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Antioxidant Status during *In vitro* Plant Regeneration in *Lucas aspera* Spreng

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ABSTRACT

Lucas aspera Spreng. (Lamiaceae) is an important medicinal plant, belongs to lamiaceae family. Till-date there have been no published reports on in vitro regeneration of L. aspera, hence, in the present study an attempt has been made to demonstrate the influence of antioxidant state during the in vitro plant regeneration in this plant. Internode explants from wild grown plants were collected, surface sterilized and cultured on MS medium supplemented with different concentrations and combinations of cytokinins and auxins for the in vitro regeneration. The highest number of shoot buds were induced on MS medium supplemented with 1.5 mg/L BAP and 0.1 mgL^{-1} NAA combination. Rooting was induced in the individual shoot regenerants on half-strength MS medium supplemented with 0.1mgL^{-1} IBA. Biochemical changes during in vitro shoot regeneration was monitored for 28 days. Changes in the levels of antioxidant enzymes like ascorbate peroxidase, catalase, superoxide dismutase and glutathione reductase were monitored. Results of the investigations confirm that the in vitro shoot regeneration event is free from the oxidative stress as the changes in antioxidant enzymes confirm the active regeneration and the plant growth regulator concentration and combination is optimal for the in vitro regeneration.

Key words: *Lucas aspera, L-ascorbate, reduced glutathione, antioxidant enzymes.*

INTRODUCTION

Leucas aspera Spreng, belongs to the family Labiatae (Lamiaceae), commonly known as White dead nettle in English, is an important medicinal plant in Indian traditional systems of medicine. The genus *Lucas* has about 80 different species[1]. In India, 43 species are available[2,3]. Throughout India the plant grows as a weed through seeds, comes out during rainy season in field borders and waste places[4]. The whole plant is fragrant and is used as a potherb in India.

L. aspera pacifies vitiated kapha, pitta, worm infestation, chronic rheumatism, cough, amenorrhea, intermittent fever, ulcer, in jaundice, anorexia, dyspepsia, asthma Singh et al.[5], conjunctivitis, diabetes, otalgia, scabies[6], Reddy et al.[7], toothache and wound healing[8], Chopra et al.[9,10,11].

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Traditionally, the decoction of the whole plant is taken orally for analgesic[6,] Reddy et al.[7], is used as anti-inflammatory[12,] Saundane et al.[13], as an insecticidal agent[14], psoriasis, chronic skin eruption and painful swellings Akhilesh Sharma et al.,[15] and also in antibacterial treatment. In India bruised leaves are applied to the bites of serpents and poisonous insects, headache, burning sensation and redness of eyes[16], boiled leaves vapours inhaled to relieve cough and colds Ganesan et al.[17], skin diseases, dhobis itch and ringworm[18], one side headache Burdyn et al.[19,3]. warmed leaf juice is used for earache and arthritic pain[3].

Plants have been an important source of medicine for thousands of years. Even today, the WHO estimates that upto 80% of the people still rely mainly on traditional remedies. It is estimated that approximately one quarter of the prescribed drugs contains plant extracts. Thus, medicinal plants are under tremendous pressure all across the globe, especially in India. *In vitro* propagation is alternative approach to overcome the problems of seasonal variation, reproductive inefficiency, self incompatibility and susceptibility to diseases. Micropropagation is not only useful for the multiplication of select clones and to produce high quantities of genetically homogeneous plant material Allan et al.[20], but also an alternative means for year-round production of secondary metabolites with uniform quality Zhao et al.[21,]Karuppanapandian et al[22].

Reactive oxygen species (ROS), like peroxide ($^{\bullet}OH$) radicals, superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are the inevitable molecules produced by the aerobic respiration and stress conditions in all living cells[23, 24]. These ROS at high levels, they cause membrane damage, lipid peroxidation, protein degradation, enzyme inactivation, DNA damage and programmed cell death. However, at lower concentrations, these reactive oxygen species induce metabolic, developmental and adaptive responses Batkova et al.[25], Varshney et al[24].

