

Biochemical changes in embryogenic and non-embryogenic callus of *Bambusa nutans* Wall. during somatic embryogenesis

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Abstract

Bambusa nutans Wall. is a clump-forming, evergreen bamboo species that is most often found in Southeast Asian forests. Comparative activities of nitrate reductase (NR), glutamine synthetase (GS) and peroxidase (POX) as well as expression of peroxidase isozymes during somatic embryogenesis (SE) were investigated in the segregated embryogenic callus (EC) and non-embryogenic callus (NEC) from the same genotype (CPC-648) of *B. nutans* Wall. The EC was compact, with large prominent nuclei and dense cytoplasm, while the NEC was fragile, with rudimentary nuclei and hyaline cytoplasm. SE in EC encompassed induction, maturation and germination stages each of 30 days on MS medium supplemented with ascorbic acid 50mg/l, citric acid 25mg/l, cysteine 25mg/l and glutamine 100mg/l + 2, 4-D-2 mg/l + coconut water 10%. EC exhibited dedifferentiation and growth leading to SE, while NEC remained amorphous loose lump throughout. EC had lower NR and POX activities and less number of peroxidase isozymes but higher GS activity than NEC during three stages of SE. NR activity followed a trend as induction > germination > maturation (EC and NEC); GS activity, maturation > germination > induction (EC) and induction > germination > maturation (NEC) and POX activity, maturation > induction > germination (EC) and induction > germination > maturation (NEC). Compared to NEC, EC exhibited less peroxidase isozymes. SE is an epigenetically regulated process that leads to the expression of enzymes involved in primary metabolism in EC and secondary metabolism in NEC. GS activity corresponding to SE process may be developed as biochemical marker. A chaotic scenario, rather from being a marker of SE, is indicated by high levels of peroxidase activity and isozyme expression.

Introduction

Somatic embryogenesis (SE) producing bipolar structures identical to zygotic embryos undertakes a complex developmental pathway that sequentially encompasses a dedifferentiation process and totipotency acquisition in somatic cells to obtain mass production of somatic embryos (Neves et al. 2021). This process is highly dependent on cell-to-cell communication in multicellular perennial plants, and epigenetic effects can have a significant impact on the potential for SE. During SE, various developmental stages occur in different generations of a plant, and all cells within the tissues, organs, or the plant as a whole simultaneously proceed on different developmental pathways. These developmental pathways are influenced by ontogenetic events, which are the changes that occur during the development of an organism over time. The dedifferentiation and totipotency constraints that arise from the blockage of the expression of pertinent genes and consequent metabolic pathways, which are commonly known as epigenetic effects, can result in the random occurrence of SE. (e.g., see review by Neves et al. (2021); Salaün et al. (2021)). Epigenetic effects are changes in gene expression that are not caused by alterations in the DNA sequence but rather by modifications to the chromatin structure or DNA methylation (Takatsuka and Umeda 2015). These modifications can be influenced by various factors, including environmental conditions, developmental stage, and tissue type, and they can have long-lasting effects on the developmental potential of somatic cells. Therefore, the success of SE is highly dependent on the ability to control the epigenetic effects that influence the developmental pathways of somatic

cells. Success or failure of the somatic embryogenesis (SE) response in in vitro depends on several factors. These factors include the specific species being studied, the origin of the explant used (i.e., the part of the plant used to initiate the culture), the composition of the culture medium used, the type and concentration of growth regulators added to the medium, the sources of nitrogen and carbon in the medium, and the in vitro environmental conditions (such as temperature, light, and humidity). Hence, it would be interesting to investigate SE in responsive and non-responsive cells isolated from the same genotype. We have isolated both types of cells from *Bambusa nutans* Wall. for which the authors have developed the *in vitro* reproducible protocol for SE (Mehta et al. 2011; Rajput et al. 2021). Hence, we carried out a preliminary investigation on changes in activities of three enzymes, namely nitrate reductase (E.C.1.6.6.1), glutamine synthetase (E.C. 6.3.1.2) and peroxidase (E.C. 1.11.1.7) in somatic embryogenic and non-embryogenic callus obtained from the same mature genotype of *Bambusa nutans* Wall. The presence and activity levels of certain enzymes involved in nitrogen metabolism, such as nitrate reductase and glutamine synthetase, have been shown in previous studies (Hirel and Gadai 1980; Kenis et al. 1992) to be indicative of orderly growth and development in plants. In other words, when these enzymes are expressed at appropriate levels, it suggests that the plant is developing normally and following a structured growth pattern. On the other hand, the expression of peroxidase, another enzyme, has been associated with chaotic and disorderly conditions (Sachdev et al. 2021). Higher levels of peroxidase activity have been observed in plants under stress, such as when exposed to extreme temperatures, high levels of light or pollutants, or when experiencing injury or disease. This suggests that peroxidase expression is a response to stressful conditions and can be an indicator of plant distress. This experimental investigation may provide understanding on the physiological and metabolic changes that occur during SE, as well as the factors that contribute to the possibility of SE.

Materials And Methods

2.1. Callus induction and embryogenesis

After 28 days of inoculation in MS medium containing ascorbic acid (50 mg/l), citric acid (25 mg/l), cysteine (25 mg/l), glutamine (100 mg/l), and 2,4-D (2.0 mg/l), nodal shoot segments (used as explants) exhibited callus induction. The resulting embryogenic callus was then multiplied up to four passages on MS medium supplemented with ascorbic acid (50mg/l), citric acid (25mg/l), cysteine (25mg/l), glutamine (100mg/l), 2,4-D (2 mg/l), and 10% coconut water, before being utilized for biochemical investigations (Rajput et al. 2021). Using a compound microscope, two-month-old embryogenic callus (EC) and non-embryogenic callus (NEC) were selected for the study based on their morphology. Throughout the induction, maturation, and germination stages of somatic embryogenesis, the activities of the following three enzymes were observed in embryonic and non-embryonic callus of *B. nutans* over a period of four weeks each.

