitro* transfer, which suggests that the species can cope with the predicted climate change.

Keywords Fabaceae, micropropagation, axillary shoots, reactive oxygen species, afforestation

Abbreviations

APX Ascorbate peroxidase
BA 6-Benzyladenine

CAT Catalase
Chl Chlorophyll
CN Cotyledonary node
DDW Double distilled water
GR Glutathione reductase
IBA Indole-3-butyric acid
Kn Kinetin
MDA Malondialdehye content
MS Murashige and Skoog medium
NAA α- Naphthalene acetic acid
PGR Plant growth regulator
SOD Superoxide dismutase

Introduction

In recent years, molecular and genetic engineering techniques have been employed in forest tree research for successful, reforestation and forest management programs. The application of tissue culture methods like clonal propagation has gained momentum to meet the growing demands for biomass and forest products. In the last decade, *in vitro* protocols to regenerate several woody species have been developed. Species of *Acacia* have been given due importance in tree tissue culture owing to their ecological and economical significance. Proper selection and collection of explants with judicious incorporation of plant growth regulators, antioxidants, additives and adsorbents during *in vitro* culture have greatly contributed in developing successful regeneration protocols for many *Acacia* species [1], [2].

Acacia gerrardii (Fabaceae), a small to medium size legume tree, is commonly found in arid river valleys or shrubby uplands. It is hardy to at least 20° F and sustained only minor damage from temperatures as low as 15° F. Plant extract has been shown to possess antioxidant and pesticidal properties [3]. Its unusual papery bark, ample shade and spring flowers make *Acacia gerrardii* an excellent tree to mix with more traditional arid landscape species in both formal and more naturalistic desert landscape designs. Conventional methods of propagation are difficult due to recalcitrant seeds as well as short seed viability. Also, vegetative propagation of *Acacia gerrardii*

through vegetative cuttings is rather slow, and good number of cuttings died during transportation and plantation.

Micropropagation through adventitious or axillary bud proliferation has the capacity to produce large numbers of plants within limited space and time irrespective of the season, under controlled conditions of temperature, light intensity, and photoperiod. Within the last four decades plant micropropagation has developed from a laboratory curiosity to a real industry. Nevertheless, its widespread use is restricted by the formation of plantlets of abnormal morphology, anatomy and physiology induced by special conditions during *in vitro* culture, *e.g.*, high air humidity, decreased air turbulence, low irradiance, low CO_2 concentration during light period, cultivation media supplemented with sugars and growth regulators [4], [5]. After *ex vitro* transfer, these plantlets might be easily impaired by sudden changes in environmental conditions. Oxidative stress is a common physiological stress. Reactive oxygen species (ROS) such as superoxide radical ($O2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are major agents causing oxidative stress [6]. Their uncontrolled production can significantly affect plant cell growth and metabolism leading to the damage of proteins, membrane lipids, nucleic acids, and chlorophyll directly or through the formation of secondary toxic substances [7], which, in turn, can lead to a decrease in plant productivity. To prevent the harmful effects of ROS, plants activate antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX), and stimulate the production of antioxidant molecules including ascorbic acid, glutathione and a-tocopherol, which scavenge ROS [8]. This integrated system prevents oxidative damage in general, and it is therefore a common component of the response of plants to numerous stresses [9].

So far, there has been no report on *in vitro* shoot multiplication of *A. gerrardii*. This paper describes a successful protocol on *in vitro* mass propagation from cotyledonary nodes of *in vitro* grown seedlings and determines the photosynthetic pigments content as well as antioxidant enzymes activity of micropropagated plants of *A. gerrardii*.

Materials and methods

Seed germination and procurement of explants

Seeds of *Acacia gerrardii* were collected from King Saud University, Riyadh, Saudi Arabia. Prior to surface sterilization, seeds were soaked in distilled water for about 24 hr, washed under

running tap water for 15 min followed by treatment with 1% (v/v) Teepol for 5 min and rinsed with double distilled water for three times. Prior to inoculation, seeds were sterilized with 0.1% (w/v) aqueous $HgCl_2$ for 2 min followed by 2-3 rinsing with double distilled water in a laminar air flow cabinet. The sterilized seeds were inoculated on MS [10] basal medium. After 2 weeks of inoculation, seed germinated and gave rise to healthy seedlings. These *in vitro* seedlings were used as a source of explant. CN explants (1-1.5cm) (Fig. 1a) excised from 15 days old aseptic seedlings were inoculated on different nutrient media.

