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ORIGINAL ARTICLE

Shoot multiplication kinetics and hyperhydric status of regenerated shoots of gladiolus in agar-solidified and matrix-supported liquid cultures

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Abstract In vitro shoot regeneration of gladiolus in three different culture systems, viz., semi-solid agar (AS), membrane raft (MR), and duroplast foam liquid (DF) cultures was evaluated following the kinetics of shoot multiplication and hyperhydricity at optimized growth regulator combinations. Compared to the AS system, matrix-supported liquid cultures enhanced shoot multiplication. The peak of shoot multiplication rate was attained at 18 days of incubation in the MR and DF systems, whereas the maximum rate in the AS system was attained at 21 days. An early decline in acceleration trend was observed in liquid cultures than the AS culture. The hyperhydric status of the regenerated shoots in the different culture systems was assessed in terms of stomatal attributes and antioxidative status. Stomatal behavior appeared to be normal in the AS and MR systems. However, structural anomaly of stomata such as large, round shaped guard cells with damage in bordering regions of stomatal pores was pronounced in the DF system along with a relatively higher K^+ ion concentration than in the AS and MR systems. Antioxidative status of regenerated shoots was comparable in the AS and MR systems, while a higher incidence of oxidative damages of lipid membrane as evidenced from malondialdehyde and ascorbate content was observed in the DF system. Higher oxidative stress in the DF system was also apparent by elevated activities of superoxide dismutase, ascorbate peroxidase, and catalase. Among the three culture systems, liquid culture with MR resulted in maximum shoot multiplication with little or no symptoms of hyperhydricity. Shoots in the DF system were more prone to hyperhydricity than those in the AS and MR systems. The use of matrix support such as membrane raft as an interface between liquid medium and propagating tissue could be an effective means for rapid and efficient mass propagation with little or no symptoms of hyperhydricity.

Keywords Poisson regression · Oxidative stress · Hyperhydricity · Matrix assisted liquid culture · Shoot regeneration

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Introduction

In vitro propagation of gladiolus in liquid media with an increased rate of shoot bud differentiation, somatic embryo induction, and corm development versus semi-solid agar media has been successfully demonstrated utilizing shake culture (Ziv 1989; Steinitz et al. 1991; Nhut et al. 2004) and bioreactor technology (Ziv 2005). Matrix-supported liquid culture has also been established (Prasad and Dutta Gupta 2006). The basic principle of matrix-supported liquid culture is to avoid the asphyxiation of the culture material which occurs as a result of immersion in the liquid medium, and to support plants in the gaseous phase while growing in liquid medium. The use of the support matrix combines the benefits of both solid and liquid media in a system bypassing the problems associated with liquid culture. In our earlier work, we compared the shoot regeneration of gladiolus in different culture systems following the routine practice of analysis of variance (ANOVA) assuming the data to possess normal distribution (Prasad and Dutta Gupta 2006). In order to evaluate the stochastic process of shoot multiplication, consideration of the distribution pattern of multiple shoots becomes

apparent. As the number of shoots increased with time, the variance also proportionally increased indicating the randomness and independence of the shoot multiplication event with time. This characteristic renders the number of shoots as a variate with Poisson distribution. A Poisson regression modeling relating the incubation time with the shoot data may provide a reliable interpretation of efficiency and productivity of the culture system.

In vitro culture conditions that promote rapid growth and multiplication of shoots often results in the formation of structurally and physiologically abnormal plants (Ziv et al. 1987). Particularly, explants cultured in liquid medium are more prone to unusual apoplastic accumulation of water resulting in physiological, anatomical, and gross morphological abnormalities than in agar-solidified medium (Ziv 1991; Ziv et al. 1987). Collectively, such abnormalities are known as hyperhydricity, and the regenerated plants had varying degrees of glassiness (Debergh et al. 1992). Commercial micropropagation requires reproducible production of large numbers of plants rapidly, and plantlets need to have high rates of ex vitro survival. Hyperhydric plantlets often do not survive acclimatization. Therefore, it is essential to assess the hyperhydric status of the regenerated plants and to adopt suitable culture conditions.

A wide range of micro-environmental culture conditions have been reported to cause hyperhydricity by modifying the morphology of tissue (Saher et al. 2005; Hazarika 2006). High relative humidity in the culture vessel has been considered to be the prime cause for hyperhydricity which is associated with reduced transpiration, hypoxia stress (Dewir et al. 2006), and in vitro recalcitrance, i.e. inability of cells and tissues to regenerate (Benson 2000; Kevers et al. 2004).