2.2. Nitrate reductase activity

Nitrate reductase (E.C.1.6.6.1) activity (NRA) was determined according to the method of Jaworski (1971). Samples of 500 mg each of embryonic and non-embryonic callus were taken and chopped into small pieces to estimate NRA. The chopped samples were placed in glass vials containing 2.5 ml each of 0.2 M phosphate buffer (pH 7.5) and 5% isopropanol, and 0.5 ml of 0.2 M potassium nitrate solution followed by incubation for two hours in dark at room temperature. The control containing all reagents, excluding callus tissues was simultaneously prepared and incubated for the same period in the dark. Following incubation, a 1 ml aliquot was taken and mixed with 1 ml of 0.02% N-1-naphthylethylenediamine hydrochloride (NED HCl) and 1% sulphanilamide. The volume was adjusted to 5 ml with distilled water and left for 20 min for maximum colour development. The absorbance of the developed pink colour was read at 540 nm using UV-Vis spectrophotometer against the control without any colour. A standard curve was plotted by taking various concentrations of sodium nitrite. The absorbance of these samples was compared with this calibration curve and NRA was computed as $\text{n mol NO}_2 \text{ h}^{-1} \text{g}^{-1}$ callus fresh weight.

2.3. Glutamine synthetase activity

Glutamine synthetase (E.C. 6.3.1.2) activity (GSA) was estimated following the method of Pateman (1969). 100 mg samples each of both embryogenic and non-embryogenic callus were homogenized with 100 mM Tris HCl + 1mM EDTA extraction buffer (pH 7.2). The resulting extract was then centrifuged at 10,000 rpm for 30 min at 0°C. 1900 μl reaction mixture – 0.2 M L-glutamine, 20 mM sodium arsenate, 3mM MnCl_2 , 50 mM hydroxylamine, 1mM ADP, 20mM Tris HCl buffer (pH 8.0) - was added to 100 μl of supernatant. The mixture was incubated at 37°C for 5 min., and the reaction was stopped by adding 1ml of ferric chloride reagent. The resulting brown colour was measured at 540 nm to determine GSA.

2.4. Preparation of acetone powder solution

100 mg embryogenic/non-embryogenic callus was taken and ground well in a chilled mortar and pestle in 80% ice-cold acetone. The resulting homogenate was filtered through Whatman filter paper no.1, and the residue on the filter paper was repeatedly washed with 80% ice-cold acetone, followed by diethyl ether and air-dried. The acetone powder was then suspended in 0.4 ml of 0.02 M sodium phosphate buffer (pH 6.4) and centrifuged for 10 min. at 10,000 rpm. The resulting supernatant was used to estimate peroxidase activity and total soluble proteins.

2.5. Peroxidase activity

The peroxidase activity was determined in the supernatant according to the method described by Rao et al. (1982). The reaction mixture comprised 0.1 ml supernatant, 0.4 ml of 20 mM guaiacol and 0.25 ml of 0.02 M sodium phosphate buffer (pH 6.4). The absorbance was set zero at 470 nm on a UV-Vis spectrophotometer with the reaction mixture to which 0.25 ml of 30% hydrogen peroxide was added and the change in absorbance every 30 second was recorded for 3 minutes. The peroxidase activity was expressed as $\Delta_{A470}^{-1} \text{min}^{-1} \text{mg}^{-1}$ protein.

2.6. Protein estimation

Protein content in the supernatant was determined using the method of Lowry et al. (1951). In a centrifuge tube, 0.1 ml supernatant was added with gentle mixing, followed by 0.1 ml buffer and 1 ml protein reagent. Finally, 0.1 ml of 50% Folin phenol reagent was added with immediate mixing and left for 30 min for maximum colour development. The absorbance of the resulting color was measured at 750 nm using a UV-Vis spectrophotometer. To quantify the protein content in the sample, a calibration curve was prepared using known dilutions of bovine serum albumin.

2.7. Peroxidase isozymes analysis

The method described by Tsala et al. (1996) was used to investigate the peroxidase isozyme pattern during the induction stage of somatic embryogenesis at 15, 21, and 28 days after inoculation. A resolving gel with an effective concentration of 12% (w/v) acrylamide-bisacrylamide, 0.4 M Tris HCl (pH 8.8), 6 mM ammonium per sulphate (APS), and 6.0 mM TEMED was used, which was 1.5 mm thick. The stacking gel had 6% (w/v) acrylamide- bisacrylamide, 0.4 M Tris HCl, 6 mM APS and 6.0mM TEMED. The gel buffer consisted included 50µl each of enzyme extract and tracking dye, which was made up of 0.4 M Tris HCl, 20% glycerol and 2 ml bromophenol. Gels were run at 4°C at 60 V until the sample crossed the stacking gel and at 120 volts until the run was complete. After electrophoresis, the gels were stained in a solution containing 0.06% H₂O₂, 0.1% guaiacol and 0.1% acetic acid. Gel photographs were taken immediately after staining.

