

The Study of Ascorbate Peroxidase, Catalase and Peroxidase During In Vitro Regeneration of *Argyrobium roseum*

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Abstract Here, we demonstrate the micropropagation protocol of *Argyrobium roseum* (Camb.), an endangered herb exhibiting anti-diabetic and immune-suppressant properties, and antioxidant enzymes pattern is evaluated. Maximum callogenic response (60 %) was observed from leaf explant at 1.0 mg L⁻¹ 1-nephthalene acetic acid (NAA) and 0.5 mg L⁻¹ 6-benzyl aminopurine (BA) in Murashige and Skoog (MS) medium using hypocotyl and root explants (48 % each). Addition of AgNO₃ and PVP in the culture medium led to an increase in callogenic response up to 86 % from leaf explant and 72 % from hypocotyl and root explants. The best shooting response was observed in the presence of NAA, while maximum shoot length and number of shoots were achieved based on BA-supplemented MS medium. The regenerated shoots were rooted and successfully acclimatized under greenhouse conditions. Catalase and peroxidase enzymes showed ascending pattern during in vitro plant development from seed while ascorbate peroxidase showed descending pattern. Totally reverse response of these enzymes was observed during callus induction from three different explants. During shoot induction, catalase and peroxidase increased at high rate while there was a mild reduction in ascorbate peroxidase activity. Catalase and peroxidase continuously increased; on the other hand, ascorbate peroxidase activity decreased during root development and acclimatization states. The protocol described here can be employed for the mass propagation and genetic transformation of this rare herb. This study also highlights the importance and role of ascorbate peroxidase, catalase, and peroxidase in the establishment of *A. roseum* in vitro culture through callogenesis and organogenesis.

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Abbreviations

ANOVA	Analysis of Variance
APX	Ascorbate Peroxidase
BA	6-Benzyl Aminopurine
CAT	Catalase
EDTA	Ethylene Diamine Tetraacetic Acid
IAA	Indol Acetic Acid
IBA	Indol 3 Butyric Acid
LSD	Least Significant Distance
MS	Murashige and Skoog
NAA	1-Nephthalene Acetic Acid
OD	Optical Density
PVP	Polyvinyl Pyrrolidone
ROs	Reactive Oxygen Species
SOD	Superoxide Dismutase
POD	Peroxidase

Introduction

Due to biochemical reactions in living organisms, reactive oxygen species (ROs) such as hydrogen peroxide (H_2O_2), super oxide radicals and singlet oxygen constantly produce. These ROs are normally utilized in various metabolic processes such as lignin formation in the cell wall [1], leaf and flower abscission, cell senescence, ripening of fruit, and flowering [2]; however, excessive production may damage cellular components such as DNA, protein, and lipids; might lead to cell death [3]. Naturally, plants have enzymatic and non-enzymatic defensive system against these molecules. Ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD) are members of enzymatic protectants which scavenge both radical and non-radicals oxygen species [4]. The metabolic processes lead to the production of ROs in mitochondria, chloroplast, and peroxisomes. In the first line of defense, superoxide and singlet oxygen radicals end up with production of H_2O_2 and oxygen [5]. Finally, a variety of enzymes such as catalase, peroxidase, and ascorbate peroxidase detoxify H_2O_2 into gaseous oxygen and water molecule. So, these enzymes scavenge H_2O_2 making the cell inclined against oxidative damage [6].

In vitro conditions situate oxidative stresses during callus induction, and shoot and root development [7]. To abide oxidative damage, catalase, peroxidase, and ascorbate peroxidase function continuously in all the cells. Many reports explain the role of oxidative stress in in vitro cultures including callogenesis [8], somatic embryogenesis [9], shoot induction [10], root formation, and acclimatization [11, 12] and even in vivo and in vitro-grown plant [13].

Argyrobium roseum (Camb.) Jaub and Spach is an important rare annual medicinal herb found in the tropical and sub temperate tracts of north western India and Margalla Hills of Pakistan. This plant has been traditionally used as an aphrodisiac, tonic [14], and antihyperglycemic. Khanna et al. [15] have reported regeneration of *Argyrobium roseum* via organogenesis from immature embryos. However, scarce availability, slow natural regeneration, and lack of effective cultivation strategies are the factors rendering it essential to establish in vitro cultures of *Argyrobium roseum* from other explants. Beside this, developing a regeneration protocol from somatic tissues is more beneficial because it does not demand a time frame to collect explants.

The present study was performed to establish regeneration protocol for *Argyrobium roseum* through callogenesis from leaf, stem, and root explants and to examine the complete pattern of antioxidant enzymes (ascorbate peroxidase, catalase, and peroxidase) during in vitro life cycle of *Argyrobium roseum*. In this study, we demonstrate the estimation of antioxidant enzymes during in vitro-grown plants from seeds; calli induced from different explants, callus proliferation, shoot and root development, and in different parts of the acclimatized plant.