Nutrient media preparations and culture conditions

The nutrient media consisted of the salts and vitamins of MS supplemented with sucrose (3% w/v, Qualigens, Mumbai, India) and different concentrations of growth hormones. For shoot induction, different growth hormones such as 6-benzylaminopurine (BA), kinetin (Kn): 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 µM and α-naphthalene acetic acid (NAA: 0.5, 1.0, 2.0 and 2.5 µM) were added alone or in combination to MS basal medium. For root induction, MS medium was supplemented with indole-3- butyric acid (IBA) and NAA at different concentrations (0.1- 5.0 µM) singly. All media were adjusted to pH 5.8 using 1N NaOH before autoclaving. The medium was supplied with 0.8% (w/v) agar (Qualigens, India). 20 ml of molten medium was dispensed into a culture tube (25 × 150 mm, BOROSIL) and plugged with nonabsorbent cotton wrapped in one layer of cheesecloth. The cultures were steam sterilized at 1.06 kg/cm^2 for 15 min. Subculturing was done after 2 weeks onto the same medium. All cultures were incubated in 16 /8 h light:dark photoperiod under light intensity of 50 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent light (Philips, India) at 25 ± 2°C with 55% relative humidity. Each treatment consisted of 10 replicates containing one explant and was repeated three times.

Acclimatization

Shoots with well developed roots were removed from the culture vessel and rinsed with running water to remove agar. Plantlets were transferred to 10-cm-diameter thermocol cups containing Soilrite^{TM} (Keltech Energies Ltd., India). Potted plantlets were covered with transparent plastic bags to ensure high humidity and irrigated regularly with tapwater. Plastic bags were opened and gradually removed over a period of 10 days. Potted plantlets were kept at 25 ± 2°C in artificial light (irradiance of 50 µmol m^{-2} s^{-1}) provided by cool and white fluorescent tubes for 4 weeks.

Thereafter, plants were transferred to garden soil in earthen pots and kept in the greenhouse. After 8 weeks, the plants were transferred to the field conditions.

Determination of chlorophyll and carotenoid contents

Chlorophyll a and b as well as carotenoid contents were determined using the methods of Mackinney (1941) [11] and Maclachan and Zalick (1963) [12], respectively. About 0.5 g fresh leaf tissues, from interveinal areas, were ground in 5 ml acetone (80%) and the suspension was filtered with Whatman filter paper Number 1. Absorbance of the solution was measured at 645 and 663 nm for chlorophyll a and b, respectively, and at 480 and 510 nm for carotenoid content using a UV–VIS spectrophotometer (UV 1700 PharmaSpec, Shimadzu, Tokyo, Japan).

Estimation of Lipid peroxidation and proline content

Leaf oxidative damage was estimated in terms of lipid peroxidation by determining the concentration of MDA, a product of the oxidation of polyunsaturated fatty acids. Measurements of MDA were performed as in Hodges et al. (1999) [13], taking into account the possible influence of interfering compounds in the assay for the 2-thiobarbituric acid reactive substances. The MDA concentration was expressed in terms of μmol g^{-1}(Fw), using an extinction coefficient of 155 mM^{-1} cm^{-1} at 532 nm. Absorbances measured at 600 and 440 nm allowed to take into account interference due to nonspecific turbidity and carbohydrates, respectively on a UV-visible spectrophotometer (Shimadzu, Japan).

Proline was determined following the ninhydrin method as described by Bates et al. (1973) [14] with some modifications according to Magne and Larher (1992) [15]. Briefly, fresh leaf tissue was extracted in 1.5 ml of 80% ethanol. After centrifugation at 10,000 × *g* for 5 min, 100 μl of the supernatant was added to 400 μl of a mixture of 1% ninhydrin and glacial acetic acid in a 60:40 (v/v) ratio. The reaction mixture was incubated in a water bath at 100ºC for 1 h, then rapidly cooled and portioned against 1 ml of toluene. After centrifugation at 3,000 × *g* for 5 min the organic phase was collected and absorbance was read at 520 nm using toluene as a blank. Proline concentration was determined against a standard curve (0.1 to 1.0 μg ml^{-1}) with L-proline (Loba, India) dissolved in 80% ethanol. The proline content is expressed in μmol g^{-1} Fw.