The ultra-structural properties of guard cells as well as the antioxidative status of the tissues are considered as vital to characterize the phenomenon of hyperhydricity (Werker and Leshem 1987; Han et al. 1992; Fontes et al. 1999; Kevers et al. 2004; Dewir et al. 2006). The change in structure and function of guard cells can alter the tissue water relations or balance (Ziv and Ariel 1994). The influx and efflux of K^+ ions in the guard cells may also affect the stomatal function (Schroeder 2003). Antioxidative enzyme activities, particularly that of superoxide dismutase (SOD), which catalyses the reduction of superoxide radical into H_2O_2 and then subsequently to water, has been largely correlated to hyperhydricity as a measure of oxidative stress (Chen and Ziv 2004; Chakrabarty et al. 2006; Dewir et al. 2006). The physiological status of the hyperhydric and non-hyperhydric leaves grown in vitro from a single culture system has been compared (Piqueras et al. 2002; Saher et al. 2005; Hazarika 2006). However, variation in hyperhydric status of the regenerated plants among the different culture systems has not yet been documented.

In our previous work, matrix-supported liquid culture was found to be more effective with superior organogenic shoot development compared to semi-solid agar medium (Prasad and Dutta Gupta 2006). However, there exists a difference in optimum shoot regeneration response with culture types and growth regulators. Apart from establishing the culture conditions for optimum shoot regeneration, it is essential to know the extent of hyperhydricity of the regenerated shoots. It has been suggested that a slower rate of shoot multiplication with limited occurrence of hyperhydricity are associated with agar cultures, whereas liquid cultures render a higher rate of shoot multiplication with a greater incidence of hyperhydricity. To test this hypothesis and to optimize the culture system that yields maximum shoot multiplication with little or no symptoms of hyperhydricity,we describe here the Poisson regression modeling of shoot multiplication kinetics of gladiolus in semi-solid and matrix-supported liquid media, and illustrate the hyperhydric status of the regenerated shoots among the different culture systems at the optimized level of growth regulators.

Materials and methods

Plant material and culture establishment

The primary leaves of the sprouted corms of *Gladiolus hybridus* Hort. cv. Wedding Bouquet were surface disinfected with 0.1% $HgCl_2$ followed by three to four rinses in sterile double distilled water. The basal portions of the innermost leaves were dissected, blotted dry, and inoculated on Murashige and Skoog (MS; Murashige and Skoog 1962) medium containing 3% (w/v) sucrose and 10.7 μM α-napthaleneacetic acid (NAA). The leaf-derived callus was then transferred onto MS medium supplemented with 1.07 μM NAA and 8.8 μM 6-benzylaminopurine (BA) for the induction of meristematic bud clusters as described previously by Dutta Gupta and Datta (2004). The regenerated bud clusters were used to study the shoot multiplication in semi-solid agar (AS), polypropylene membrane raft (MR), and duroplast foam (DF) culture systems. Shoot multiplication refers to the differentiation of new buds as well as the proliferation of buds into shoots, and is measured by the number of shoots produced. The description of the establishment of culture systems with various concentrations of NAA and BA were detailed in Prasad and Dutta Gupta (2006). The optimized conditions for shoot regeneration obtained from the previous study were used to study the shoot dynamics and hyperhydric status of the regenerated shoots. For the AS culture, 0.8% agar (Hi-Media, Mumbai)

was used for the MS medium containing 1.07 μM NAA and 8.8 μM BA. For liquid cultures, pre-sterilized MR (Osmotek^{TM}; Rehovat, Israel) with 0.3 μm pore size and DF of 0.3 cm thickness (polyurethane foam; Sheela Foam, Ghaziabad, India) were used as an interface between tissue and liquid media. The liquid media consisted of MS basal salts supplemented with 5.3 μM NAA and 8.8 μM BA for MR, and 2.6 μM NAA and 17.6 μM BA for DF. Five meristematic bud clusters each comprising three to five shoot buds were inoculated per Magenta GA-7 (Sigma, India) culture vessel, and incubated up to a period of 35 days. There were three vessels per system and the experiment was repeated twice. The pH of the media was adjusted to 5.6 prior to autoclaving. The cultures were incubated at 25 ± 1°C for 35 days under a 16-h photoperiod provided by cool-white fluorescent lamps (60 μmol m^{-1} s^{-2}).

were excised during the photoperiod, gently washed, and blotted dry. A thin layer of clear nail varnish was coated on the surfaces of each leaf blade and allowed to dry for 10 min. A strip of transparent cellophane tape was placed over the dried varnish, and pressure was applied to obtain an imprint. The cellophane tape with its varnish imprint was peeled off and placed onto a clean glass microscope slide. Replicas were examined under a Carl-Zeiss Jena light microscope and then stomatal density was determined by counting the number of stomata and epidermal cells per microscopic field. Stomatal imprints were photographed using an Olympus camera C7070 fitted to microscope model No. CKX41. Care was taken so that fields of view were not overlapped. Percentage of stomatal index (SI%) was calculated using the following equation (Salisbury 1927):

$$\mathrm{SI}\% = \left[\frac{\text{No. stomata per field view}}{(\text{No. of stomata per field view} + \text{No. of epidermal cells per field view})}\right] \times 100$$

Shoot regeneration kinetics of different culture systems by Poisson regression modeling

Shoot counts were recorded at 7-day intervals by placing meristematic bud clusters onto a sterile Petri dish, after which the cultures were transferred back to the same culture system until 35 days. The data were subjected to Poisson regression analysis utilizing NCSS Software (Utah), as described by Mendes et al. (1999). Rate of shoot multiplication and acceleration were calculated from the fitted regression models. Rate is defined as the number of shoots that differentiated per unit time, whereas acceleration is defined as change in the rate of shoot multiplication per unit time.