Materials and Methods

Collection of Seeds and In Vitro Germination

The seeds of *Argyrobium roseum* (Camb.) Jaub and Spach were collected from Margalla hills, Islamabad, Pakistan. The dormancy of seeds was broken by placing the seeds in sand at 4 °C for 48 h. Thereafter, under aseptic conditions, the seeds were surface sterilized by immersing in 1.0-ml clorox (5.25 % sodium hypochlorite) for 10 min and rinsed thrice with autoclaved distilled water. Sterilized seeds were inoculated on half-strength Murashige and Skoog (MS) medium [16]. The flasks were kept at 25±2 °C in complete darkness until seed germination initiated. Then they were placed under 16-h photoperiod.

Callus Induction

Eight-week-old seedlings were used as explant source. The hypocotyl, leaf, and root sections (1.0 cm²) were separated. The leaf explants were also cut at the margins. The MS basal medium supplemented with sucrose (30 g L⁻¹) as carbon source and Noble agar (7 g L⁻¹) as solidifying agent were used for all the experiments performed in this study. For callus induction, the basal medium also contained different concentrations of 6-benzyl aminopurine (BA) and 1-nephthalene acetic acid (NAA) either alone or in combination. The medium was adjusted at pH 5.7 with 1.0-M KOH. A volume of 30-ml media was dispensed in 100-ml flask and autoclaved at 121 °C and 15 kPa for 20 min. The cultures were maintained in the growth room at 25±2 °C under a 16-h photoperiod unless specified otherwise. A constant photosynthetic photon flux density of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was provided by cool white fluorescent lamps as measured at the culture level. Callogenic response from all explants was recorder after 4 weeks of inoculation of explants. For proliferation, the calli were transferred on the same media after every 14 days. The test was performed in triplicate and each replicate contained eight explants. Callus induction response (percentage) was transformed to validate the one-way analysis of variance (ANOVA) assumption of normality of data distribution, and then subjected to statistical analysis.

Effect of Additives on Callus Induction

Due to low percentage of callus induction response and callus proliferation rate, all three explants were inoculated on MS medium containing 30 g L⁻¹ sucrose, 1.0 mg L⁻¹ NAA, and 0.5 mg L⁻¹ BA along with different concentrations of additives; polyvinyl pyrrolidone (PVP) and silver nitrate (AgNO₃) either alone or in combination. The additives (0.5–2 mg L⁻¹) were added in the medium before pH was adjusted at 5.7. The environmental conditions were same as described before. The media was refreshed after 14 days and the data was recorded after 4 weeks. The test was performed three times and each replicate contained six

explants. Percentage callus induction response was transformed to validate the one-way ANOVA assumption of normality of data distribution, and then subjected to statistical analysis.

Shoot and Root Induction

To develop shoot, the calli induced at 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA with AgNO_3 and PVP (0.5 mg L^{-1} each) were shifted on MS medium containing different concentrations of BA and NAA. The shoot induction medium also contained 0.5 mg L^{-1} PVP and AgNO_3 each. The shoot regeneration media was supplemented with sucrose (30 g L^{-1}) as carbon source and agar (7 g L^{-1}) as solidifying agent. The growth room conditions were maintained as described before. Each treatment was replicated thrice and each replicate contained 10 explants. The combined data from the three separate experiments were used for statistical analysis. Data of percent shoot induction ((responding calli/total number of calli inoculated) $\times 100$), number of shoots per flask (per callus inoculated in flask) and shoot lengths were recorded after 4 to 6 weeks. The data obtained was statistically analyzed with ANOVA and LSD to confine difference in treatments.

The shoots, when attained heights ~ 1 to 2 cm approximately, were separated from the base and transferred to MS medium supplemented with indol 3 butyric acid (IBA) at different concentrations (0.1 – 1.0 mg L^{-1}). The rooting media also contained sucrose and agar, 30 and 7 g L^{-1} , respectively. Each treatment was replicated twice and each replicate contained six explants. The rooted plantlets were thoroughly washed under running tap water to remove excessive medium and planted in plastic containers containing vermiculite and sand mixture (1:1). The pots were placed in a growth chamber for 1 week for acclimatization. The plastic pots were covered with transparent polythene bags to retain high humidity. After 2 weeks, the polythene bags were gradually removed and the plantlets were transferred to greenhouse.