2.8. Statistical analysis

The data obtained in all experiments were subjected to one-way analysis using ANOVA table. The significance of the data was evaluated at a p-value of 0.05 using the F-test. If the calculated F-value was significant at $p \leq 0.05$, the critical difference at a p-value of 0.05 (CD0.05) was calculated for the comparison of treatments using the SPSS Statistics for Windows, Version 24.0 (Armonk, NY: IBM Corp.)

Results

3.1. Morphology

We obtained both embryogenic (Fig1a) and non-embryogenic (Fig.1b) calli from the same genotype of *Bambusa nutans*. The embryogenic callus (EC) appeared compact and granular with dense cytoplasm and large prominent nuclei (Fig.1a, a1), while non-embryogenic callus (NEC) was mucilaginous and fragile wooly with underdeveloped nuclei (Fig1b, b1). Somatic embryos were developed from EC and passed through three stages i.e., induction, maturation (Fig. 1b2) and germination (Fig. 1b3-4) each of 30 days cycle. Each germinated somatic embryo developed into a healthy plantlet (Fig.1b5) which was then acclimatized and hardened ready to field transfer (Fig.1b6).

3.2. Enzyme activity

During three stages of somatic embryogenesis (induction, maturation, and germination), the activities of three enzymes were investigated: nitrate reductase and glutamine synthetase, which are involved in

nitrogen metabolism, and peroxidase, which is part of the antioxidant pathways. The activities of these enzymes were measured at intervals of 7 days (Figs.2-4).

3.12.1 Nitrate reductase activity (NRA)

NRA exhibited a significant variable trend between EC and NEC as well as across three stages of somatic embryogenesis (Fig.2). The average NRA value (nmol h⁻¹ g⁻¹ fresh weight) $\pm$ SE for EC and NEC was 0.131 ± 0.023 and 0.281 ± 0.052 , respectively, during induction; 0.075 ± 0.012 and 0.252 ± 0.021 , respectively, during maturation; and 0.045 ± 0.008 and 0.193 ± 0.019 , respectively, during germination. Additionally, the trend of mean NRA activity for EC and NEC was induction > germination > maturation.

During induction, NRA activity significantly declined at 28 days after inoculation (Fig. 2a), whereas it peaked at 7 days after inoculation during maturation (Fig. 2b) and remained relatively stable during germination (Fig. 2c). Conversely, during induction, NRA activity attained its highest value at 28 days after inoculation (Fig. 2a) and fluctuated during maturation and germination stages (Fig. 2b, c).

3.2.2. Glutamine Synthetase activity (GSA)

GSA also exhibited a significant variable trend both between EC and NEC as well as across three stages of somatic embryogenesis (Fig.3). The average GSA value (μ mol GH mim⁻¹ mg⁻¹ protein) $\pm$ SE was 218.60 ± 65.96 for EC and 36.30 ± 15.35 for NEC during induction, 327.17 ± 152.13 for EC and 19.19 ± 1.28 for NEC during maturation and 237.83 ± 46.25 for EC and 29.29 ± 6.31 for NEC during germination. Notably, EC had significantly higher GSA than NEC at all three stages of somatic embryogenesis. Additionally, EC exhibited a trend of GSA as maturation > germination > induction, while NEC exhibited a trend of induction > germination > maturation.

In EC, GSA showed a cyclic pattern during induction stage (Fig.3a), with a peak value observed at 21 days after inoculation during germination (Fig.3b) and an initial high followed by continuous decline till 28 days after inoculation during germination stage (Fig.3c). In contrast, NEC maintained comparable non-significant pattern of GSA throughout all three stages of somatic embryogenesis (Fig.3).

3.2.3. Peroxidase activity

Figure 4 shows that peroxidase activity had a significant variable trend both between EC and NEC, as well as across three stages of somatic embryogenesis (Fig.4). The average peroxidase activity value (Δ_{A470} mim⁻¹ mg⁻¹ protein) $\pm$ SE was 2273 ± 89 for EC and 323637 ± 136781 for NEC during induction, 2783 ± 118 for EC and 180126 ± 70414 for NEC during maturation and 1909 ± 169 for EC and 284209 ± 113233 for NEC during germination. Remarkably, NEC had over 1000 times more peroxidase activity than EC at all three stages of somatic embryogenesis. Peroxidase activity exhibited the trend as maturation > induction > germination in EC and induction > germination > maturation in NEC.

EC had statistically similar, albeit very low, peroxidase activity at all stages of somatic embryogenesis. However, NEC displayed significantly highest peroxidase activity at 14 days after inoculation during both

the induction and germination stage, and at 0 days after inoculation during the maturation stage (Fig. 4).

3.3. Peroxidase isozyme pattern

The peroxidase isozyme pattern in both EC and NEC was analyzed at 15, 21 and 28 days after inoculation for induction stage of somatic embryogenesis only. At each of these three sampling intervals, EC and NEC expressed three and five isozymes of peroxidase, respectively. However, the molecular weight of peroxidase isozymes was greater in EC than in NEC (Fig. 5).