Evaluation of hyperhydric status in different culture systems

The in vitro regenerated shoots were collected from the different culture systems at the end of 35 days and used to determine the hyperhydric status as follows.

Determination of stomatal densities of regenerated leaves

Stomatal imprints were obtained from 35-day-old leaves from the three culture systems following a modified method of Sampson (1961). The fully expanded leaves

Scanning electron microscopy

Regenerated leaves were collected from the AS, MR, and DF culture systems after 35 days of incubation and freeze-dried for 12 h at −40°C. Samples were positioned on stub prior to gold sputtering (Olmos and Hellin 1998). Photomicrographs were taken under a JEOL JSM-600 scanning electron microscope (SEM) operating at 20 keV.

Electron probe elemental microanalysis of guard cells

Regenerated leaf segments from the three culture systems were freeze-dried as mentioned previously. Elemental analysis of the guard cells was performed using an energy-dispersive X-ray microanalysis system (LINK ISIS; Oxford Instruments) fitted to a JEOL JSM-600 scanning electron microscope. The acceleration voltage was set at 10 keV. A standard quantitative analysis program was used for the quantitative elemental estimation in the guard cells. At least three energy dispersive spectra (EDS) were obtained from leaves representing each culture system.

Assessment of antioxidative status of the regenerated shoots in different culture systems

The shoots regenerated in the AS, MR, and DF culture systems with respective optimal media compositions at

35 days were excised for estimation of oxidation stress and assay of the antioxidative enzyme activities as follows.

Estimation of malondialdehyde and ascorbate content

Malondialdehyde (MDA), the end product of lipid peroxidation, was measured using the thiobarbituric acid (TBA) test (Hodges et al. 1999). Shoot tissues (1 g) were homogenized in 1 ml of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was cleared at 10,000*g* for 20 min, and 0.5 ml of the supernatant was added to 1 ml 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min and the reaction terminated in an ice bath. The samples were centrifuged at 10,000*g* for 5 min, and the absorbance of supernatant was read at 532 nm. The experimental values were corrected for non-specific absorption at 600 nm. The MDA-TBA complex (red pigment) was quantified using the extinction coefficient of 155 mM^{-1} cm^{-1}.

For determination of ascorbic acid content (ASA), shoot tissues were homogenized in a mortar with a solution of 5% metaphosphoric acid (1:2; w/v) and centrifuged at 14,000*g* for 20 min. The oxidation of ASA was prevented by adding 10 mM sodium azide. The quantitative determination was carried out according to Law et al. (1983). To quantify ascorbate, 150 mM phosphate buffer (pH 7.4) was added to the supernatant. Then, 10% TCA (w/v), 44% H_3PO_4 solution (v/v), 4% α–α′dipyridyl (w/v) in 70% methanol, and 3% $FeCl_3$ (w/v) were added in that order. After vigorous stirring, the samples were kept at 37°C for 60 min and then the absorbance was read at 525 nm. The concentration of reduced ascorbic acid was then estimated from the standard curve.

Superoxide dismutase, ascorbate peroxidase, guiacol peroxidase and catalase activity assay

Shoot tissues (0.5 g) were frozen in liquid nitrogen along with polyvinylpyrrolidone (PVP), ground to a fine powder, and homogenized with 1.0 ml 100 mM Tris–HCl buffer (pH 7.0) containing 1 mM EDTA. The extraction buffer for ascorbate peroxidase (APX) activity assay contained 1 mM ASA in addition to the above mentioned components. The homogenate was centrifuged at 15,000*g* for 20 min at 4°C and the supernatant kept at −20°C until further use.

Superoxide dismutase (EC 1.15.1.1) activity was determined by measuring the inhibition rate of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971). One unit of SOD activity is equivalent to the amount of enzyme required to inhibit the reduction of extract free reaction mixture by 50%. The activity of APX (EC 1.11.1.11) was determined according to Heath and Packer (1968), measuring the decrease in absorbance at 290 nm due to ascorbate oxidation ($E = 2.8$ mM^{-1} cm^{-1}). A unit of APX was defined as the amount necessary to oxidize 1 µmol of ascorbate per min at 25°C. Guiacol peroxidase (GPX) (EC 1.11.1.7) activity was measured by following the increase in absorbance at 420 nm as a result of oxidation of guaiacol ($E = 26.6$ mM^{-1} cm^{-1}) (Gasper et al. 1975). Activity of catalase (CAT) (EC 1.11.1.6) was estimated by monitoring the decrease in absorbance because of H_2O_2 reduction ($E = 0.0435$ mM^{-1} cm^{-1}) at 240 nm (Beers and Sizer 1952). One enzyme unit was defined as 1 µmol H_2O_2 reduced per minute at 25 ± 2°C. Soluble protein content of the crude enzyme extracts were estimated by using bovine serum albumin fraction V as standard (Lowry et al. 1951).