Determination of Antioxidant Enzyme Activities

Antioxidant enzyme concentrations were determined during the *in vitro* germination of seeds, callogenesis, organogenesis, and acclimatization stages. Ascorbate peroxidase, catalase, and peroxidase enzymes were studied in leaf, stem, and roots parts of *in vitro*-grown plants from the first week of germination till the eighth week. The same enzymes were also studied during callus development from leaf, hypocotyls, and root explants on MS medium containing plant growth regulators (1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA) and additives (AgNO_3 and PVP 0.5 mg L^{-1} each) from 2nd week of callus initiation till the 18th week. Furthermore, during shoot induction, the shoot produced on MS medium supplemented with 2.0 mg L^{-1} BA and 1.5 mg L^{-1} NAA with AgNO_3 and PVP (0.5 mg L^{-1} each) were selected to study enzymes. During this step, both leaf and stem explants were preceded for enzyme assay starting from the 1st week of shoot initiation till the 10th week. The leaf, stem, and root explants of plants rooted on 0.5 mg L^{-1} IBA were subjected to enzyme assay during root development stage. These three explants were also subjected to study enzymes concentrations during acclimatization stage.

To study enzyme activity, 1.0-g plant material (leaf, stem, and root) or calli was ground in pre-chilled pestle and mortar in extraction medium. The extraction medium comprised of 0.1-M phosphate buffer (pH 7.0), 10-mM KCl, 1.0-mM MgCl_2 , and 10-mM EDTA. The ground material was centrifuged at $10,000g$ for 20 min at 4°C . The supernatant was used for enzyme assays. Total soluble protein determination was also performed following the

method of Bradford [17]. All the readings were drawn from Agilent 8453 UV-Visible Spectrophotometer.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined by the method developed by Nakano and Asada [18]. In a test tube 0.1-ml enzyme extract was taken and 0.9-ml of reaction mixture was added. Reaction mixture contains 50-mM potassium phosphate buffer (pH 7.0) and 0.5-mM ascorbic acid. The reaction was started by the addition of 0.06 ml of 1.0-mM H₂O₂ and a decrease in absorbance was observed at 290 nm. Under the assay conditions, a decrease in 0.01 absorbance corresponded to 3.6-mM ascorbate oxidized. The enzyme activity was calculated as micromole ascorbate decomposed per minute.

Catalase (CAT; EC 1.11.1.6) activity was determined by the method reported by Aebi [19]. A volume of 300- μ l supernatant was treated with 0.5 ml of 10-mM H₂O₂ and 600 μ l of 30-mM potassium phosphate buffer (pH 7.0). Optical density (OD) was recorded at 240 nm. The enzyme activity was calculated as micromole H₂O₂ decomposed per milligram protein per minute using H₂O₂ extinction coefficient 36 μ M⁻¹ cm⁻¹.

Peroxidase (POD; EC 1.11.1.7) activity was determined by the method as described by Zhou and Leul [20]. In a test tube, 0.1-ml enzyme extract was treated with 3.0 ml of 0.1-M phosphate buffer, 0.05 ml of 20-mM guaiacol and 0.03 ml of 12.3-mM H₂O₂. Cuvette was inserted in spectrophotometer chamber and OD was recorded at 436 nm using guaiacol as an extinction coefficient 6.39 μ M⁻¹ cm⁻¹.

Experimentation and Data Analysis

For in vitro study, the data was recorded for callus induction, shoot multiplication, and root generation. The experiments were arranged in a completely randomized design and each experiment was repeated twice or thrice. Percentage data were transformed to validate the one-way ANOVA assumption of normality of data distribution, and then subjected to statistical analysis. For all the tested parameters, ANOVA was used to find differences among treatments, Duncan multiple range test for separating mean values, and significant effects were accepted at $p < 0.05$. Data computations were done using SPSS for Windows (version 15.0, SPSS, Chicago, USA). All the enzyme assays were performed in triplicate and values are presented as mean \pm standard deviation. Percent variation in enzyme concentration at each stage was calculated as:

$$\text{Percent variation} = ((\text{final concentration} - \text{initial concentration}) / \text{initial concentration}) * 100$$

Results

Callogenesis and Organogenesis

All three explants (leaf, hypocotyls, and root) exhibited significant callogenic response when inoculated on MS medium supplemented with BA and NAA in combination. Maximum callogenic response (60 %) was observed from leaf explant at 1.0 mg L⁻¹ NAA and 0.5 mg L⁻¹ BA in combination following hypocotyl and root explants (48 % each) at the same combination (Table 1). The texture and morphology of calli derived from hypocotyl and leaf explants was green and soft (Fig. 1a–d); however, the callus derived from root explant was yellowish green in color. In the presence of 1.0 mg L⁻¹ NAA, the hypocotyls and leaf explants responded 40 and 35 %, respectively. Increase in the concentration of BA or NAA

either alone or in combination (1.5 and 2.0 mg L⁻¹) drastically decreased the callogenic response. In the present study, release of phenolic compounds from callus was one of the reasons that decreased callus induction and proliferation rate. To control the release of phenolic compounds; PVP and AgNO₃ were added in the culture media. Addition of PVP in the medium as compared to AgNO₃, influenced better on callus induction and proliferation rate (Table 2). Low concentration of PVP and AgNO₃ (0.5–1.0 mg L⁻¹) either alone or in combination in MS medium established better callus induction response from all three explants. Combination of both additives (0.5 mg L⁻¹ each) in the medium increased callogenic response up to 86 % from leaf and 72 % from hypocotyl and root explants.

