Anatomical and biochemical responses to oxidative stress in shoots of Bambusa vulgaris Schrad. ex Wendl during the in vitro-ex vitro transition

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Research Article

Keywords: Bamboo, Hperhidricity, MEB, ROS

Posted Date: August 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1886347/v1

Abstract

Hyperhydricity can affect the development of plant morphology. A better understanding of the anatomical and physiological changes of hyperhydric plants is needed to predict and control the occurrence of hyperhydryc conditions. The aim of this study is to demonstrate the role of oxidative stress in hyperhydricity. To this end, the anatomical, physiological and biochemical responses to oxidative stress in shoots of *Bambusa vulgaris* Schrad. ex Wendl were compared during the in vitro-ex vitro transition. For this propose, we used shoots grown in two different culture systems: liquid static culture medium and temporary immersion system. Our results showed that hyperhydricity was associated with oxidative stress in the shoots. In hyperhydric shoots chlorophyll content decreased when cultured in liquid static culture medium. Moreover, hydrogen peroxide content and malondialdehyde, as well as the activities of catalase and enzymes of ascorbate-glutathione cycle (ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase) were increased in these shoots. On the other hand, scanning electron microscopy showed that the leaves of hyperhydric shoots exhibited anatomical changes in the stomata of the plants, whereas the leaves of normal shoots showed normal structural development. Finally, normal shoots showed high survival rate and allowed better adaptation of the plantlets in the greenhouse.

Key Message

Knowledge of the morpho-physiological, anatomical, and biochemical changes associated with hyperhydricity could prevent this abnormality in in vitro shoots of *B. vulgaris*.

Introduction

Bambusa vulgaris Schrad. ex Wendl (*B. vulgaris*), is considered one of the most important bamboo species with industrial and environmental importance (Tewari et al., 2014; Yeasmin et al., 2015; Krishnakumar et al., 2017). Traditionally, plants are propagated by vegetative methods, (Pattanaik et al., 2004; Ray et al., 2017). However, due to the low efficiency of propagation, this method cannot meet the high demand. The use of organogenesis in in vitro plant regeneration of *B. vulgaris* provides an alternative to increase the number of plants for reforestation in a short period of time (Larekeng et al., 2020). Several factors can affect the in vitro propagation of shoots, such as hyperhydricity, which is one of the most important factors in in vitro propagation in this species.

Hyperhydricity is considered a disorder of plants in which leaves become translucent and stems become swollen, deformed, and brittle. Various environmental conditions prevail in tissue culture vessels that affect normal growth and morphological and physiological responses of plants (Chakrabarty et al. 2006; Dewir et al. 2006, 2015). In several bamboo species this disorder could be a response to environmental stress when explants are exposed to inefficient gas exchange, or high cytokinin concentration (Ramanayake et al. 2006; Beena and Rathore, 2012; Venkatachalam et al. 2015).

Several studies suggest that the abnormal morphology of hyperhydrated leaves is related to changes at the cellular level caused by oxidative stress and altered membrane composition. In plant cells, oxidative stress responses are associated with toxic free radicals generated during the reduction of molecular oxygen to superoxide radicals, singlet oxygen, hydroxyl radicals and hydrogen peroxide. To counter the dangerous effects of reactive oxygen species (ROS) under stress, plants have evolved a complex antioxidant defense system consisting of both antioxidant enzymes and metabolites such as catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Muneer et al. 2016; Tian et al. 2017; Naz and Perveen, 2021). To clarify these hypotheses and to better understand the stress and morphological responses in hyperhydric shoots (HS), the main objective of this work was to elucidate the role of oxidative stress in hyperhydricity. We compared the anatomical, physiological and biochemical responses to oxidative stress in shoots of *B. vulgrais* during the in vitro-ex vitro transition.

Materials And Methods

Plant material and culture conditions

B. vulgaris shoots grown in vitro in static liquid culture medium according to the protocol described by García-Ramírez et al. (2010) were inoculated into 70 ml of proliferation medium in Magenta jar (Sigma Aldridge Company Ltd.). Shoots were continuously subcultured at 3-week intervals. The resulting shoot clusters were divided into smaller clusters of 3 shoots. After the sixth and seventh subcultures of shoot multiplication, with subcultures every 20 days, homogeneous shoots were selected for the experiments. Shoots were grown in two different culture systems: liquid static culture medium (LSCM) and temporary immersion system (TIS).