In vitro plant cultures are subjected to high air humidity, decreased air turbulence, low irradiance, low CO_2 concentrations during light period, culture media supplements like sugar and growth regulators, create stress to produce ROS[26]. Recent studies have linked the activity of auxin in root and shoot development with changes in redox balance. High levels of auxin are correlated with the generation of ROS, and it was suggested that redox sensing could be responsible for the arrest of the stem cells in the quiescent center in G_1 [27,] Zehava et al[28]. Increase in ROS production was found to be correlated with and associated with shoot primordium formation and suggested that, a certain level of ROS is indeed required at early stages of shoot organogenesis and for shoot regeneration. It is suggested that uncontrolled reduction of free radicals, by employing antioxidants that scavenge ROS, can lead to the disruption of metabolic pathways that are essential for tissue and organ differentiation[29].

Oxidative damage occurs when there is a serious imbalance in any cell compartment between the production of ROS and antioxidant defense, oxidative stress Meloni et al[30]. ROS increase, initiates chain reactions in which SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and molecular oxygen and Meloni et al[31]. Hydrogen peroxide is then detoxified in the ascorbate – glutathione cycle[32,30], which involves the oxidation and re-reduction of ascorbate and glutathione through the ascorbate peroxidase and glutathione reductase action[33,] Mittler et al.[34], thus providing protection against different stress factors, which has become an interesting research subject in recent years Mittler et al [34].

In view of the medicinal importance of *Leucas aspera* and the abundant occurrence of secondary metabolites, the main goal of the present study was aimed at to investigate the biochemical changes taking place during regeneration of plantlets from the explants of *Lucas aspera* Spreng., by monitoring the efficiency of certain antioxidant enzymes and certain metabolites so as to confirm whether *in vitro* regeneration is active regeneration or hampered by oxidative stress. The present

study was designed to carry out biochemical assays in such a way that, starting from initiation of explants proliferation (competence stage), shoot induction and development. Later, at every 7 days interval the samples were collected up to rooting stage. So the samples were collected at 7th, 14th, 21th and 28th days.

MATERIALS AND METHODS

Explant preparation and *in vitro* plant regeneration:

Lucas aspera Spreng. plants were collected from the local fields nearby Davanagere University, Shivagangothri campus, Davanagere, Karnataka, India. Internodal explants (2.5 cm long) were thoroughly washed under running tap water, treated with Tween-20 (5% v/v) and Bavistin (1% w/v) for 10 min, rinsed with double distilled water to remove detergent and fungicide. Further surface sterilization was done in 70% (v/v) ethanol for 30 sec, rinsed with sterile distilled water and then treated with 0.1% (w/v) mercuric chloride for 2-3 min and finally rinsed 3-4 times in sterile double distilled water to remove traces of mercuric chloride. Surface sterilized explants were trimmed at cut ends (1-1.2 cm) and aseptically inoculated onto MS medium[35] supplemented with 1.5 mgL^{-1} BAP and 0.1 mgL^{-1} NAA. All the cultures were maintained in culture room under cool fluorescent lamps (Philips, India) for 16-h light photoperiod 40 $\mu mol\text{-}m^{-2} s^{-1}$, at 25 °C ± 2 °C and 60% - 70% relative humidity as described by Poornima et al[36].

Acclimatization of regenerated plants:

Plantlets with well-established roots and expanded leaves were removed, washed and subsequently transferred to sterile thermal cups filled with garden soil:sand:dry cow-dung manure (1:1:1), Plantlets were maintained inside the culture room for 2 weeks and later transferred to greenhouse. After 4-5 weeks, plantlets were successfully established in the field.

Antioxidant enzyme extraction and assays:

To determine antioxidant enzyme activity, enzyme extract was prepared by first freezing weighed amount of (1g) fresh leaf tissue collected from 0, 7, 14, 21 and 28 days old *in vitro* plantlets in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 ml extraction buffer (0.1 M phosphate buffer (pH 7.5) containing 1% polyvinylpyrrolidone (PVP), 1% Triton X-100, and 0.5 mM EDTA in case of SOD, CAT, and GR, and 0.5 mM EDTA and 1 mM ascorbic acid in case of APX) using pre-chilled mortar and pestle. Briefly, was passed 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, India) and UV-visible spectrophotometer (Elico, India) were used.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was performed by recording the decrease in optical density of formazone made by superoxide radical and nitro blue tetrazolium dye by the enzyme (Dhindsaw et al.,[37] in a reaction mixture consisting of 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13.33 mM methionine, 50mM Na_2CO_3, 75 µM nitroblue tetrazolium (NBT), 2 µM 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[38]. The assay mixture contained 50 mM phosphate buffer (pH 7.0), and 50 µl enzyme extract in a total volume of 3 ml, and the reaction began by adding 0.5 ml of 75 mM H_2O_2. Glutathione reductase (GR; EC 1.6.4.2) activity was measured using the protocol on glutathione-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 412 nm[39]. The assay mixture contained 66.67 mM phosphate buffer (pH 7.5), 0.33 mM EDTA, 0.5mM DTNB in 0.01 M phosphate buffer (pH 7.5), 66.67 µM (NADPH), and 666.67 µM glutathione disulfide (GSSG). The enzyme extract (0.1 ml) was added to begin the reaction, and the increase in absorbance was