The green-colored and soft calli were transferred to MS medium containing auxin and cytokinin for shoot induction (Table 3). Maximum shooting response (50 %) was observed at 1.0 mg L⁻¹ NAA with average shoot length 1.9±0.16 cm and 5±1.2 average numbers of shoots per flask (Fig. 1e). However, number of shoots per flask and shoot length was highest in the presence of BA. BA at 1.5 mg L⁻¹ produced 6±0.6 average numbers of shoots per flask with average shoot length of 2.1±0.24. Combination of BA and NAA could not promisingly promote shoot initiation as compared individually in the MS medium. Numerous root formations were observed when the regenerated shoots were cultured on three different concentrations of IBA (0.1, 0.5, 1.0 mg L⁻¹). The rooted plantlets were transferred

Table 1 Effect of growth regulators (mg L⁻¹) on callus induction response from hypocotyl, leaf, and root explants of *Argyrolobium roseum*

Plant growth regulator		Callus induction response (%)		
NAA	BA	Hypocotyl	Leaf	Root
0.5		25±3 b	32±4 b	17±2 b
1		40±6 a	35±6 a	20±5 a
1.5		15±2 c	12±2 c	5±1 c
2		7±2 d	12±2 c	5±2 c
	0.5	35±7 a	32±7 a	17±4 a
	1	32±4 ab	12±3 b	8±2 c
	1.5	12±3 c	12±4 b	11±3 b
	2	5±1 d	0	8±3 c
0.5	0.5	20±4 bc	24±4 c	10±2 c
0.5	1.0	15±4 cd	20±5 cd	8±1 d
0.5	1.5	12±3 d	16±2 d	6±1 de
0.5	2.0	7±2 e	12±2 e	9±2 cd
1.0	0.5	48±6 a	60±7 a	48±6 a
1.0	1.0	25±3 b	36±5 b	17±3 b
1.0	1.5	17±3 c	16±3 d	8±2 d
1.0	2.0	12±1 d	12±2 e	2±1 e
1.5	0.5	17±3 c	20±4 cd	8±2 d
1.5	1.0	12±6 d	24±3 c	6±2 de
1.5	1.5	5±1 e	0	0
1.5	2.0	0	0	0

The test was performed in triplicate and each replicate contained eight explants. The small letters on the bars represent statistical difference in callus induction response from all three explants at $p < 0.05$. Data is presented as mean ± standard deviation



Fig. 1 Callogenesis and organogenesis of *Argyrolobium roseum*; **a** Callus formation from hypocotyl explant on 1.0 mg L^{-1} NAA; **b** callus from hypocotyl explant formation on 0.5 mg L^{-1} BA; **c** callus formation from hypocotyl explant on 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA; **d** callus formation from leaf explant on 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA; **e** Shoot organogenesis from calli on 1.0 mg L^{-1} NAA in the MS medium; **f** In vitro acclimatization of *Argyrolobium roseum* (arrows in **c** and **d** show initiation of shoot primordial from callus)

to in vivo conditions; initially in growth room for a period of 2 weeks and then planted in pots containing soil under green house conditions (Fig. 1f). A total of 47 plants were rooted; out of those, 34 (72.3 %) plants were successfully acclimatized in greenhouse.

Table 2 Effect of additives (mg L⁻¹) on callus induction response from hypocotyl, leaf and root explants of *Argyrobium roseum*

Plant growth regulator		Callus induction response (%)		
AgNO ₃	PVP	Hypocotyl	Leaf	Root
0.25		6±2 d	5±1 d	8±2 e
0.5		12±3 b	60±4 a	52±5 a
1		65±6 a	20±3 b	36±4 b
1.5		10±3 c	8±2 c	28±3 c
2		10±3 c	0	16±3 d
	0.5	25±2 c	56±6 a	48±6 a
	1	55±4 a	23±4 b	16±3 b
	1.5	30±3 b	14±2 c	16±3 b
	2	10±2 d	8±1 d	8±2 c
0.5	0.5	72±2 d	86±7 a	72±8 a
0.5	1.0	40±5 b	36±4 b	20±3 b
0.5	1.5	10±2 e	24±3 c	16±2 c
0.5	2.0	5±1 f	8±2 f	4±1 f
1.0	0.5	25±3 c	36±4 b	20±3 b
1.0	1.0	85±7 a	24±2 c	16±3 c
1.0	1.5	35±4 bc	20±3 d	4±1 f
1.0	2.0	0	0	0
1.5	0.5	8±2 ef	8±2 f	12±2 d
1.5	1.0	12±2 de	12±3 ef	8±2 e
1.5	1.5	4±1 f	4±1 g	0
1.5	2.0	0	0	0