The TIS used in this experiment was based on the two-vessel system described by García-Ramírez et al. (2016). The frequency and length of the immersion time was set at 2 min every 6 h. For liquid static culture medium (LSCM) treatment, 3 explants were cultured in 10 magentas for 3 weeks. In this experiment, twelve explants with two pairs of fully developed leaves were inoculated into the TIS with 250 ml of liquid culture medium per vessel. For the liquid static culture medium (LSCM) treatment, 3 explants were cultured in 10 magentas for three weeks under conditions similar to those described above and simultaneously evaluated using TIS. The rooting medium consisted of MS salts (Murashige and Skoog 1962), half MS salts (Murashige and Skoog 1962), 10 µM Indole-3-butyric acid (IBA) at pH 6.0. Shoots were incubated in a sunlight growth chamber at 28 ± 2 °C with a 14-h photoperiod at 38.0-45.7 µmol m^{-2} s^{-1} irradiance.

Scanning Electron Microscopy

Anatomical characterization by scanning electron microscopy (SEM) of leaves of *B. vulgaris* shoots was evaluated according to the method described by García-Ramírez (2019).

Biochemical parameters

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, and total chlorophyll content were quantified according to the method described by García-Ramírez (2021). Absorbance was measured at 440, 532, and 600 nm using a spectrophotometer (UV 1800, Shimadzu, Japan).

Enzyme activity

For the determination of antioxidant enzyme activities, 50 µL of the leaves were homogenized in 1.5 ml of the respective extraction buffer in a preboiled mortar and pestle with liquid nitrogen. The homogenizate was filtered through four layers of gauze cloth and centrifuged at 22 000×*g* for 20 min at 4 °C. The supernatant was centrifuged again at 22 000×*g* for 20 min at 4 °C to determine antioxidant enzyme activities. The preparation was applied to a column of sephadex G-25, equilibrated with the same buffers and kept in an ice bath until the assays were completed. The protein concentration of the enzyme extract was determined according to Bradford (1976).

APX activity (EC 1.11.1.11) was determined in 1.0 mL of reaction mixture containing 50mM K-phosphate (pH 7.0), 0.1mM ascorbate (extinction coefficient, 2.8mM^{-1} cm^{-1}) and 0.3mM H_2O_2. The decrease in absorbance was measured for 3 min at 290 nmfor (Chen and Asada, 1989).

Catalase activity (EC 1.11.1.6) was determined by tracking the consumption of H_2O_2 (extinction coefficient, 39.4mM^{-1} cm^{-1}) at 240 nm for 3 min (Aebi, 1974).

MDHAR activity (EC1.6.5.4) was determined from the decrease in absorbance at 340nm due to NADH oxidation using an extinction coefficient of 6.22mM^{-1} cm^{-1} (De Gara et al. 2000).

DHAR activity (EC 1.8.5.1) was measured by measuring the reduction of dehydroascorbate at 265nm for 4 min (De Gara et al. 2000).

Ex vitro acclimatization

Shoots were transplanted to a greenhouse according to the method described by García-Ramírez (2021). Ex vitro survival was assessed after 7 days.

Experimental design and statistical analysis

Morphometric variables (20 explants per replicate), biochemical parameters and ex vitro survival (80 explants per replicate) were assessed. Statistical analysis was performed using SPSS version 22 for Windows. Normality of the data was analyzed using the Kolmogorov-Smirnov test. The significance of differences was determined by analysis of variance (ANOVA). Means were compared with the Tukey test and are expressed as mean ± standard error.

Results And Discussion

Scanning electron microscopy

Significant differences in stomata morphology were observed between hyperhydric and normal shoots. SEM showed low stomata density and closed stomata of larger size, with larger stomata pore on the leaf surface of HS compared to normal shoots (Fig. 1). This could explain that the presence of stomata with larger area and size on HS of *B. vulgaris* could indicate low development of the stomatal complex compared to normal shoots.

Dutta and Prasad (2010) demonstrated a positive correlation between normal leaf development and a higher rate of stomata occurrence. Picoli et al. (2001) also reported, based on studies (SEM), that stomatal differentiation is impaired in hyperhydric plants. These changes are consistent with the characteristics exhibited by hyperhydric plantlets of other species, such as *Tectona grandis* L. (Quiala et al.2012) and *Vaccinium* spp. (Gao et al. 2018).