Discussion

Somatic embryogenesis is an orderly process of dedifferentiation, development and growth (Méndez-Hernández et al. 2019). Presumably, a single diploid (somatic) cell conditioned to take cue from the surroundings participates in the process and is distinguished from the neighboring mass of cells in an eucaryotic multicellular plant body i.e., bamboo nodal explants in the present investigation. Consequently, two types of cells exist in multicellular somatic plant body; specific cells and non-responsive cells for somatic embryogenesis. Morphologically, the progenitor cells of SE exhibit large prominent nucleus and dense cytoplasm (Xue et al. 2022) as has also been observed in case of *B. nutans*, here. The referred specific cells are juvenile being maintained at homeostasis to take a course of dedifferentiation and growth leading to SE. The non-responsive cells are well differentiated mature cells and do not participate in the process of dedifferentiation and growth. Considering these points in view, EC and NEC obtained in the present investigation represent 'system' and 'chaos', respectively, and are subjected to the enzymes of system's metabolism such as nitrate reductase and glutamine synthetase, and chaotic conditions, such as peroxidase. The former represents gateway for nitrogen in the system as well as biosynthesis of nitrogenous compounds which are responsible for maintenance of central dogma and labyrinth of metabolism indispensable for somatic embryogenesis. The latter is expressed under chaotic metabolic conditions and acts as scavenger of superoxide.

As a result, SE requires steady supply and utilization of nitrogen for transcription and translation of pertinent genes, as well as biosynthesis of phytohormones, that cooperatively and synergistically operate during the process (Yue et al. 2022). However, the excess consumption of nitrogen (nitrate) from the EC culture medium accounts for low NRA and concurrent high GSA during the *in vitro* progress of SE. On the contrary, NEC culture maintains high NRA and low GSA due to non-utilization but accumulation of nitrogen (nitrate) from the culture medium. This is because of the fact that nitrate reductase is an inducible enzyme and its expression linearly depends upon availability of nitrate in the biological system (Bian et al. 2020; Sivasankar and Oaks 1996). A faulty and rudimentary metabolism in NEC fails to utilize nitrate-nitrogen from the medium, accumulating excess nitrate in the NEC system, thereby high induction of nitrate reductase activity during the course of the present investigation.

In fact, the entrant nitrate in the EC system needs a linear sequential expression of nitrite reductase, glutamine synthetase and glutamate synthase (Fan et al. 2022) for its efficient incorporation and utilization in *de novo* nitrogenous metabolites including biosynthesis of transcriptomes, proteins and

regulatory/ signaling molecules like phytohormones. That is why in the present study of *B. nutans*, glutamine synthetase activity in EC is consistently higher than that in NEC across all three stages of somatic embryogenesis, suggesting that enzymes (nitrate reductase and glutamine synthetase) of nitrogen metabolism are coupled in the former and decoupled in the latter. By and large, the NRA trend in EC is in agreement with that obtained during various stages of the *in vitro* SE in *Daucus carota* (Kamada and Harada 1984) and *Dalbergia latifolia* (Shirin et al. 2020). On the contrary, NRA trend obtained in NEC has not been mentioned in the literature. As for GSA more in EC than NEC in the present investigation, Rodríguez et al. (2006) have demonstrated that two glutamine synthetase genes are specifically expressed during SE in *Pinus pinaster* and *Pinus sylvestris*, prompting to recommend them as marker genes for the process. Further, an exogenous supply of glutamine facilitates SE in Norway spruce, *Picea abies* L. Karst (Carlsson et al. 2019; Carlsson et al. 2017). In fact, GSA is responsible for the production of L α -glutamine by absorbing NH_4^+ , which is the end result of nitrate assimilation. During transamination, glutamine donates, amino group for the biosynthesis of essential amino acids and other vital nitrogenous compounds through diverse metabolic pathways (Bernard and Habash 2009).

During the SE process in *Bambusa nutans*, NEC has stronger peroxidase activity and expresses a bigger number of its isozymes than EC. Earlier, Bajaj et al. (1973) found that the non-differentiating callus had much greater peroxidase activity than the differentiating callus. Changes in peroxidase and esterase isozyme patterns have also been linked to different phases of somatic embryogenesis in monocots (Bapat et al. 1992; Coppens and Dewitte 1990; Rao et al. 1990). By and large, our findings on peroxidase activity/ isozyme are in agreement with the previously published literature on different species (Bonfill et al. 2003; Sarkar et al. 2010; Somleva et al. 2000; Thorpe et al. 1978). Interestingly, a recent investigation on EC and NEC reports higher peroxidase activity in the former than the latter during the *in vitro* SE in *Olea europaea* and recommends peroxidase to be considered as SE marker (Oulbi et al. 2021). The elevated peroxidase activity indicates that the biological system is experiencing disorderly stress conditions due to ROS generation (Sachdev et al. 2021). The “chaotic situation” may occur as a momentarily transition stage preceding emergence/ establishment a *de novo* orderly biological system such as somatic embryogenesis or organogenesis that accounts for changes in peroxidase activity or its isozymes in several investigations. However, none of the previously published investigations incorporates EC and NEC isolated from the same genotype for a comparative peroxidase activities and isozyme patterns during the *in vitro* SE as has been the case of the present investigation on *Bambusa nutans*. Therefore, our findings reiterate that the elevation in peroxidase activity and expression of more peroxidase isozymes indicate either stress conditions preceding the orderly de-differentiation and growth of the *de novo* organogenesis or persistency of “chaos” in the biological system.

Conclusion

The isolation of EC and NEC from the same genotype of *B. nutans* offers an excellent opportunity to investigate physio-morphological and biochemical mechanism of SE within the similar genetic set up. SE appears to be an epigenetically controlled phenomenon, expressing enzymes of primary metabolism in

EC and secondary metabolism in NEC. This investigation can provide insight into the physiological and biochemical changes that occur during SE and the factors that contribute to the potential for SE. Further research can build upon these findings and explore the epigenetic effects and other factors that contribute to the potential for SE. GSA has a strong correlation with the process of SE and might be used as a potential biochemical marker for the process. On the contrary, peroxidase activity and expression of its isozymes denote chaotic situation rather than marker of SE. Time lapse transcriptome analysis and comparison in EC and NEC systems may help trace sequence of gene expression and corresponding metabolic pathways culminating SE.