The data was recorded after 4 weeks. The test was performed in triplicate and each replicate contained six explants. The small letters on the bars represent statistical difference in callus induction response from all three explants at $p < 0.05$. Data is presented as mean ± standard deviation

Antioxidant Enzyme Pattern During In Vitro Seedling

Ascorbate peroxidase, catalase, and peroxidase enzyme activities were determined in leaves, stem, and root parts of in vitro-grown *Argyrobium roseum* plants (Fig. 2). In case of APX, a short fall (27.6 %) was observed in leaf parts while 44.5 and 62.9 % decline was evaluated in stem and root parts, respectively, of in vitro-grown *Argyrobium roseum* plants. An increase in catalase activity was observed in all three explants from the first week up to the eighth week. Leaf and stem explants presented 45.8 and 41 % increase, respectively, while 26 % increase was observed in root parts. A high increase in POD activity was observed in leaf, stem, and root parts, i.e., 108.9, 109.2, and 89.2 %, respectively.

Antioxidant Enzymes Pattern During Callus Induction and Proliferation

Explants of 8-week-old in vitro seedlings, i.e., root, stem and leaves were cut into small pieces and transferred to the callus induction medium. APX, CAT, and POD enzymes were

Table 3 Effect of plant growth regulator (mg L^{-1}) on shoot induction response from callus of *Argyrobium roseum*

Plant growth regulator		Response (%)	Shoot length (cm)	No. of Shoots/flask
NAA	BA			
0.5		10±3 d	1.6±0.3 b	2±1 c
1		50±4 a	1.9±0.16 ab	5±1.2 a
1.5		25±2 b	1.7±0.2 b	4±1 b
2		15±2 c	2±0.14 a	2±0 c
	0.5	0	0	0
	1	10±2 b	1.2±0 b	3±1 b
	1.5	40±4 a	2.1±0.24 a	6±0.6 a
	2	0	0	0
0.5	0.5	0	0	0
0.5	1.0	5±1 ef	0.8±0 d	1±0 e
0.5	1.5	5±1 ef	1.1±0.1 d	2±0 d
0.5	2.0	0	0	0
1.0	0.5	5±1 ef	1±0.2 d	1±0 e
1.0	1.0	7.5±2 e	1.3±0.3 cd	2±0 d
1.0	1.5	12.5±3 cd	1.5±0.3 c	2±1 d
1.0	2.0	7.5±2 e	2±0.5 a	1±0 e
1.5	0.5	2.5±1 f	1.7±0.2 bc	1±0 e
1.5	1.0	7.5±2 e	1.4±0.1	3±1 c
1.5	1.5	7.5±2 e	1.8±0.3 b	1±0 e
1.5	2.0	15±4 c	1.2±0.1 d	4±1 b
2.0	0.5	10±3 d	1.9±0.2 b	2±0 d
2.0	1.0	25±5 b	1.8±0.2 b	3±1 c
2.0	1.5	45±6 a	1.6±0.4 bc	5±2 a
2.0	2.0	15±3 c	1±0.1 d	2±0 d

Data of percent shoot induction, number of shoots per flask, and shoot length were recorded after 4 to 6 weeks in culture. The small letters on the bars represent statistical difference in response at $p < 0.05$. Data is presented as mean \pm standard deviation

studied from the 2nd week of callus induction to the 18th week (Fig. 2). A continuous increase in APX was observed in the calli originated from leaf, stem, and root explants. In leaf-originated calli, 163.9 % increase was observed from the 2nd week of callus initiation to the 18th week. While in the case of stem and root explants, there was a linear increase during the initial week and later, fast increase in APX activity was observed. Average percent increase in APX activity was higher (477.8 %) in stem-derived calli as compared with root-derived calli (363.5 %). During callus induction and proliferation, a continuous decline was observed in CAT and POD activities irrespective to type of explant used for callus initiation. Approximately equal drop in CAT activity was calculated in the calli derived from leaf and root explants (66.3 and 64.1 %, respectively), while the decline was little bit more in case of stem explant (73.9 %). The same pattern was observed in the case of POD where decline was 41 to 44 % in calli derived from any explant.