Antioxidant enzymes extraction and assay

To determine antioxidant enzyme activitiy, 0.5 g fresh leaf tissue, collected from 0, 7, 14, 21 and 28 days old micropropagated plantlets, was homogenized in 2.0 ml extraction buffer containing 1% polyvinylpyrrolidone (PVP), 1% Triton X-100, and 0.1g ethylene diamine tetra-acetic acid (EDTA) using pre-chilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 rpm for 20 min. The supernatant was used for protein determination and enzyme assays. Extraction was carried out in the dark at 4°C. A high speed centrifuge (Remi Instruments Ltd., India) and UV-visible spectrophotometer (Shimadzu, Japan) were used. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium in a reaction mixture consisting of 0.5 M Phosphate buffer (pH 7.5), 0.1 mM EDTA, 13 mM methionine, 63 mM nitroblue tetrazolium (NBT), 1.3 mM riboflavin and 0.1 ml enzyme extract. The reaction mixture was irradiated for 15 min and absorbance was measured at 560 nm against the non- irradiated blank. Catalase (CAT; EC 1.11.1.6) activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease of absorbance at 240 nm, following the method of Aebi (1984) [16]. The assay mixture contained 50 mM phosphate buffer (pH 7.0) and 100 µl enzyme extract in a total volume of 3 ml, and the reaction began by adding 10 mM H_2O_2. Glutathione reductase (GR; EC 1.6.4.2) activity was measured using the protocol based on glutathione-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. The assay mixture contained 50 mM phosphate buffer (pH 7.5), 1.0 mM EDTA, 0.2 mM (NADPH), and 0.5 mM glutathione disulfide (GSSG). The enzyme extract was added to begin the reaction, and the reaction was allowed to run for 5 min at 25 °C. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined as described by Nakano and Asada (1981) [17]. The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA, and 0.1 ml enzyme extract. The activities of each enzyme were expressed in enzyme units (EU) mg^{-1} protein min^{-1}. The protein content in enzymatic extracts was determined following the Bradford assay (Bradford 1976) [18] using bovine serum albumin as a standard.

Scoring of Data

All experiments were repeated thrice. Data on percent response, total number of shoots and shoot length were determined after culture initiation (4 weeks) and multiplication (8 weeks) of shoots

respectively. Similarly, data on percent rooting, total root number and root length were determined after 4 weeks of culture. The data of different treatments was quantified and subjected to statistical analysis using One Way Analysis of Variance (ANOVA) and pair wise means compared using Duncan's Multiple Range Test procedure ($p < 0.05$). The results were represented as mean ± standard error (SE). All the statistical analyses were performed using the SPSS statistical package software, version 10 (SPSS Inc., Chicago, USA).

Results and discussion

Multiple shoot induction and proliferation

The efforts have been intensified to initiate direct multiple shoot regeneration in *Acacia gerrardii* from CN explants. The media that did not contain PGR were virtually incapabale of eliciting any response from explants whereas media that contained increasing concentrations of any of the cytokinins assayed were able to promote shoot induction producing differentiated shoots with normal architecture. After 2 weeks in shoot induction medium containing growth regulators, explants swelled and differentiation of multiple axillary shoots was observed. The statistical difference was observed between the two cytokinins in terms of overall average results recorded for CN explant (Table 1). For each cytokinin type, the applied concentrations significantly influenced the formation of multiple shoots. Of the different concentrations of BA tested, 5.0 μM BA elicited maximum number of shoots (5.50 ± 0.28) per explant in 90 % cultures after 4weeks of induction (Table 1, Fig. 1b). On contrary, MS medium supplemented with Kn (5.0 μM) showed lower response with the induction of 2.5 ± 0.28 shoots per explant in 60 % cultures after 4 weeks. Our result showed that BA was able to induce the maximum number of shoots in CN explant compared to Kn (Table 1). However, increasing the concentrations of both BA and Kn beyond 5.0 μM did not evoke shoot proliferation and the percentage response got declined. The superiority of BA has also been reported for other *Acacia* species [19], [20], [21], [22], [23], [24], [25], [26].