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recorded spectrophotometrically. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured by monitoring the decrease in absorbance at 290 nm within 1 min[40]. 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}. Protein content in enzymatic extracts was determined following the Bradford assay[41] using bovine serum albumin as a standard.

Statistical analysis

All experiments were conducted in three replicates. Results presented as means of replications with the standard error (±SE) and data were processed by analysis of variance (ANOVA) and comparisons between the mean values of treatments were done according to Tukey-Kramer (HSD) principle range test at $P \leq 0.05$ Assaad et al[42].

RESULTS AND DISCUSSION

The present study reports for a very simple, rapid and reliable protocol for the *in vitro* multiplication of *Lucas aspera*. We have achieved i*n vitro* regeneration in internodal explants cultured on MS medium[35] supplemented with different plant growth regulators either alone or in combination (Fig. 1a). MS medium[35] supplemented with BAP in combination with NAA enhanced the rate of shoot multiplication within 10 days. Maximum number of shoots (6.9±0.33 shoots/explants) (Fig. 1b) and shoot length (3.48±0.25) were obtained on MS medium[35] supplemented with 1.5 mgL^{-1} BAP with 0.1 mgL^{-1} NAA Poornima et al[36]. Shoot elongation was achieved by transferring the *in vitro* shoots to half-strength medium during subculture, and subsequent rooting was also achieved on the same medium. Maximum percentage of rooting (83.2±1.71) was obtained with IBA (0.1 mgL^{-1}), number of roots were more (6.6±0.74) with 4.64±0.51 cm length (Fig. 1d and 1e) Poornima et al[36].

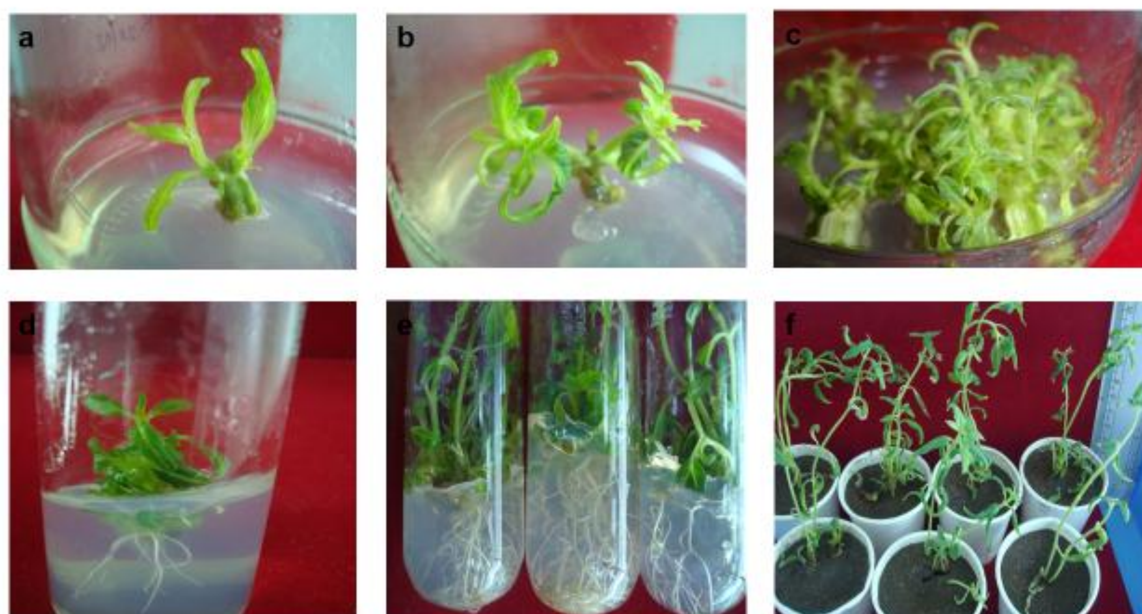

Fig. 1: Micropropagation of *Lucas aspera* Spreng.