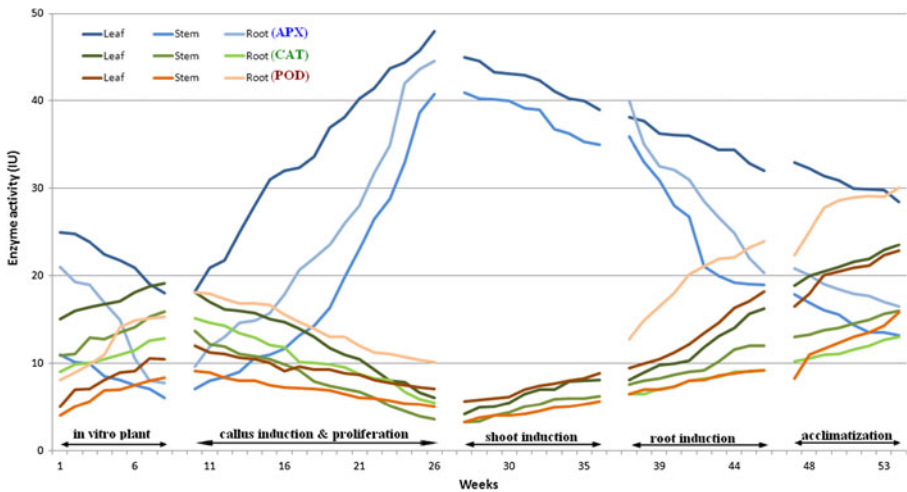


Fig. 2 Complete pattern of antioxidant enzymes; ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD) during in vitro life cycle of *Argyrolobium roseum* (the gaps between the lines are created to separate different phases)

Antioxidant Enzyme Activities in Regenerate Leaves and Stem

Shoot originated on MS medium containing 2.0 mg L^{-1} NAA and 1.5 mg L^{-1} BA with 0.5 mg L^{-1} PVP and AgNO_3 were subjected for the analysis of antioxidant enzymes from the first week to the tenth week. A mild descending pattern was observed for APX activity while CAT and POD activities increased continuously with time course in leaf and stem parts (Fig. 2). During shoot development, only 13.2 and 14.4 % decrease was observed in leaf and stem, respectively. CAT increased up to 92 % both in leaves and stem while POD increased 56.6 % in leaves and 74.6 % in stem.

Activities of Antioxidant Enzymes during Root Development and Acclimatization

The shoots, when attained sufficient heights, were transferred to different concentrations of IBA for rooting response. From the first week of root initiation, antioxidant enzymes were studied in leaf, stem, and root parts (Fig. 2). A mild decrease was observed in APX activity in leaves (16 %), while it was 47 and 49 % in case for stem and root parts, respectively. Increase in CAT activity was observed more in leaf (101.8 %) following stem (58.8 %) and roots (42.8 %) in a 10-week time period. The same pattern was observed in case of POD where 92.3, 42.7, and 87.4 % activity increased in leaf, stem, and roots, respectively. The same descending pattern of APX and ascending pattern of CAT and POD continued during acclimatization in leaf, stem, and root parts (Fig. 2). However, the increase of POD in the stem was much higher (91.2 %) as compared to leaf and root explants.

Discussion

Micropropagation of *Argyrolobium roseum*

The growth regulators stimulated the cells of leaf, hypocotyl and root explants of *Argyrolobium roseum*; developing calli. Callus induction initiated from the cut ends of the

explants; however, the variation in callogenic response depends upon the explant type, growth media, hormone type, and concentration as well as on the uptake of hormone by explant [15, 21]. The results also demonstrate that the addition of cytokinin and auxin in combination is valuable for callus induction and good quality callus. Hypocotyl, leaf, and root explants presented better callogenic response at combination of BA and NAA. This might be due to BA acting in conjunction with NAA eventually stimulating growth of cell and also flourishing it [22–24]. Combination of BA with auxin (NAA or IAA; indol acetic acid) has been reported essential for *Argyrobium roseum* callus induction from immature embryos [15].

Release of phenolic compounds associates with ethylene production [25]. Ethylene in the culture vessel inhibits cell proliferation and organogenesis in different plant species [26]. Addition of PVP in the medium showed significant influence on callus induction and proliferation rate when compared to AgNO_3 . Hypocotyl, leaf, and root explant showed better callogenic response in the presence of 0.5 mg L^{-1} PVP and/or AgNO_3 . Use of PVP as phenolic attractive substance [27] and addition of AgNO_3 as ethylene inhibitor has already been reported in the tissue culture medium [28].

Maximum shooting response from green-colored soft calli was observed in the presence of NAA. However, a number of shoots per flask and maximum shoot length was achieved when BA was incorporated in the MS medium. The regeneration of shoots from callus is a programmed process and shoots emerge even at hormone-free medium [29]. Number of shoots and shoot characteristics varies depending on the type and concentration of plant growth regulator. Khanna et al. [15] also reported the presence of cytokinin either alone or with auxin for shoot induction from callus of *Argyrobium roseum*. BA alone or with NAA has been reported for shoot induction from different explants of family Fabaceae plants, i.e., *Astragalus nezaketiae* [30], *Mucuna pruriens* [31], *Clitoria ternatea* [32]; and *Thermopsis turcica* [29]. Numerous root formations were observed when regenerated shoots were cultured on three different concentrations of IBA. Rooting efficiency of this plant species in the presence of IBA has been reported by Khanna et al. [15]. The rooted plantlets were transferred to in vivo conditions; initially in growth room for a period of 2 weeks and then planted in the pots containing soil under greenhouse conditions.