Statements And Declarations

Author contribution ALL authors contributed to this study. Material preparation, data collection, and analysis were performed and first draft of the manuscript by [DSR] and [TSR]. SAA, MF, AAA and EMA checked the data analysis, provided interpretation and finalized the manuscript to be submitted to the journal.

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Ethical standard Research work reported in the MS complies all ethical responsibilities.

Conflict of interest the authors declare that they have no conflict of interest.

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Figures

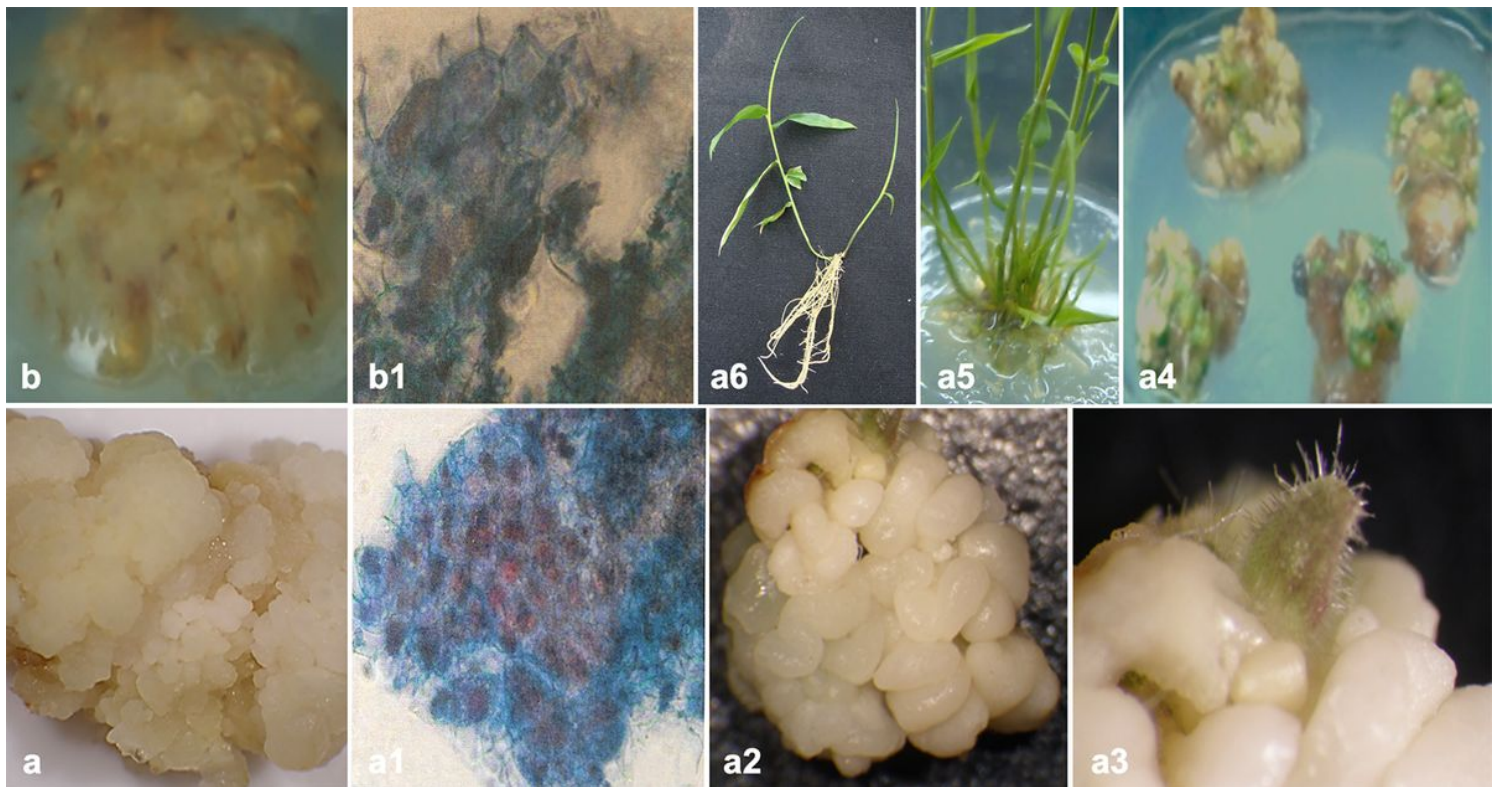


Figure 1

Somatic embryogenesis (SE) in *Bambusa nutans* Wall. (a) Compact granular embryogenic callus with (a1) prominent nucleus and dense cytoplasm, (a2) SE induction, (a3) SE maturation, (a4) SE germination, (a5-a6) SE plantlet, (b) Fragile amorphous non-embryogenic callus with (b1) rudimentary nuclei and cytoplasm.