(a) *Ex vitro* nodal explant in culture medium. (b) Shoots regeneration in medium with 1.5 mgL^{-1} BAP and 0.1 mgL^{-1} NAA. (c) Multiple shoots induced on half-strength medium after 20 weeks of culture. (d) Shoot elongation and rooting in half-strength medium. (e) *In vitro* regenerated plant with well-developed roots. (f) Acclimatized plants in cups and ready for field transfer.

Rooted plantlets were thoroughly washed and transferred to thermal cups containing sterile mixture of sand:soil:dry cow-dung mixture (1:1:1). For the first 15 days of transplantation, plantlets were covered with polythene bags to ensure high humidity and sufficient light.

Nearly 85% of the plants were acclimatized and survived in culture room conditions (Fig. 1f). The growth of *in vitro* plants did not show any significant morphological variations from those of wild grown ones.

Changes in antioxidant enzymes levels: In the present study we have observed the time dependent variation of antioxidant enzyme activities during the acclimatization of *Lucas aspera.* The activity of SOD was increased after 7 days of acclimatization, but the SOD activity got reduced at 28th day of transfer (Fig. 2).

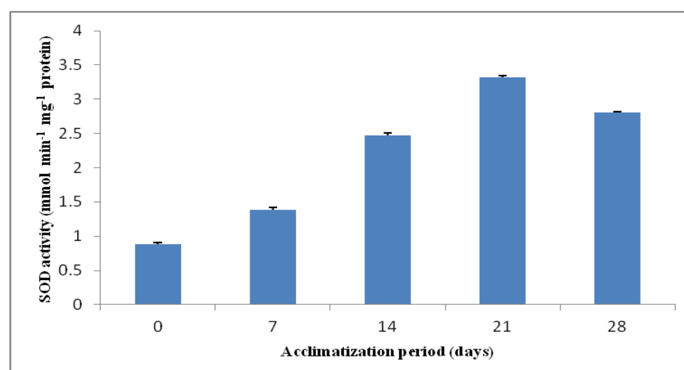

Fig. 2: Changes in superoxide dismutase (SOD) activity

Superoxide generates DNA reactive hydroxyl radicals[43,44] that oxidize cell membrane and damage to biological molecules. In response to this the cellular machinery generates ROS scavengers like SOD and prevents cell damage. In addition, the CAT activity increased and reached highest value at 28th day of acclimatization (Fig. 3). This increased activity of CAT suggests the plant protective mechanism to scavenge H_2O_2 by converting it into O_2 and H_2O in peroxisomes (Dat et al.[45], Mitrović et al.[46,47].

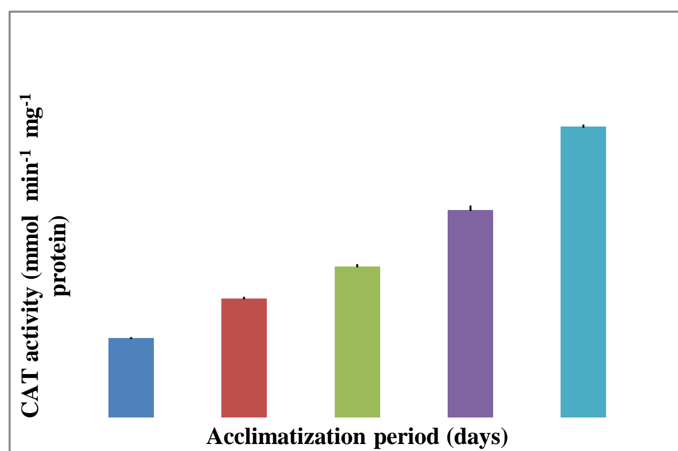

Fig. 3: Changes in catalase (CAT) activity

Similarly, the activities of APX (Fig. 4) and GR (Fig. 5) also increased from 0 to 28 days of acclimatization.