Pattern of Antioxidant Enzymes during *Argyrobium roseum* Life Cycle

The ROSs radicals produced in cellular compartments can denature protein, mutation of DNA, and/or peroxidation of lipids. Enzymatic mechanism to scavenge such ROSs is powerful tool to abide the destruction [33, 34].

During the germination of seeds of *Argyrobium roseum* on half-strength MS medium, APX showed decline behavior while CAT and POD increased as the seedlings developed into whole plant. CAT activity was more profound in leaf following stem and root parts while POD activity was higher in roots. Seed germination associates with many metabolic, cellular, and molecular events, rendering the radicle able to emerge from the seed and develop into whole plant. The activation of metabolism following seed inoculation on the medium provides the basis for production of ROSs. Production of H_2O_2 at the early imbibitions and germination period has been observed in soybean [35], radish [36], maize [37], sunflower [38], wheat [39], and tomato [40]. Other ROSs, such as NO [41], hydroxyl radicals, and superoxide radicals [36] also accumulate during the germination of seeds of various plant species. These antioxidant enzymes have importance for the completion of germination following development. Normally, high activity of CAT and POD has been recorded in leaves as compared to stem and root [42]. As long as plant development

proceeds, metabolically active portion of the tissues increases following the production of H_2O_2 and finally, enzymatic antioxidant system. However, the above results demonstrate that the POD performs a major role to protect developing plants against oxidative stress as compared with CAT. CAT and POD scavenge H_2O_2 and catalyze the oxidation of various kinds of hydrogen donors [43]. APX isoforms play a defensive role against ROSs produced in surplus under environmental stress. Therefore, their expression level often reflects the occurrence of stress conditions [44]. APX also differs significantly in reactivity and physiochemical properties with typical guaiacol-type peroxidase. The activities of these enzymes, as well as the content of ascorbate and glutathione increase parallel to the activities of glyoxysomal marker enzymes during the course of germination [45].

As long as the explants from in vitro-grown seedlings were inoculated on callus induction medium, the pattern of antioxidant enzymes varied. These results are in agreement with Wakamatsu and Takahama [46] that excision of tissue for callogenesis constitutes a physical stress, which may not only trigger some of the biosynthetic processes (e.g., lignin synthesis) but also cell division. In tobacco leaves, 20-fold increase in POD activity has been observed due to wounding [47].

APX enzyme level started to increase as the callus formation started from root, stem, and leaf explants. This increase in pattern continued even during proliferation phase. During callus induction and proliferation, metabolic processes increase the endogenous level of H_2O_2 [48]. Increase in APX activity after a few weeks of callus initiation might also be due to the accumulation of high amount of H_2O_2 that caused browning or necrosis of callus. APX scavenge the excess of H_2O_2 to prevent the browning of callus. In response to wounding, the cytosolic APX expression in sweet potato also attained maximum level after a few days [49]. On the other side, activity of CAT and POD started to decrease as calli formation started from the leaf, stem, and root explants of *Argyrolobium roseum* cultured on MS medium. Same decline level continued during proliferation stage. Decline in the CAT and POD activities, which constitutes the cells' first line of antioxidative defense system, suggests that cells are progressively heading towards the oxidative state, as callus formation and proliferation requires oxidized conditions. Gupta and Datta [48] also reported that POD activity decrease during callus initiation and proliferation. H_2O_2 also plays an important role in transduction of defense signals in plants [50]. The calli, as proliferated in the presence of cytokinin and auxin, might start transformations into shoot. Therefore, it can be suggested that H_2O_2 might act as novel cellular "messenger", capable of effecting gene expression and stimulating formation of shoot or embryo [8].

The activities of antioxidant enzymes reversed in regenerated shoots as observed during callus initiation and proliferation. APX activity started to decrease while CAT and POD initiated elevation. This variation in antioxidant activities might be due to organ formation or development. The organogenesis from the callus is a complex process, accompanied with a variety of genes expression, protein synthesis [51], and role of H_2O_2 [8]. Totipotency of plant cells has also been related to the activity of the cell antioxidant machinery, since a direct correlation exists between high ROSs content and repressed expression of totipotency [52]. CAT activities also increase during the development of horse chestnut somatic embryos [53] and during the development of oak microcuttings [54]. Developing embryos generate significant amounts of ROSs, necessitating tight control by antioxidant mechanisms. The ascorbate system seems to play a central role in embryogenesis and cell growth [55], mainly because ascorbate controls cell-cycle progression [56]. It has also been proposed that ascorbate content influences cell growth by modulating the expression of genes involved in hormonal signaling pathways [57]. CAT also plays a significant role in growth and cell differentiation. High activity of catalase in regenerated plantlet might also be due to the