In general, normal shoots had elliptically shaped and closed stomata (Fig. 1). Therefore, we conclude that normal shoots had normal development of the stomata complex compared to HS. According to Tian et al. (2015) in hyperhidric plants leaves are one of the most affected and sensitive organ during the ex vitro survival in the greenhouse. Thus, stomata morphology is a valuable tool for predicting ex vitro survival in plants. Deformation stomatal complex development in hyperhydric plants negatively affects physiological processes such as photosynthesis, tissue drying, and plant death during the acclimatization phase (Palma et al. 2011).

Therefore, stomata structure and density, as well as stomata movement, play a very important role in regulating the water content of a plant (Apóstolo and Llorente 2000; Picoli et al. 2001). These changes can cause more stomata to malfunction and stomata to movement abnormally. Abnormal stomata can affect the overall physiology of the plant, especially the relationship between cell and water. Therefore, changes in stomata structure and stomata movement may be critical for the induction, promotion and development of HS in *B. vulgaris*.

Changes in chlorophyll content

The highest values for total chlorophyll content were obtained in shoots of *B. vulgaris* cultured in TIS (Fig. 2). HS, however, those cultured in static liquid medium showed a significant decrease in the content of this photosynthetic pigment. Saher et al. (2004) suggested that hyperhydricity causes inhibition of electron transport, increasing the likelihood of generation ROS, which can damage the membrane components of PSII, leading to causing oxidative damage in the photosynthetic apparatus. These results may suggest that the HS may have damage to photosynthetic pigments due to hyperhydricity. According to Tian et al. (2015), HS showed lower total chlorophyll content compared to shoots without hyperhydricity, which might be due to the harmful effects of oxygen species (ROS). In addition, they suggested that these conditions could cause an alteration in cellular redox homeostasis and damage to chloroplasts.

Similarly, Chakrabarty et al. (2006) reported that hyperhydric leaves had significantly lower chloroplast number per cell and chloroplasts showed reduced thylakoid stacking compared to healthy leaves. Also,

the chlorophyll content in *Allium sativum* L., decreased which affected the physiological response of the plants (Wu et al. 2009). Petruş-Vancea et al. (2018) indicated that hyperhydricity leads to a decrease in chlorophyll content in *Beta vulgaris* var. Conditiva. These authors shared that low chlorophyll content in shoots is one of the most common physiological responses induced by hyperhydricity. In this study Pathares et al. (2018) found similar results in hyperhydric shoots of *Lippia grata* Schauer, which reduced chlorophyll content.

Biochemical response

Comparison of hyperhydric shoots with normal plants showed a significant increase in H_2O_2 and MDA content in HS (Fig. 3A and B). The lowest values were obtained in normal shoots with significant differences compared with HS. The highest values for MDA content were obtained in HS, suggesting that lipid peroxidation might be increased in these shoots, (ROS). Oxidative stress could be induced in these shoots and affect their morphology. According to Hamed et al. (2013), lipid peroxidation is a consequence of oxidative damage to cell membranes and leads to the formation of MDA. MDA is an indicator oxidative stress response in plants (Perveen et al. 2013; Sen and Alikamanoglu, 2013; Mittler, 2017).

The activity APX and CAT was significantly lower in normal shoots compared with hyperhydric tissue (Figs. 3C). According to Malik et al. (2014), oxidative stress is defined by an imbalance between oxidants and reductants in the cell. This imbalance is due to excessive production of ROS and the cell is unable to counteract the harmful effects of ROS. This means that the increase in H_2O_2 accumulation normally occurs at a low level, due to the presence in the plant of several antioxidant systems are present that allow eliminating the excessive production of H2O2 and maintaining the optimal levels of ROS to achieve a normal dynamic balance.

The morpho-physiological, anatomical, and biochemical changes differ among plants species and depend on the specific responses of each species and the culture conditions used. Shoots of *B. vulgaris* cultured in static liquid culture responded strongly to hyperhydricity. Knowledge of the changes associated with hyperhydricity could prevent this anomaly in bamboo shoots cultured in vitro.