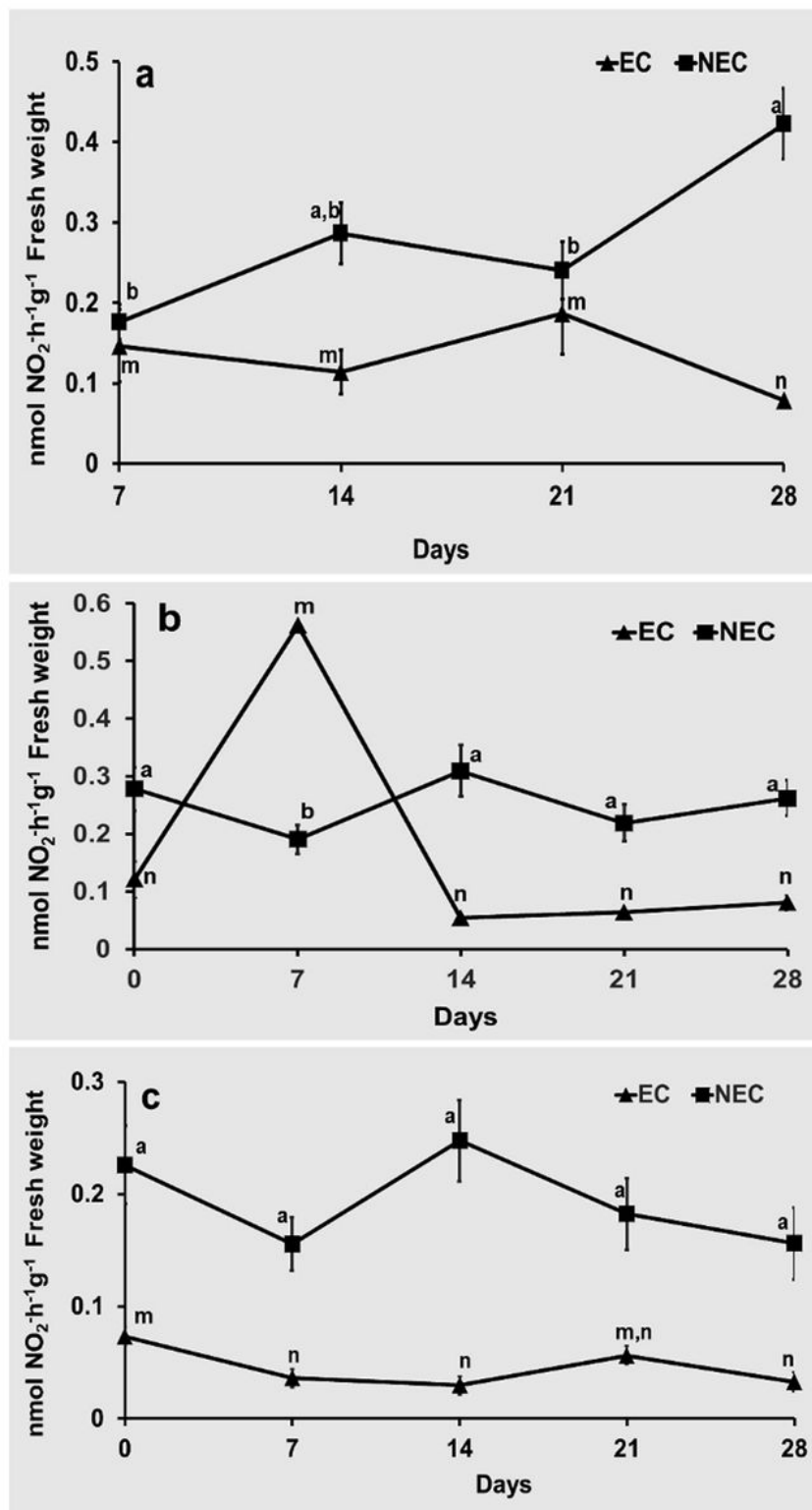


Figure 2

Nitrate reductase activity in *Bambusa nutans* Wall. during (a) induction, (b) maturation and (c) germination stage of somatic embryogenesis. Data (significant at $p < 0.05$) at each point are mean of four replicates with vertical bar representing $\pm$ SE. Embryogenic callus (EC) and non-embryogenic callus (NEC).