different growth hormones used in the medium to enhance regeneration [58]. Transcriptional activation of CAT and POD continuously increased in leaves and stem of regenerated plants, while a slight decrease in APX was observed. Similar observations have already been reported for gladiolus [48] and ice plant [59]. Regenerated leaves presented high levels of APX activity as compared to regenerated stem. It seems that at this stage, catalase and peroxidase play a significant role to scavenge H_2O_2 . However Shigeoka et al. [60] reported that cAPX gene expression upregulates in response to cellular H_2O_2 level. Increase in catalase activity suggests its effective scavenging of H_2O_2 produced during regeneration and development. As opposite to the embryogenesis, organogenesis requires redox state for the development of organogenic culture. The reverse pattern of POD was noticed during the regeneration stage as compared to APX; also reported by others [42, 48]. Various POD isoenzymes are involved in different physiological processes such as lignifications and suberization [61]. The high activity of POD might be due to hormonal balance inducing organogenesis. Increase in POD activity leads to an increase in auxin/cytokinin catabolism, to the diminution of auxin level and to the modification of auxin/cytokinin ratio, in the favor of cytokinin, thus resulting in shoot appearance [62]. Oxidants produced during different metabolic processes demands synthesis of this enzyme to scavenge O_2 (pro-oxidants) and H_2O_2 (peroxides) [63].

The activity of POD was noticeably higher in regenerated roots as compared to regenerated stem and leaves in the present study. POD is considered as a marker of rhizogenesis. It plays an important role in root formation as it is involved in cambial cell division and cell differentiation during rooting [59]. Addition of phytohormone in the medium enhances the POD activity. Basal medium without the addition of phytohormone showed no increase in POD activity as compared to medium having plant growth regulators [64]. APX enzyme showed opposite pattern in enzyme activity as compared to catalase and POD.

Transferring of regenerated plant from *in vitro* to *in vivo* condition produces stress so changing concentration of APX. High H_2O_2 content in early stages of acclimatization process reflected a similar process of oxidative stress [65]. Our work suggests that tissue-cultured plants develop antioxidant enzymatic protective system which determines the ability to survive in oxidative stress and the regulation of these enzymes would help to reduce the built up of reactive oxygen species. It suggests that APX may merely act as scavengers of hydrogen peroxide as produced during shifting. During the acclimatization stage, leaves showed a high level of CAT activity as compared to stem and roots also reported by others [66]. CAT involves in peroxides detoxification produced in a series of metabolic reactions in peroxisomes. These observations are in accordance with reports that high levels of H_2O_2 and free radicals are associated with the promotion of senescence whereas lipid peroxidation contributes to membrane degradation [67, 68]. Shifting of *in vitro* developed regenerated plants to *in vivo* conditions showed stress environment during initial weeks. Activity of CAT increased as the plants were shifted to acclimatized condition [65]. The high activity of POD was also noticed in root, stem, and leaves during acclimatization. In roots, much high activity was noticed as compared to leaves and stem in these experiments. POD was not only taking part in the induction of root primordia but also involved in the elongation of roots. These results are in agreement with Zheng and Van Huyster [69] that high POD activity in root was related to the oxidation reaction and membrane stability. Transferring the regenerated plantlets to acclimatized conditions reported initially decline in enzyme level in leaf, stem, and roots. After 2 weeks, POD activity was increased at much extent, POD respond was quite fast for cutting, shifting, and even rubbing, but soon regains its normal metabolism. As the regenerated plantlets shifted to acclimatized state, low activity of POD might be due to that acclimatized conditions are moving towards

oxidized status. Tissue-cultured plant developed antioxidant system, which determines the ability to survive and upregulation of these enzymes would help to reduce the built up of ROSs [65].

Information regarding micropropagation of *Argyrobium roseum* using leaf, hypocotyl, and root explants was not available. In the present study, these explants were inoculated on MS medium containing different concentrations of BA and NAA for callus induction. However, the release of phenolic compounds in the medium delayed the callus induction response that was counteracted with the addition of AgNO₃ and PVP in the culturing medium. Low concentration of NAA was optimum for shoot induction from calli, while the presence of BA produced a maximum number of shoots per flask and shoot length. The protocol reported can be used for mass propagation to meet the demands of pharmaceutical industries and genetic transformation of *Argyrobium roseum* using different explants. Results provide evidence that there were distinct patterns of enzyme activity under different stages of tissue culture (Fig. 2). Activity of different enzymes observed in in vitro plant development demonstrates change in behavior during callogenesis. Activity of ascorbate peroxidase increases during callogenesis. This is in contrast to activity of in vitro plant development where there was a decrease during callogenesis of catalase and peroxidase. Activity of these enzymes also show variation in enzyme level in regenerated plantlets as compared to in vitro plants, as regenerated plants faced the callogenesis stage during their development that is a highly stressful condition as compared to organogenesis. This study reflects the behavior of antioxidant enzymes at different tissue culture stages and highlights the importance of these enzymes in the developmental process.

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