Usually a combination of two or more different types of growth regulators is required for successful *in vitro* shoot proliferation, with the cytokinin- auxin interaction considered to be the most important for regulating plant growth. In accordance, overall mean results of the study indicated that *A. gerrardii* CN explants respond better when the medium is enriched with different concentrations of auxin NAA tested here (Table 2). Addition of NAA with Kn did not

improve the parameters evaluated as 4.33 ± 0.88 shoots per explant was produced on MS medium containing Kn (5.0 µM) and NAA (0.5 µM) combination in comparison to BA-NAA combination after 8 weeks of culture (Table 2). Among all BA-NAA combination tested, the maximum number of shoots (9.40 ± 0.70) per CN explant with an average shoot length of 6.00 ± 0.00 cm was recorded in the MS medium amended with BA (5.0 µM) and NAA (0.5 µM) in 90 % cultures after 8 weeks (Fig. 1c). Moreover, the shoots, on average, were longer than those obtained in the presence of 5.0 µM BA alone. In contradiction to our results, Vengadesan et al. (2002) [2] in *Acacia sinuata* and Khalafalla and Daffalla (2008) [25] in *Acacia senegal* have reported that when CN explants were cultured on MS medium containing a combination of BA and auxins (IBA, NAA or IAA), the number of shoots was reduced and produced basal callus. The majority of literature has also reported BA and NAA as the most synergistic combination for shoot multiplication in various plant species [27], [28], [29], [30], [26].

In vitro root induction and acclimatization of plantlets

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil. Rooting occurred from microshoots cultured on a medium containing auxins. Excised shoots failed to produce roots when cultured on MS medium without any growth regulator even after 4 weeks of culture. To determine which auxin would be effective for rooting induction, microshoots were employed to evaluate the effects of different concentrations of IBA or NAA in the medium. After 4 weeks of culture, the root architecture was analyzed. The results shown in the Table 3 demonstrate that a PGR free medium cannot sustain spontaneous adventitious rooting from regenerated shoots. However, supplementation of the MS medium with IBA at different concentrations was the best method for inducing well formed roots than the use of NAA. On NAA supplemented medium, basal callus was formed and no root formation was observed. Similar observation has also been documented by Rout et al. (2008) [31] in *in vitro* shootlets of *Acacia chundra.* Roots were initiated between 15 and 20 days after culture on IBA containing media. Among the various concentrations of IBA tested, IBA (2.0 µM) induced maximum number of roots (5.40 ± 0.30) per shoot with an average root length of 2.00 ± 0.05 cm after 4 weeks of culture (Table 3, Fig. 1d). Similarly, Vengadesan et al. (2002) [2] reported that rooting of *in vitro* shootlets of *A. sinuata* was better on MS medium supplemented with 7.36 µM IBA. Our results are also in accordance with other reports where IBA has been reported to have stimulatory effect on root induction in many tree species including *Acacia sinuata* [2], *Acacia*

mangium [32], *Acacia senegal* [25], *Prosopis cinerea* [33], *Stereospermum personatum* [34] and *Balanites aegyptiaca* [30].

The regenerated plantlets were successfully acclimatized in soilrite after 4weeks of transfer (Fig. 1e) and established in soil with 70% survival rate. All the plantlets were morphologically uniform and exhibited normal characteristics similar to those of the source plant.

Photosynthetic pigments

A significant increase in both Chl a (1.83 ± 0.12 mg g^{-1} Fw) and Chl b (2.10 ± 0.11 mg g^{-1} Fw) was observed in acclimatized plantlets compared to day 0 (0.50 ± 0.11 mg g^{-1} Fw Chl a and 0.50 ± 0.05 mg g^{-1} Fw Chl b) (Fig. 2). Likewise, carotenoid content in plantlets at 28th day was 1.73 ± 0.12 mg g^{-1} Fw higher than that at day 0 (0.41 ± 0.05 mg g^{-1} Fw) (Fig. 2). Similar observations have also been documented for *Rauvolfia tetraphylla* [35] and *Tylophora indica* [36] where increases in chlorophyll pigments and carotenoids contents were detected in plantlets during acclimatization. The observed differences in chlorophyll content indicated that plantlets of *A. gerrardii* were under stress, and perhaps these were associated with likely impairment in photosystem II and carotenoids are reported to be involved in protecting the photosynthetic machinery from photo-oxidative damage [37], [38], [39].