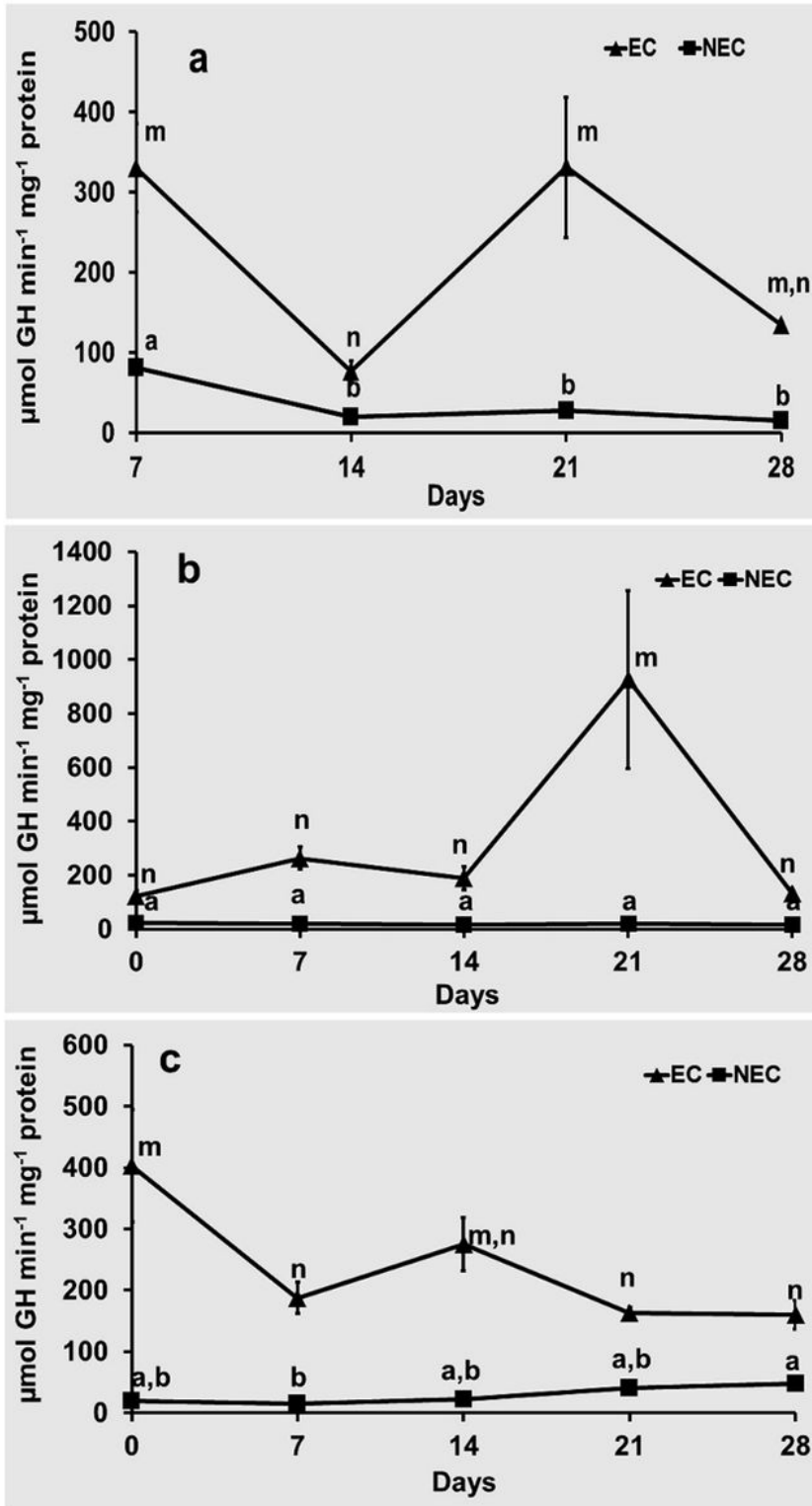


Figure 3

Glutamine synthetase activity in *Bambusa nutans* Wall. during (a) induction, (b) maturation and (c) germination stage of somatic embryogenesis. Data (significant at $p < 0.05$) at each point are mean of four replicates with vertical bar representing $\pm$ SE. Embryogenic callus (EC) and non-embryogenic callus (NEC).