Electrophoretic profiles of SOD in the regenerated shoots obtained from the AS, MR, and DF cultures

Superoxide dismutase enzyme profile was characterized by native page following the procedure of Laemmli (1970) in standard Tris–Glycine buffer (pH 8.3). Defined amount of protein were loaded in each well and then electrophoresed at 80 V through the stacking gel (5%) and 180 V through the separating gel (12%) using a vertical slab gel electrophoresis system (Bangalore-Genei 05; Bangalore Genei, Bangalore, Karnataka, India). Electrophoresis was carried out at 4°C. After electrophoresis, a photochemical method of Beauchamp and Fridovich (1971) was followed to locate SOD activities on gel. The gel was first soaked in 25 ml of 1.23 mM NBT for 15 min, briefly washed, and then soaked in the dark in 30 ml 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine) and 2.8×10^{-2} mM riboflavin for another 15 min. The gel was briefly washed again, and then illuminated under two 40-W fluorescent lamps for 15 min to initiate the photochemical reaction. SOD activity was visualized as colorless bands as a result of inhibition of NBT that would otherwise form blue colored formazan.

Results and discussion

Shoot regeneration kinetics in different culture systems by Poisson regression modeling

The number of shoots produced in each culture system at different incubation periods, and the adjusted Poisson regression models are presented in Table 1. The variances

Table 1 Number of shoots produced at different culture period and estimated Poisson regression models with coefficient of determination (R^2)

Culture system	Incubation period (days)[a]					Model	R^2
	0	7	14	21	28	35	
AS	2.43 ± 0.965	3.57 ± 1.235	7.35 ± 3.259	18.58 ± 3.973	21.47 ± 4.106	24.79 ± 4.153	$Y = \exp[0.6419 + 1.2635X - 0.1032XX]$	0.967
MR	2.68 ± 0.863	5.87 ± 1.425	16.36 ± 3.948	23.43 ± 4.157	28.88 ± 5.269	32.00 ± 5.632	$Y = \exp[0.2417 + 1.3253X - 0.1168XX]$	0.993
DF	2.73 ± 1.463	5.56 ± 2.982	14.29 ± 2.996	19.54 ± 4.873	24.9 ± 5.442	28.5 ± 5.921	$Y = \exp[0.0658 + 1.09799X - 0.0922XX]$	0.984

[a] Values represent mean number of shoots ± SD

of the number of shoots over a period of 35 days increased proportionally with incubation time in all the culture systems (data not shown). Hence, the shoot multiplication characteristically followed a Poisson distribution rather than a normal distribution. A close fit between the observed and model-expected mean number of shoots was observed.

The first derivative of the fitted Poisson regression models suggests a higher rate of shoot multiplication in the MR culture than in the AS and DF cultures. In the MR- and DF-supported liquid culture, the maximum shoot multiplication was observed at 18 days of incubation, whereas in the AS system, maximum multiplication was attained at 21 days of incubation (Fig. 1). Enhanced nutrient uptake might have promoted such an early response in liquid cultures. An inverse correlation between plant biomass, and residual concentrations of sugar, ammonium, nitrate, potassium, and calcium, was observed in watermelon shoot cultures on polypropylene membrane rafts (Desamero et al. 1993; Adelberg 2006). The time frame when multiplication rate decreases is shown in Fig 1. The transition from a positive to a negative acceleration (*y*-axis) represents the time of decline in shoot multiplication rate. In matrix-supported liquid cultures, a decline in multiplication rate was observed during 17–18 days of culture, while the AS system sustained the multiplication rate to 21 days. It appears that the shoots in the MR and DF systems exhibited deceleration in shoot multiplication earlier than the AS system, although the actual number of shoots continued to increase with time. The cause of such a decline may be a result of early and rapid utilization of most of the available nutrients in the liquid medium. The shoot development pattern in the DF culture was different from the AS and MR systems. Shoots in the DF system grew at a faster rate than those in the AS and MR systems. Presumably, high concentration of BA in the DF system prevents axillary bud breaks resulting in decreased bud multiplication with increased shoot proliferation (Prasad and Dutta Gupta 2006).

Poisson regression modeling of shoot count data suggests the efficacy of liquid culture systems with rapid and increased shoot multiplication when compared to AS. Among the liquid culture systems, maximum efficiency was noted with the MR system. The kinetics of shoot regeneration through Poisson regression modeling has also been well illustrated in optimizing banana micropropagation (Mendes et al. 1999).

Hyperhydric status in different culture systems

Hyperhydric status of the regenerated shoots was assessed through various morphological and physiological means.