Lipid peroxidation and proline content

Figure 3A shows that the MDA content increased significantly following 7 to 21 days of acclimatization and reached maximum at 28th day from the start of acclimatization in *A. gerrardii*. Bailly et al. (1996) [40] documented that MDA, a decomposition product of polyunsaturated fatty acids hydroperoxides, results in oxidative damage and has been frequently used as a biomarker for lipid peroxidation. The increase in the amount of MDA observed immediately after *ex vitro* transfer of *A. gerrardii* is an indirect indicator of the generation of ROS, similar to those findings in *Phalaenopsis* [39], *Rauvolfia tetraphylla* [35], *Tylophora indica* [36] and *Ulmus minor* [41] during acclimatization. Moreover, the proline content increased significantly from the first week after *ex vitro* transfer and stabilized thereafter at 28th day of acclimatization (Fig. 3B). Hence, the trend of variation in the proline content with the duration of acclimatization was similar to that of MDA content in *A. gerrardii* plantlets. Ashraf

and Foolad (2007) [42] reported that proline is compatible solute that accumulate in plants under diverse stress conditions, and that have the potential to play an important role in protecting membranes and proteins from the deleterious effects of environmental stress. Moreover, proline may stabilize antioxidant enzymes and it may directly stimulate the ROS production in the mitochondria due to its effect on the electron transport processes in Pro-pyrroline-5-carboxylate cycle [43]. Therefore, we can speculate that in *A.gerrardii* the anti-ROS enzymatic system is triggered by the accumulation of an appropriate amount of proline when environmental stress is severe. The accumulated pool of proline can also serve as a carbon and nitrogen reserve for growth after stress relief, or it can provide the reducing agents to support the levels of mitochondrial oxidative phosphorylation and ATP generation needed for fast recovery from stress and repairing of stress-induced damages [44].

Antioxidant enzyme activities

The changes in the antioxidant enzymatic activities of micropropagated plants during acclimatization were observed in our study. SOD and CAT activities increased with increasing duration of acclimatization. On day 28, SOD and CAT activities was highest in the persistent leaves than the control plants (0 day) (Fig.4A and B). Similarly, significant increase in the activities of APX and GR were observed at 28th day of acclimatization than the control plants (0 day) (Fig.4C and D). According to Apel and Hirt (2004) [45], plant cells display nonenzymatic and enzymatic antioxidant systems to mitigate the oxidative damages caused by ROS. A balance between oxidant and antioxidant intracellular systems is hence vital for normal cell function, growth regulation and adaptation to diverse growth conditions. In accordance with this work, Van Huylenbroeck et al. (2000) [46] reported that micropropagated *Calathea* plantlets developed an antioxidant mechanism during acclimatization. Moreover, the authors suggested that the increase of SOD, APX, and GR activities revealed a protection against photo-oxidative stress linked to photoinhibition. Increases in SOD, CAT, GR and APX activities were also reported after *ex vitro* transfer of *Phalaenopsis* [39], *Rauvolifia tetraphylla* [35], *Zingiber officinale* [47], *Tylophora indica* [36] and *Ulmus minor* [41] micropropagated plantlets against oxidative stress. The rise in many of the antioxidant activities, that are located in different cellular compartments, suggest that formation of activated oxygen species can augment with increasing light intensity not only in chloroplasts, but also in mitochondria and peroxisomes. Since approximately 90% of

total leaf ascorbate peroxidase activity is localised in the chloroplast, this activity can represent the best estimation of chloroplast-based detoxification of reactive oxygen species via the Mehler-peroxidase pathway [48]. The observed pattern of activity for CAT in relation to light intensity strengthens the significance of the acclimatory changes observed for the predominantly chlorplastic ascorbate peroxidase. It is possible that the pattern observed for CAT is related to its role in photorespiration and/ or detoxification of H_2O_2 formed as a result of mitochondrial electron transport [49]. The ascorbic acid-dependent antioxidants enzymes, *e.g.* APX and GR, are predominantly localized in the chloroplast and GR is considered a key enzyme responsible for maintaining the reduced form of glutathione pool [50]. In this study, the increase in GR activity in the early stages of acclimatization suggests that GR may play an important role in scavenging H_2O_2 that is produced in chloroplast of persistent leaves, under both light intensities, during the period. These findings also corroborate those reported for *Calathea louisae* [46] and *Vitis vinifera* [51] during the hardening process. Our results indicate that micropropagated *A. gerrardii* plantlets develop an antioxidant enzyme system after *ex vitro* transfer which suggests that the environment at which plants are exposed during its hardening determinate their ability to tolerate ROS.

Conclusions

The present study was conducted to develop a complete *in vitro* propagation protocol for *Acacia gerrardii* with the flexibility of starting tissue culture with seedling derived explants. During optimization, various PGRs were tested, alone or in combination, to develop a high proliferation rate and to obtain healthy, multiple shoots. Using the protocol, it has been possible to obtain about 10 plants per CN explants after 8 weeks of transfer. Our observations also suggest that tissue-cultured plants developed a functional photosynthetic apparatus and antioxidant enzymatic protective system during acclimatization. A good climatic control during the critical days of the acclimatization process will lead to better results. The procedure outlined above may facilitate improvement, conservation and mass propagation of this tree in afforestation programs.