This increase in the activities of APX and GR indicates chloroplast based detoxification of free radicals via Mehler pathway and NADPH-dependent reduction of the disulfide bond of oxidized glutathione[47]. The results suggest that our *in vitro* propagated plants have developed functional photosynthetic machinery during acclimation. These results are in concurrence with other studies showing similar findings in the antioxidant enzyme activities in the micropropagated *Cassia alata* L.[47], *Tecomella undulate* Batkova[25], *Rauvolfia tetraphylla*[48], and *Dianthus caryphyllus* L. Varshney et al.,[24] during acclimatization.

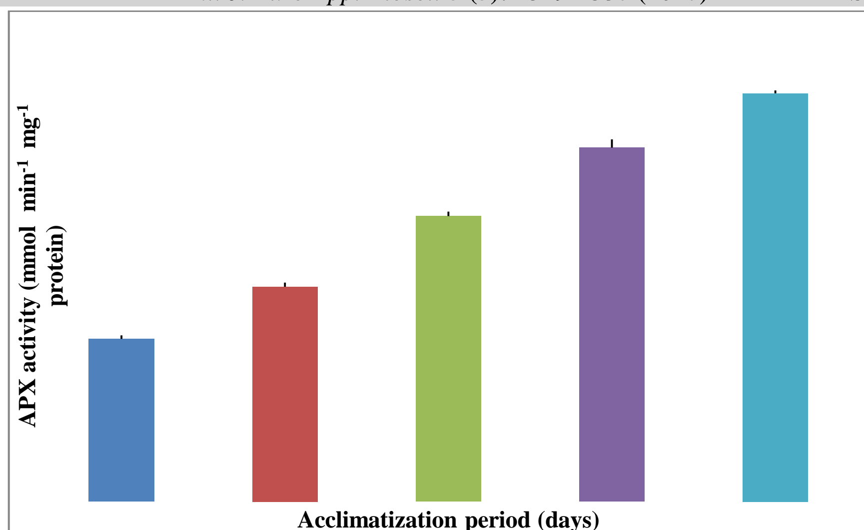

Fig. 4: Changes in ascorbate peroxidase (APX) activity

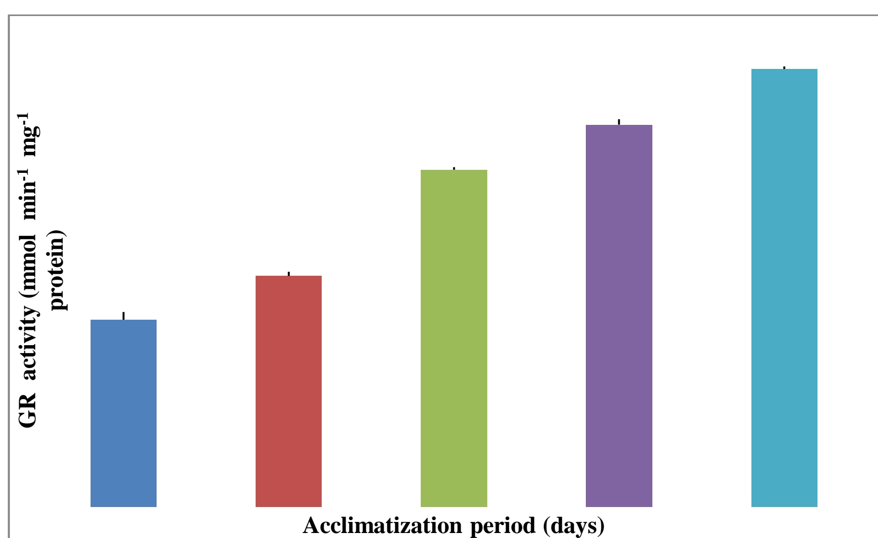

Fig. 5: Changes in glutathione reductase (GR) activity

Finally, the present study reports a reliable protocol for the *in vitro* propagation and multiple shoot regeneration in internodal explants of *L. aspera* and were successfully established in the field condition with 92% survival rate resulted from changes in their physiological characteristics triggered by gradual acclimatization. Therefore, acclimatization described here and previously reported Poornima et al.,[36] micropropagation protocol are going to reduce dependency on natural *Lucas* plants for the production of its useful metabolites for pharmaceutical purposes and also helps in conservation.

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