Several studies have been conducted to investigate the relationship between hyperhydricity and oxidative stress (Gupta and Prasad 2010; Balen et al. 2011; Dewir et al. 2014; Tian et al. 2017). The time course of H_2O_2 generation in hyperhydric tissues of *Dianthus caryophyllus* confirmed the close relationship between hyperhydricity and oxidative stress in this species (Saher et al. 2004). In this context, these authors described that HS could cause greater oxidative stress, due to the accumulation of ROS in cells. They also reported that ROS is one of the first cellular responses that stimulate defense mechanisms against oxidative stress in several plant species. However, high concentrations of ROS can cause uncontrolled oxidation of various cellular components and degradation of chlorophyll proteins. To counteract these effects, the cell must activate various antioxidant defense mechanisms to eliminate the harmful effects of these ROS.

According to Dewir et al. (2006) HS, a typical stress-induced change in physiological state is evident. They note an oxidative stress characterized by markedly increased MDA content in HS of *Euphorbia millii* Des Moul.. At the same time, oxidative stress was show to be reduced in normal shoots by increased activities of SOD, POX and CAT and enzymes of the ascorbate-glutathione cycle (APX, GR, MDHAR and DHAR). These enzymes play a crucial role in the elimination of H_2O_2 from plant cells.

In this context, Balen et al. (2009) showed higher oxidative stress in *Mammillaria gracilis* Pfeiffdue to an increase in MDA and ROS content compared to shoots without hyperhydricity. They also suggested that the increased activities of the antioxidant enzymes SOD, APX, CAT in shoots indicate activation of a defense mechanism against the increased production of ROS in these tissues. Therefore, oxidative stress could be responsible for the deficient morpho-physiology and biochemistry of HS in this species.

Tian et al. (2015) indicate that under stressful conditions in an in vitro environment, the increased production and decreased degradation of ROS lead to oxidative damage. The antioxidant capacity may be the crucial factor in the production of excessive ROS. Therefore, the remarkable change in antioxidant enzyme activity and antioxidant molecule content at the subcellular level may be a hallmark for the induction of hyperhydricity. In this work, the results confirm the aspects previously reported.

Isah (2019) share that plants have developed an antioxidant defense machinery against ROS that could cause membrane damage to cellular structures; it consists of antioxidant compounds and enzymes that include CAT and APX. If the enzymes did not detoxify the accumulation of ROS and H_2O_2 in the cells or if they were absent this could lead to hyperhydricity in the in vitro cultures. They reported that the activity of CAT and APX regulates the accumulation of ROS that result from cellular oxidative stress.

Ex vitro acclimatization

After 7 days of acclimatization, a decreased ex vitro survival rate of *B. vulgaris* hiperhidric plantlets was observed. The highest survival rate 94.23% was observed in the normal plantlets after transfer to the greenhouse (Fig. 4).

The lowest percentage of survival was obtained in hyperhydric plants, which coincided with the highest number of HS and the highest water content in the shoots. In addition, these shoots had the highest number of open and probably non-functioning stomata and the highest content of antioxidant. The highest percentage of surviving plants from TIS without symptoms of hyperhydricity could be due to the fact that they showed a better morpho-physiological response in vitro. This response was related to the highest values of total chlorophyll content and better development of the stomatal complex. On the other hand, these plants showed less oxidative stress. All this indicates that they were better prepared to prevent water loss by transpiration and to adapt to environmental changes in vitro and ex vitro; this confirms the description of Zobayed et al. (2000).

This could lead to better morpho-physiological, anatomical and biochemical development of plants. These characteristics could facilitate the adaptation response during acclimatization of *B. vulgaris* plants

in the culture house, as reported by Kozai (2010). In agreement with Hazarika (2006), the results obtained show that defining the morpho-anatomical and physiological characteristics of plants propagated in vitro allows early selection of those plants that can survive during acclimatization.

The results obtained in the present study show that the plants from TIS have a higher anatomical and biochemical response ex vitro compared to the SLM plants. In addition, Vidal and Sánchez (2019) confirmed the positive effect of TIS on morphological quality and ex vitro adaptation in different woody species. These authors indicated that these conditions reduced stress during ex vitro acclimatization and increased the growth of these plants. These results were consistent with those obtained in *B. vulgaris* plants in the present study. However, other authors described a different response to this variable for this species only when they used a different culture system. For example, Gajjar et al. (2017) achieved a lower survival percentage (80%) in *B. vulgaris* than in this study when they used a semisolid culture medium. Similarly, Desai et al. (2019) achieved a survival rate of 85%. dos Santos Ribeiro et al. (2020) determined an ex vitro survival rate of 88.88% in SLM in this species. However, in this work the percentage of survival (94.23%) increased by using TIS.