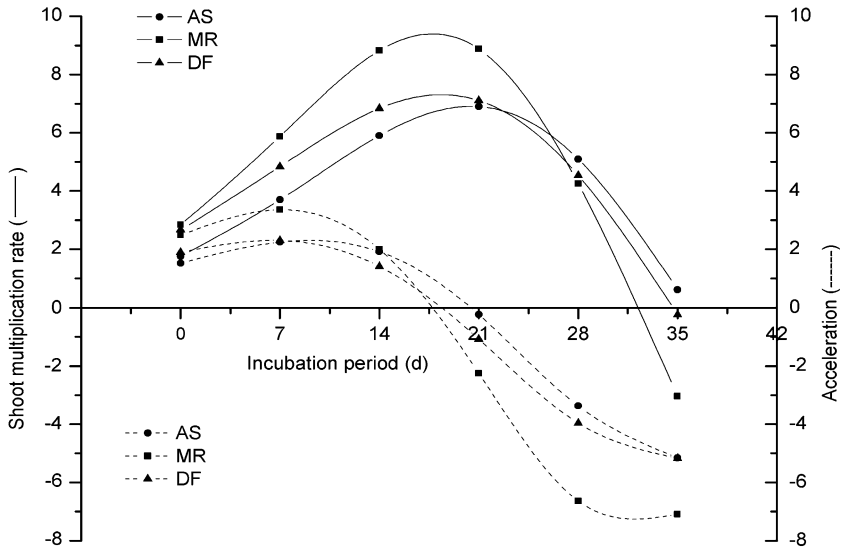

Fig. 1 Graphical display of the adjusted Poisson regression models expressing the rate (*solid line*) and acceleration (*dashed line*) of shoot multiplication of gladiolus in different culture systems

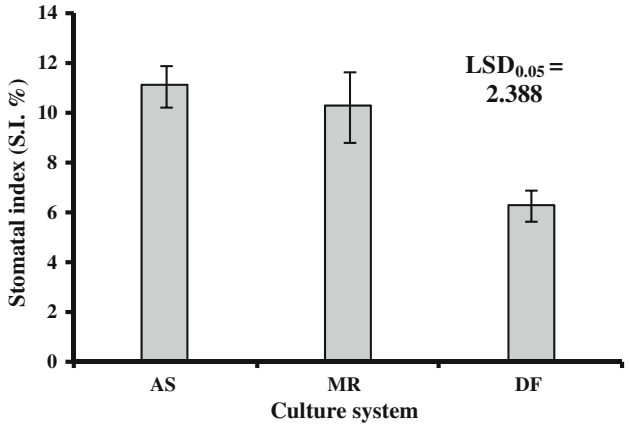

Fig. 2 Stomatal imprints and distribution in different culture systems. *Bars* represent standard error of 15 observations from three representative regenerated leaves of each culture system

Stomatal properties in different culture systems

The percentage of stomatal indices (SI%) of the regenerated leaves derived from the AS, MR, and DF culture systems were 11.06, 10.22, and 6.24, respectively (Fig. 2). The stomatal index of regenerated leaves in the DF culture system was significantly lower than the AS and MR culture systems. No significant difference in stomatal distribution was observed between the AS and MR cultures.

Stomatal distribution among the various culture types indicated that the shoots in AS and MR are less prone to hyperhydricity than those in DF. A positive correlation between normal leaf development and higher rate of stomatal occurrence has been suggested by Olmos and Hellin (1998). Based on SEM studies, Picoli et al. (2001) also reported that the stomatal differentiation was affected in hyperhydric plants. However, there exist differences in the stomatal density even from one hyperhydric plantlet to another (Werker and Leshem 1987). Therefore, estimation of hyperhydric status based solely upon stomatal density would be inappropriate, and further ultrastructural and physiological observations need to be considered

Ultrastructure of stomata in different culture systems

Stomata in the AS and MR systems have normal kidney-shaped guard cells (Fig. 3a, b), while round-shaped guard cells were observed in the DF culture (Fig. 3c). The guard cells of leaves from the DF culture were larger in size than those of the AS and MR cultures, and appeared to be torn in bordering regions of the stomatal pore. The stomatal pore surrounded by guard cells in the DF cultures was more open and rounded in contrast to the elliptical pore in the AS and MR cultures. The deposition of epicuticular wax in DF was found to be sparse than that seen in AS and MR. The findings indicate a hyperhydric nature of regenerated shoots in the DF culture. Stomatal features in the AS and MR systems appeared to be normal without any symptoms of hyperhydricity. Similar observations were recorded in carnation, where the pore surrounded by the guard cells in hyperhydric leaves was more rounded in contrast to the elliptical pore in normal leaves (Ziv et al. 1987). Anomalies in the size and shape of one or both guard cells and the opening of the stomata as seen in DF have also been observed in hyperhydric leaves of a variety of species (Werker and Leshem 1987; Olmos and Hellin 1998; Ticha et al. 1999; Picoli et al. 2001).