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Table 1: Effect of cytokinins on multiple shoot induction from CN explants of *A. gerrardii* after 4 weeks of culture

Cytokinins (µM)		% Response	Mean no. of shoots/explant	Mean shoot length (cm)
BA	Kn			
0.0	0.0	--	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
0.5	--	70	2.66 ± 0.66^{bc}	1.43 ± 0.12^{cd}
1.0	--	75	3.03 ± 0.60^{b}	1.83 ± 0.08^{b}
2.5	--	60	1.26 ± 0.14^{d}	1.83 ± 0.12^{b}
5.0	--	90	5.50 ± 0.28^{a}	2.50 ± 0.17^{a}
7.5	--	30	1.33 ± 0.33^{d}	1.00 ± 0.00^{e}
10.0	--	27	1.00 ± 0.00^{d}	1.00 ± 0.00^{e}
--	0.5	20	1.90 ± 0.10^{cd}	1.06 ± 0.12^{e}
--	1.0	40	1.90 ± 0.57^{cd}	1.16 ± 0.08^{de}
--	2.5	50	1.86 ± 0.13^{cd}	1.50 ± 0.11^{c}
--	5.0	60	2.50 ± 0.28^{bc}	2.00 ± 0.00^{b}
--	7.5	20	1.00 ± 0.00^{d}	1.00 ± 0.00^{e}
--	10.0	--	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}

Values represented means ± S.E (n=3). Means followed by the same letter within column are not significantly different (P = 0.05) using Ducan's multiple range test.

Table 2: Effect of optimal concentration of BA (5.0µM) and Kn (5.0µM) with NAA at different concentrations on shoot proliferation in MS medium from CN explants of *A. gerrardii* after 8 weeks of culture

Plant growth regulators (µM)			% Response	Mean no. of shoots/explant	Mean shoot length (cm)
BA	Kn	NAA			
5.0	--	0.5	90	9.40 ± 0.70^{a}	6.00 ± 0.00^{a}
5.0	--	1.0	80	5.00 ± 0.57^{b}	3.00 ± 0.57^{bcd}
5.0	--	2.0	75	3.00 ± 0.57^{bcd}	2.93 ± 0.47^{bcd}
5.0	--	2.5	60	1.33 ± 0.57^{d}	3.03 ± 0.57^{bcd}
--	5.0	0.5	71	4.33 ± 0.88^{bc}	3.66 ± 0.33^{b}
--	5.0	1.0	66	3.00 ± 0.57^{bcd}	3.33 ± 0.16^{bc}
--	5.0	2.0	66	3.00 ± 1.15^{bcd}	2.33 ± 0.03^{cd}
--	5.0	2.5	60	2.66 ± 0.33^{cd}	1.93 ± 0.88^{e}

Values represented means ± S.E (n=3). Means followed by the same letter within column are not significantly different (P = 0.05) using Ducan's multiple range test.

Table 3: Effect of IBA on root induction from *in vitro* raised microshoots of *A. gerrardii* in MS medium after 4 weeks of culture

IBA (µM)	% Response	Mean no. of roots/shoot	Mean root length (cm)
0.0	00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
1.0	20	1.00 ± 0.00^{b}	0.73 ± 0.14^{b}
1.5	30	1.33 ± 0.33^{b}	0.86 ± 0.08^{b}
2.0	70	5.40 ± 0.30^{a}	2.00 ± 0.05^{a}

Values represented means ± S.E (n=3). Means followed by the same letter within column are not significantly different (P = 0.05) using Ducan's multiple range test.

Fig. 1. **(a-e) *In vitro* morphogenic responses of cotyledonary node explant in *Acacia gerrardii*.**

a. 15 d old CN explant excised from aseptic seedling.

b. Multiple shoot induction on MS medium supplemented with BA (5.0 µM) after 4 weeks.

c. Proliferation and elongation of multiple shoots on MS medium containing BA (5.0 µM) and NAA (0.5µM) after 8 weeks**.**

d. Rooted plantlet *in vitro* on MS medium containing IBA (2.0µM) after 4 weeks.

e. Acclimatized plantlet in the soilrite showing healthy shoot after 4 weeks of *ex-vitro* transfer.