Conclusions

In conclusion, this study is the first review in the literature describing the anatomical and biochemical responses to oxidative stress in shoots of *Bambusa vulgaris* Schrad. ex Wendl during the transition in vitro to ex vitro. The results our experiment showed that the anatomy, biochemistry and physiology of shoots cultured in TIS were not affected. Moreover, an increased percentage of ex vitro survival and growth of the plantlets in the greenhouse was obtained. On the other hand, HS, which was cultured in static liquid culture, showed low morpho-physiological quality and higher oxidative stress due to increased H_2O_2 and MDA content, as well as low stomata development of leaves and lower ex vitro survival. Temporary immersion systems could be an effective tool to improve physiological and biochemical quality in in vitro mass propagation of bamboo.

Declarations

Funding: Funding for this research was provided by the Instituto de Biotecnogía de las Plantas and International project EDUNABIO—Educational Network in Agrobiodiversity.

Conflict of interest: The authors declare that they have no conficts of interest.

Ethics approval: All experiments performed in this study comply with the applicable laws of the country in which they were performed.

Data availability: All data generated or analysed in this study are included in this published article.

Author Contributions This statement confirms that all authors have seen and approved the submitted manuscript. The present idea was developed by YG-R with the assistance of GPB and STG. All authors

discussed the results and contributed to the final manuscript.

Acknowledgements This research was supported by the international project EDUNABIO—Educational Network in Agrobiodiversity. Our special thanks to EDUNABIO for financial support and grant for this research.