X-ray microanalysis of guard cells

X-ray microanalysis revealed the relative distribution of K^+, Ca^{++}, and Mg^{++} ions in the guard cells of regenerated leaves derived from the AS and liquid culture systems

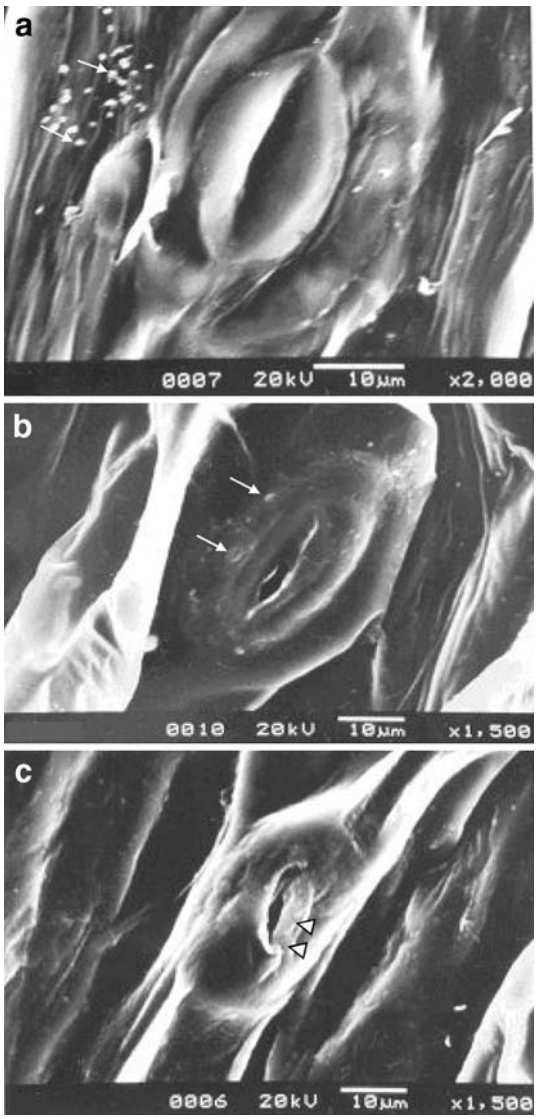

Fig. 3 Scanning electron micrographs of stomatal apparatus in **a** AS, **b** MR, and **c** DF culture systems. Deposition of cuticular wax in the AS and MR systems is marked with *arrows*. Damaged bordering regions of stomatal pore in DF system is indicated by the *arrowheads*

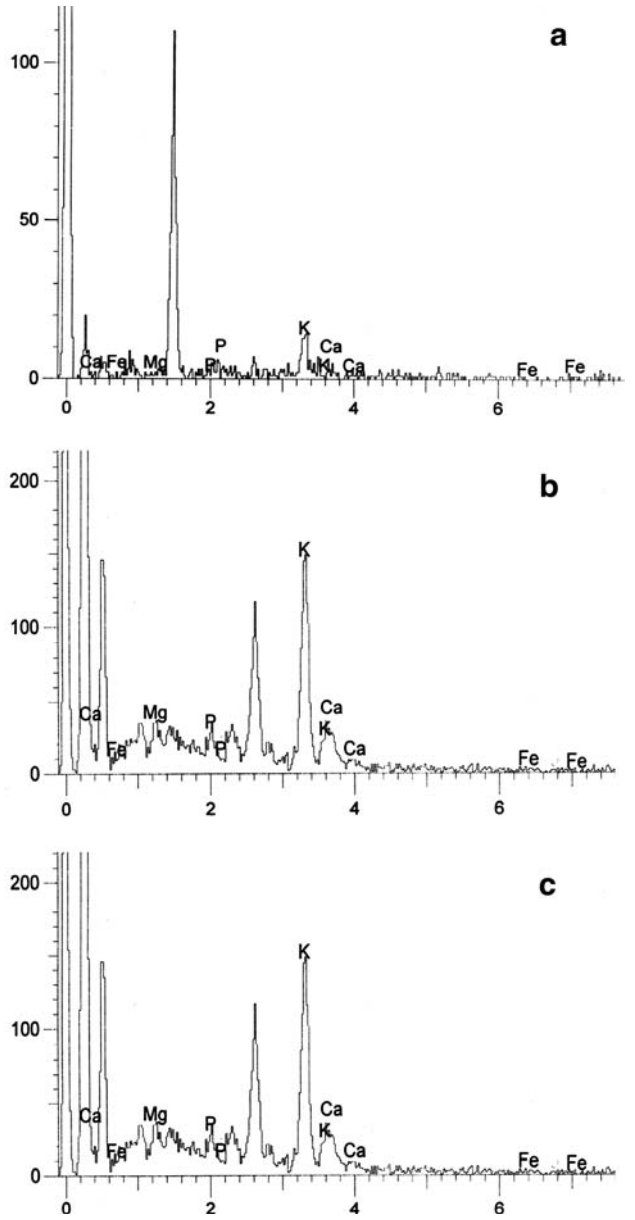

Fig. 4 EDS spectra showing relative distribution of K^+, Ca^{++}, and Mg^{++} in guard cells of regenerated leaves derived from **a** AS, **b** MR and **c** DF culture systems. *X* and *Y* axes represent energy (keV) and counts per second (cps), respectively