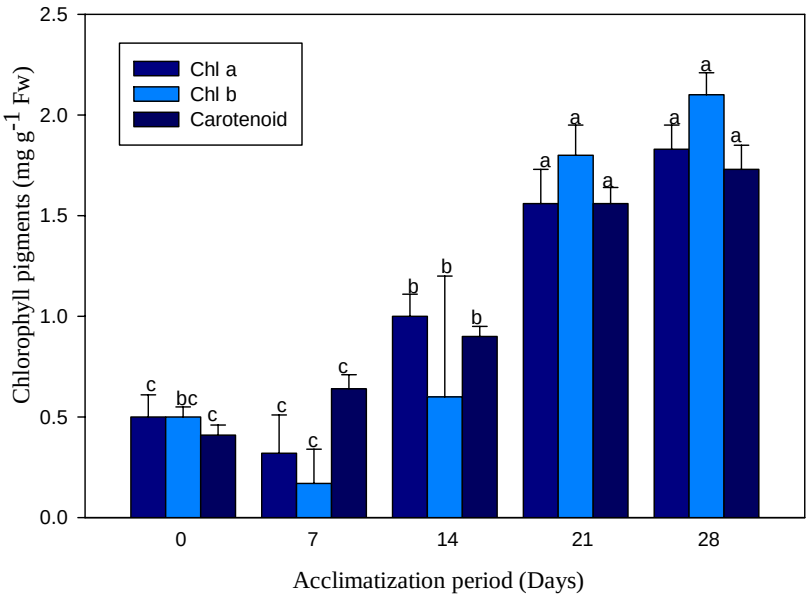

Fig.2: Changes in the levels of Chl a, Chl b and carotenoid contents in micropropagated plantlets of *A. gerrardii* acclimatized at 50µmol m^{-2} s^{-1} PPFD for 28 days. Bars represented means ± S.E (n=3). Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test.

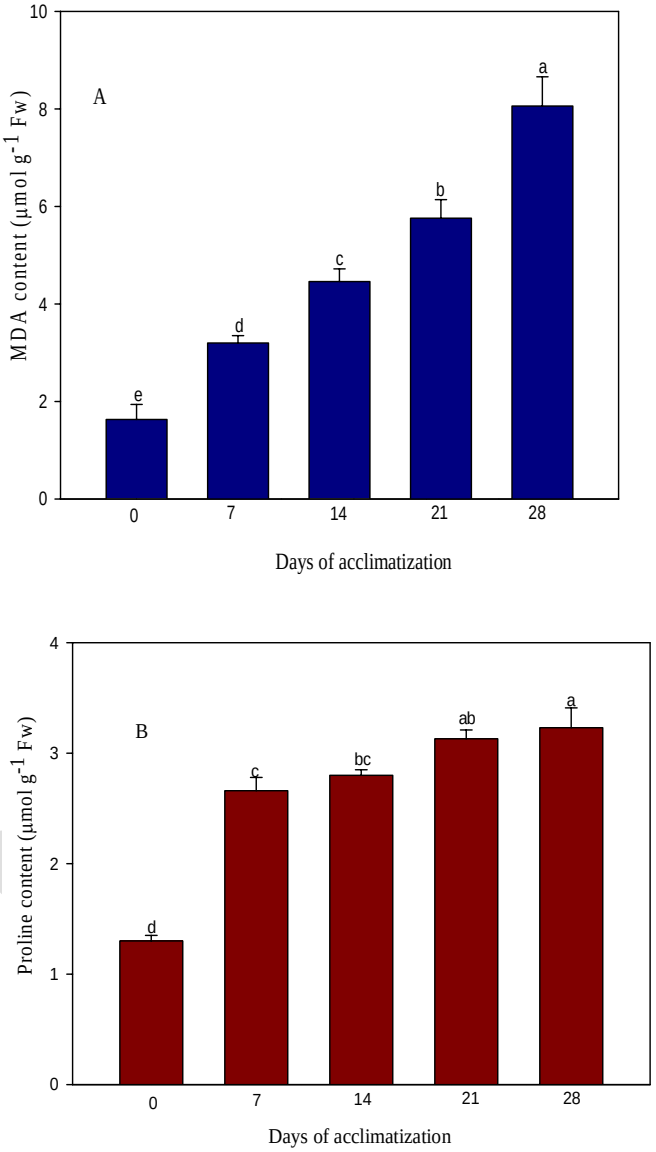

Fig. 3: Changes in (A) MDA and (B) Proline content (µmol g^{-1} Fw) of *A. gerrardii* plantlets during acclimatization at 50µmol m^{-2} s^{-1} PPFD for 28 days. Bars represented means ± S.E (n=3). Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test.