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Figures

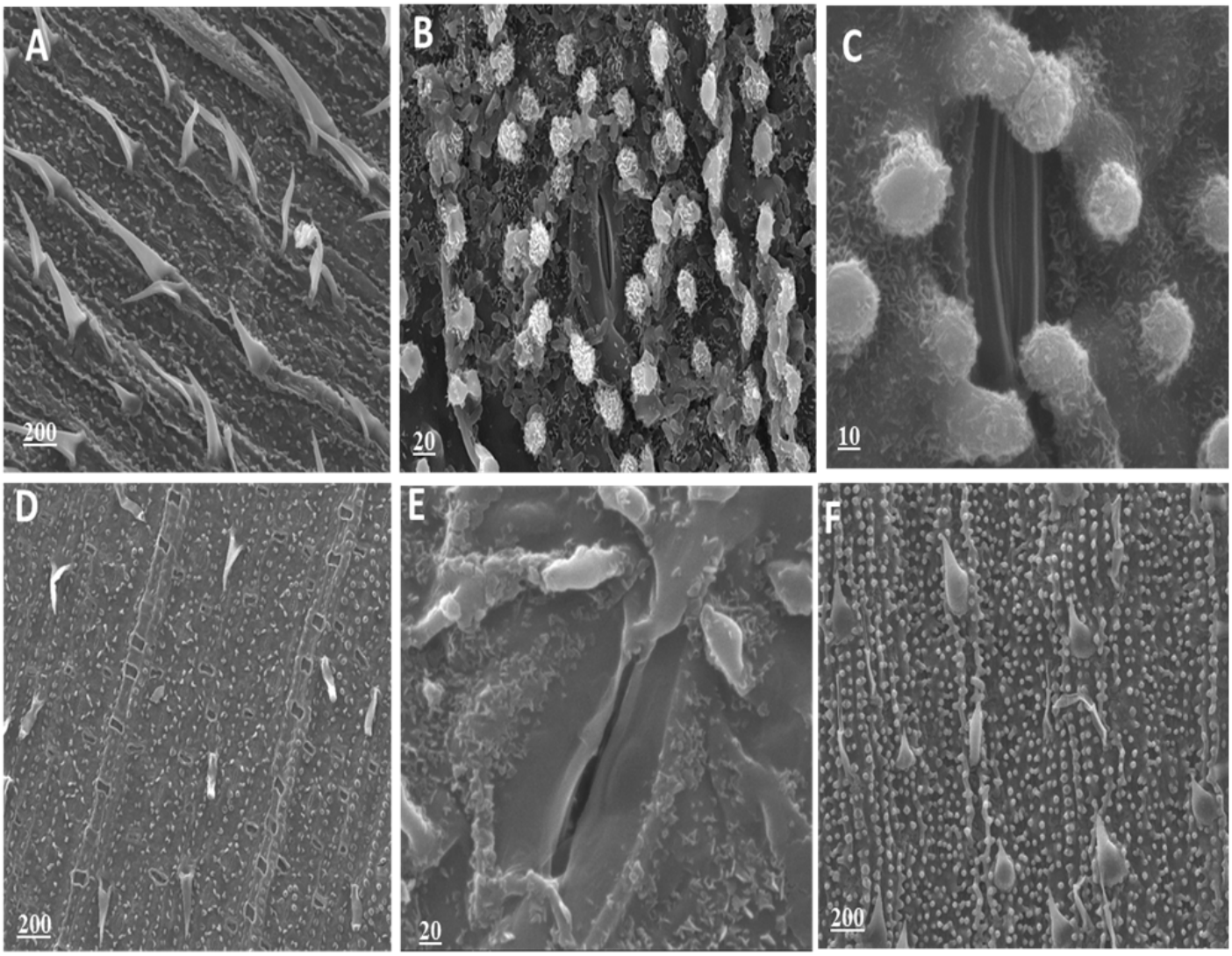

Figure 1

Stomatal surface of abaxial leaf of *Bambusa vulgaris* Schrad. Ex Wendl shoots developed under different culture systems. A, B Normal shoots. D, E Hyperhydric shoots. C, F Plants in acclimatization phase. (a,d, f bars = 200 µm) b, e, bars = 20 µm) (c bars = 10 µm).