(Fig. 4). The stomata in the liquid culture system with DF support exhibited a predominant accumulation of K^+ ions than that in AS and MR. The distribution of K^+ was found to be 13.3, 156.2, and 318.6 cps, respectively, for the DF, MR, and AS systems. Among the three culture systems, a higher amount of Ca^{++} was noted in DF with 55 cps, whereas in the MR and AS systems, the Ca^{++} accumulation was found to be 37.3 and 6.7 cps, respectively. Mg^{++} was higher in liquid culture systems with 38.6 and 36.5 cps in MR and DF, respectively, than in AS with 5.8 cps. The ratio of K^+ to Ca^{++} in the AS, MR, and DF culture systems was 1.9, 3.8, and 5.7, respectively. Proportionate relative content of K^+ and Ca^{++} indicates more inward influx of K^+ in guard cells that helps in stomatal opening. Accumulation of higher K^+ was observed in guard cells of hyperhydric leaves of *Dianthus caryophyllus* than that in normal leaves (Olmos and Hellin 1998). With respect to relative distribution of ions, the MR system is somewhat intermediate to AS and DF.

The regulatory role of K^+ uptake by the guard cells in stomatal opening has been well documented, whereas the role of intracellular Mg^{++} was found to be insignificant in stomatal opening (Schroeder 2003; Pandey et al. 2007). The wide stomatal opening observed in the regenerated leaves of DF may be a consequence of such high level of

K^+ accumulation as well as greater water absorption in the guard cells leading to turgidity and changes in cell wall structures.

Antioxidative status of the regenerated shoots in different culture systems

Malondialdehyde and ascorbate content

The MDA content of the regenerated shoots in the three culture systems ranged from 2.04 to 4.09 μM per gm fresh weight. The MDA content was found to be highest in shoot cultures of the DF system and lowest in the AS system, while the MR system contained an intermediary amount, suggesting distinct oxidative stress levels in the three systems in the following order: AS < MR < DF. A marked increase in the MDA content was also noted in hyperhydric shoots of carnation (Saher et al. 2004) and *Euphorbia millii* (Dewir et al. 2006). MDA, one of the toxic lipid peroxidation products of polyunsaturated fatty acids hydroperoxides, was generally accepted as a suitable biomarker or indicator of membrane damage under oxidative stress conditions (Bailly et al. 1996). This hypothesis was also confirmed by the higher lipoxygenase (LOX) activity in hyperhydric shoots (Dewir et al. 2006). It appears that regenerated shoots in the DF system suffered greater amounts of oxidative damage than those in the AS and MR systems. However, Franck et al. (2004) argued for the probable stress response of the hyperhydric shoots since there were no signs of oxidative damage of lipid membrane.

Ascorbic acid, a major antioxidant compound, acts as a substrate for APX. APX reduces H_2O_2 to water while oxidizing ascorbate to monodehydroascorbate. An elevated level of ASA was noted in the regenerated shoots of the AS system with 3.2 μM/gm FW, whereas those in the MR and DF systems differentially anabolized 3.09 and 2.01 μM ASA/gm FW, respectively. A lower concentration of ASA in the regenerated shoots of the DF system compared to the AS and MR systems may be due to the higher activity of APX. Increased ASA indicated a mechanism of enhanced antioxidant defence (Dewir et al. 2006).

SOD, APX, GPX, and CAT activities

Enzyme activities of APX, CAT, and SOD were found to be significantly higher in shoots regenerated in the DF culture than those in the AS and MR culture systems, except for GPX which showed a reverse trend (Table 2). The activities of APX, GPX, CAT, and SOD enzymes were comparable among the AS and MR shoot cultures suggesting similar, but reduced, stress level than that in the DF system. Stress enforced increments in the reactive oxygen species (ROS) concentration activate the genes encoding the antioxidative enzymes as a defence mechanism (Gressel and Galun 1994; Arora et al. 2002). However, the antioxidative defence system of plants was quite limited in its capacity to respond to stress, as the activities of component enzymes usually increase in response to many stress situations (Arora et al. 2002). Therefore, it appears that SOD, APX, and CAT activities increased in the DF system to overcome increased stress levels than that induced in the AS and MR cultures. High levels of SOD activities were observed in hyperhydric shoots of *Prunus avium* L. (Franck et al. 1998, 2004) and *Nicotiana tabacum* (Piqueras et al. 1998). High SOD activity in shoots of the DF system suggests a stronger defence mechanism against the increased production of superoxide radicals while resulting in increased accumulation of H_2O_2. Similarly, higher activities of the antioxidative enzymes except lipoxygenase were observed in the hyperhydric shoots (Saher et al. 2004). Dewir et al. (2006) also reported that SOD, peroxidase (POD), and CAT activities were significantly higher in hyperhydric tissue as compared to non-hyperhydric normal leaf tissue. The spectrophotometric activity of APX was also found to be high in hyperhydric shoots (Dewir et al. 2006). Lin et al. (2004) also reported high SOD activity and MDA levels in vitrified shoots; however, the POD, CAT, APX, and ASA activities were low.