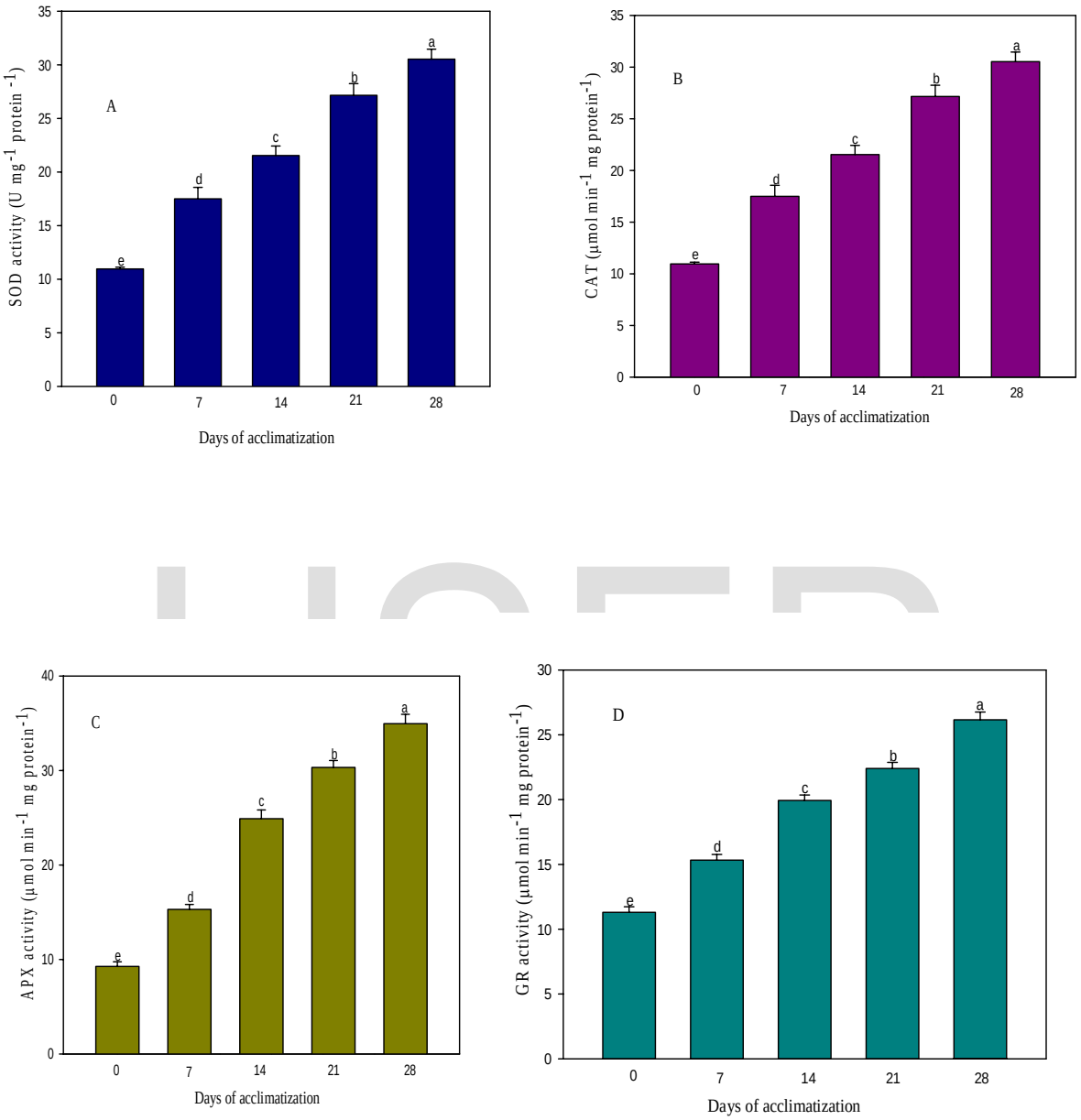

Fig. 4: Changes in the activities of antioxidant enzymes (A) SOD, (B) APX, (C) CAT and (D) GR in micropropagated plantlets of *A. gerrardii* acclimatized at 50µmol m^{-2} s^{-1} PPFD for 28 days. Bars represented means ± S.E (n=3). Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using Duncan's multiple range test.