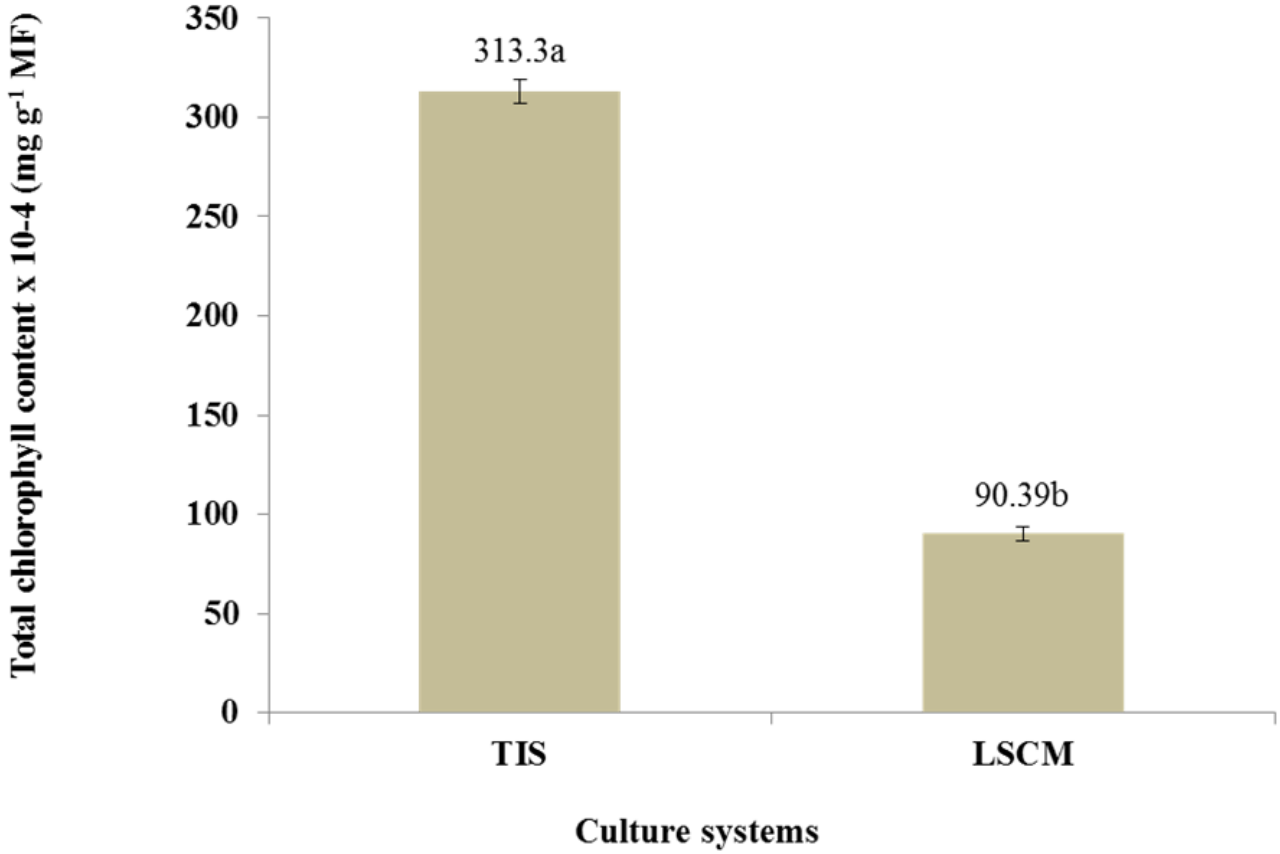

Figure 2

Chlorophyll content in shoots of *B. vulgaris* in two culture systems after 30 days of culture. Values represent the means ± standard errors (n = 80). Different letters indicate significant differences as determined by Tukey test at P = 0.05. Legend: TIS: Temporary Immersion System. LSCM: Liquid Static Culture Medium. (n=80)

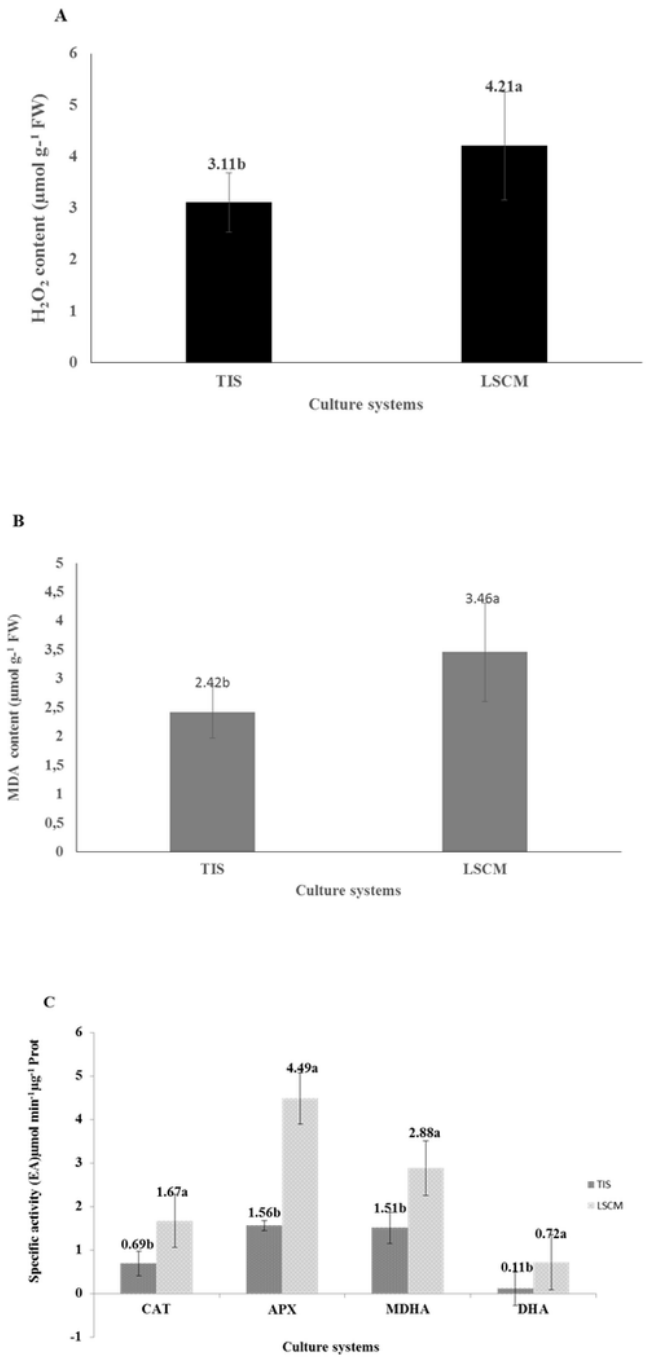

Figure 3

Specific activity (SA) of antioxidant enzymes in different culture systems (30 days). (a). $H_2O_{2:}$ Hydrogen peroxide content. (b). MDA: Malondialdehyde. C-Enzyme: CAT (Catalase); MDHAR (monodehydroascorbate reductase); DHAR (dehydroascorbate reductase); APX (Ascorbate peroxidase). Bars with mean values with different letters are statiscally different (Simple Anova, Tukey p≤0,05) (n=80). Legend: TIS: Temporary Immersion System. LSCM: Liquid Static Culture Medium. *(n=80)*