Table 2 Specific activities of various antioxidative enzymes in different culture systems

Antioxidative enzymes	Activity (U/mg protein)		
	AS	MR	DF
APX	7.01 ± 0.94 a	8.77 ± 1.20 a	14.44 ± 0.67 b
GPX	1.09 ± 0.09 b	0.88 ± 0.06 b	0.42 ± 0.04 a
CAT	9.14 ± 0.77 a	10.78 ± 0.8 a	14.09 ± 0.74 b
SOD	36.16 ± 2.47 a	37.58 ± 1.98 a	61.22 ± 3.46 b

Different letters in the same row represent significant differences between columns

Higher oxidative damage in the DF cultures can be ascribed to hydroxyl, peroxy, and alkoxy radical mediated membrane degradation. Further, a rise in SOD activity coupled with antioxidant activities of CAT and APX was found to be insufficient to ameliorate the oxidative damage in the DF system (Table 2). This can be due to the potential of peroxidases for not only catalyzing the hydrogen peroxide detoxification, but also to catalyze the production of extremely reactive and toxic hydroxyl radicals from hydrogen peroxide in the presence of superoxide radicals (Chen and Schopfer 1999; Piqueras et al. 2002).

APX and CAT activities were correlated with hyperhydricity and meristematic cluster formation in liquid cultured *Narcissus* (Chen and Ziv 2001, 2004). High MDA

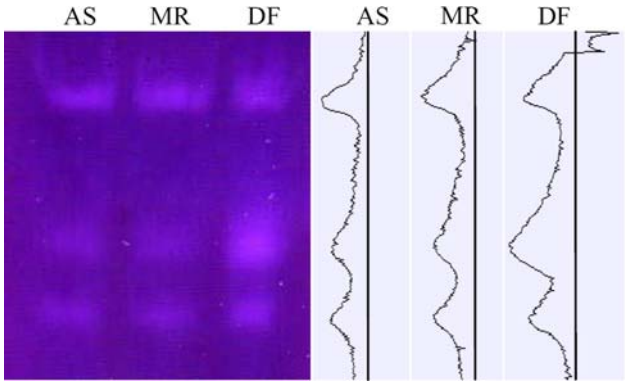

Fig. 5 Activity profiles of SOD in three different culture systems with corresponding densitograms

content in the DF cultures and increased peroxidase activities suggest such a pathway of $OH^{\cdot}$ radical production. GPX was involved in hardening the cell wall during development and growth. In the DF culture, the GPX activity was found to be substantially low indicating a possibility of impaired cell wall development. The DF cultures showed comparatively lesser regeneration capacity than the MR cultures. The oxidative stress was found to be significantly higher among the regenerated shoots of the DF culture system than the MR system. Oxidative stress-enforced recalcitrance has been well documented in in vitro cultures (Benson 2000; Papadakis and Roubelakis-Angelakis 2002). Higher oxidative stress level and low multiple shoot regeneration in the DF culture system when compared to the AS and MR systems implies a tendency towards hyperhydration.

Electrophoretic profiles of SOD in regenerated shoots

Electropherograms of SOD in regenerated shoots revealed three active isozymes of SOD in all the three culture systems. When compared to AS, the isozyme of band 1 in MR and the isozymes of band 1 and 2 in DF were distinctly prominent. Highest activity was noticed with isozyme in band 2 of DF (Fig. 5).

The activity profile intensities suggest that large amounts of superoxide anions were present in the DF system which were mostly scavenged by the isozyme visualized in band 2 of DF. In the case of MR and AS, radicals were largely scavenged by the isozyme in band 1. Apparently, there was no difference in the activity level of isozymes in both the MR and AS systems. Native PAGE analysis by Dewir et al. (2006) revealed seven SOD isozymes, whereas three SOD isozymes were identified by Chakrabarty et al. (2006) with similar increase in activity in hyperhydric shoots.

The present study detailed the kinetics of shoot multiplication revealing the rate, the maximum peak of shoot production, and the time where the trend in multiplication rate changes among the culture systems at the optimized level of growth regulators. Differences in shoot regeneration response with NAA and BA were found to be associated with culture types (Prasad and Dutta Gupta 2006). Thus, differential behavior of the culture systems could also be a result of the hormonal differences. The high concentration of BA used for shoot multiplication in the DF culture may also affect the hyperhydric status of the regenerated plants. However, the present study established the optimum condition for shoot multiplication including the hormonal requirements and culture types. Rapid and increased shoot multiplication was evident in the liquid culture systems compared to the AS culture. The hyperhydric status of the regenerated shoots in MR and AS was comparable without any predominant symptoms of hyperhydricity. In contrast to MR, the DF culture was prone to hyperhydricity. Liquid culture with MR support was found to be the most effective in inducing multiple shoots with little or no symptoms of hyperhydricity. The use of matrix support such as MR as an interface between liquid medium and propagating tissue could be a viable alternative technique for rapid and efficient means of mass propagation with few or no symptoms of hyperhydricity.

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