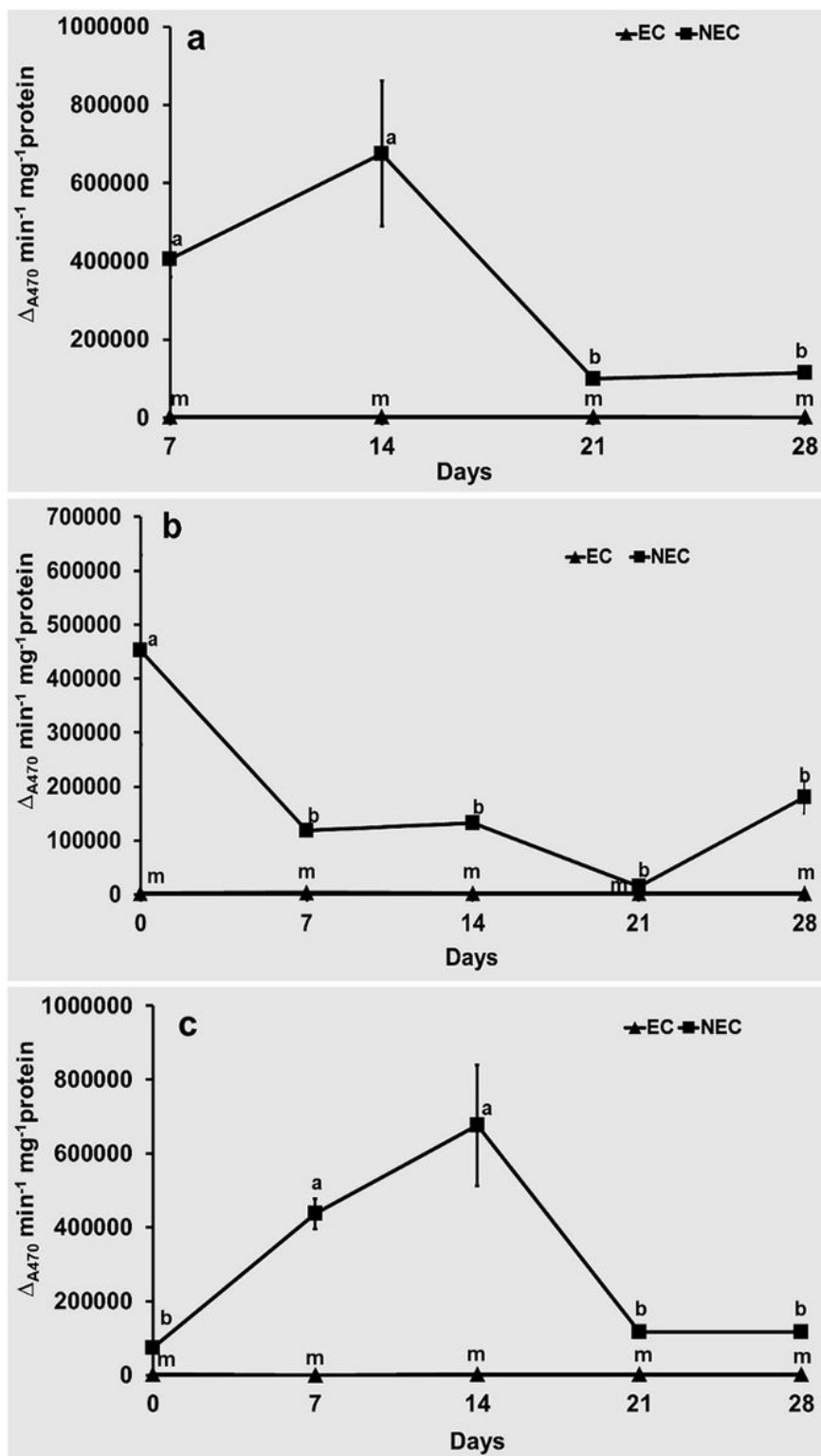


Figure 4

Peroxidase activity in *Bambusa nutans* Wall. during (a) induction, (b) maturation and (c) germination stage of somatic embryogenesis. Data (significant at $p < 0.05$) at each point are mean of four replicates with vertical bar representing $\pm$ SE. Embryogenic callus (EC) and non-embryogenic callus (NEC).

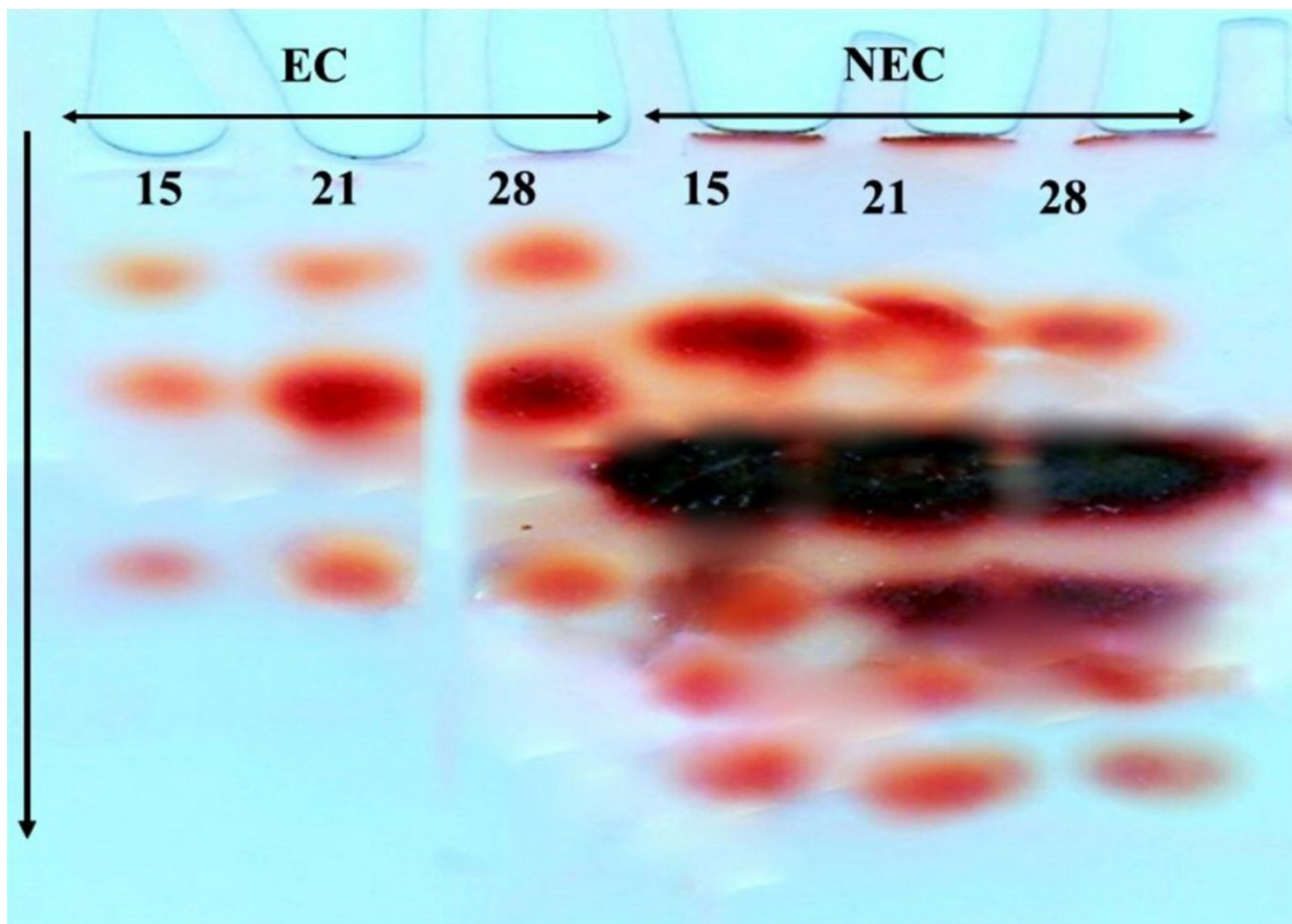


Figure 5

Pattern of peroxidase isozymes obtained at 15, 21 and 28 days after inoculation for induction stage of somatic embryogenesis in embryogenic callus (EC) and non-embryogenic callus (NEC) of *Bambusa nutans* Wall. Expression of peroxidase isozymes was less in number in EC (3) than NEC (5).