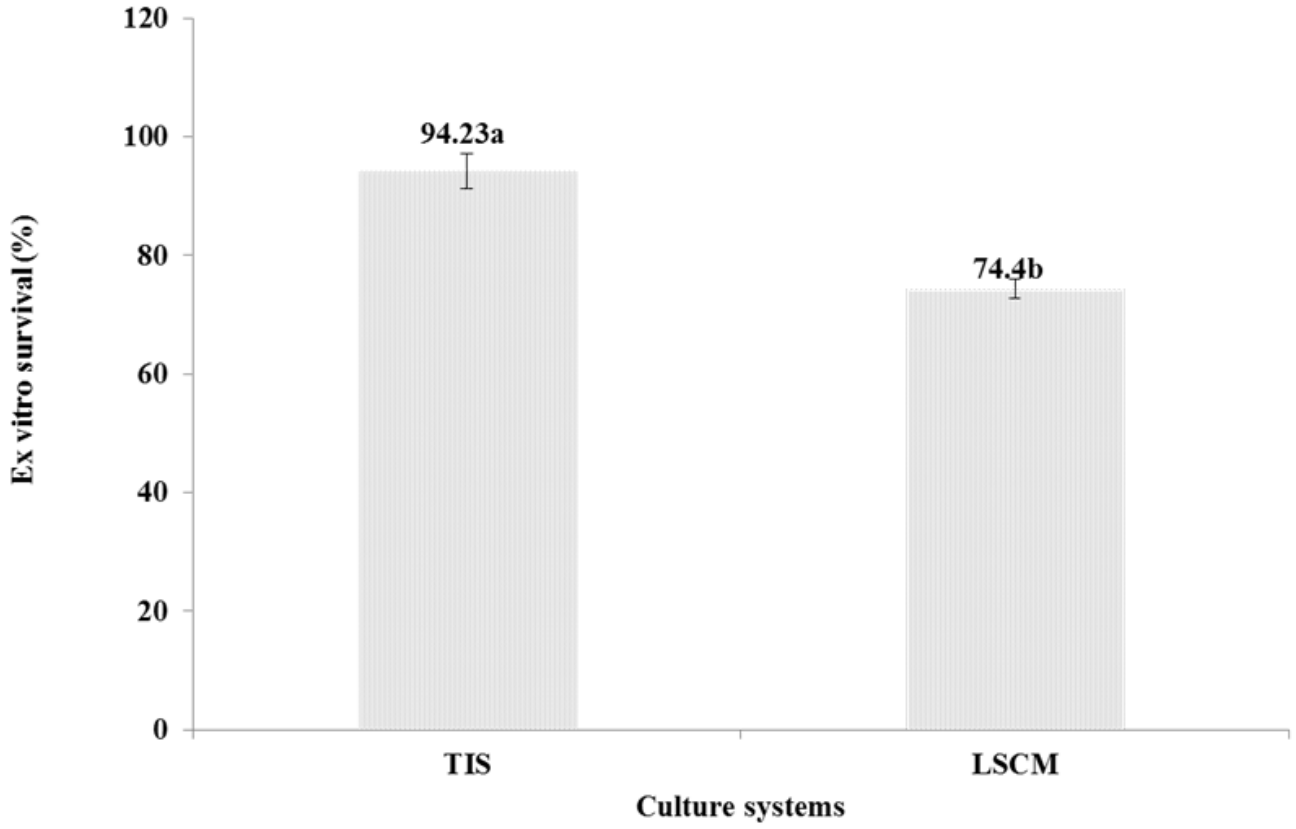

Figure 4

Percentage of survival of *Bambusa vulgaris* Schrad. Ex Wendl plantlets in the greenhouse. Different letters on bars indicate significant differences assessed at 5% using Tukey test. (n = 80). Legend: TIS: Temporary Immersion System. LSCM: Liquid Static Culture Medium. *(n=80)*