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Hyperhydricity-Related Morphologic and Biochemical Changes in Vanilla (*Vanilla planifolia*)

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Abstract Shoot cultures of vanilla (*Vanilla planifolia*) showed a progressive change toward hyperhydricity syndrome (HHS) leading to the necrosis of shoot buds when transferred to liquid medium of shake-flask type from solid (gelled) medium (S). HHS was also associated with severe damage at cellular and subcellular levels, an increase in free polyamines (PAs) and accumulation of water, a decrease in quantities of chlorophyll and protein, and drastic changes in reducing and nonreducing sugars. Spermine was by far the major polyamine in all the analyzed cultures. The progression toward and onset of HHS showed higher activities of antioxidant enzymes, indicative of the shoots' defensive efforts against oxidative stress. The specific enzyme activities of normal and H2 stages were 342.6 and 350.35 U mg^{-1} protein for peroxidase (POD, EC 1.11.1.11), 38.4 and 30.38 U mg^{-1} protein for superoxide dismutase (SOD, EC 1.15.1.1), and 71.3 and 82.75 U mg^{-1} protein for catalase (CAT, EC 1.11.1.6), respectively. The kinetic parameters of the culture medium suggested that nutrient utilization was normal in HHS and that the severe biochemical alterations and cellular damage were mainly due to oxidative stress.

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Keywords *Vanilla planifolia* · Complete immersion system · Hyperhydricity syndrome · Morphologic changes · Biochemical changes

Introduction

Switching over to submerged cultivation is the first step toward automation of shoot cultures in a micropropagation industry. Initial trials for this are normally conducted using shake-flask cultures, often known as a complete immersion system (CIS) because the shoots are continuously bathed in liquid medium. During cultivation in vitro, the plantlets are exposed to a wide range of stress conditions caused by high relative humidity, gas accumulation in the headspace, altered nutrient/hormonal combinations, and noncongenial osmotic levels of the culture medium. Although most plant cultures adapt to changes in environmental conditions, some become abnormal with a turgid, translucent, less green, watery, hypolignified, wrinkled, and brittle appearance. This phenomenon, known as hyperhydricity syndrome (HHS), can lead to irreversible loss of multiplication and regenerative potential. HHS has also been a generic problem in continuous cultivation or scale-up of plant organs in vitro. Such a shift toward hyperhydricity has been linked to various metabolic disorders, changed array of proteins, and altered stress responsive pathways.

Under stressful conditions cells undergo a surge of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The generation of ROS has been associated with oxidases in plasma membrane and the electron transport of chloroplast and mitochondrion (Laloi and others 2004; Ye and others 2006). It has also been known to affect photosynthetic pigments, membranes, and cell ultrastructure (Xu and

others 2008). A higher concentration of intracellular ROS has been shown to create cytotoxic conditions, including oxidative damage to lipids, proteins, and nucleic acids and disruption of cellular functions, finally leading to cell death.

Plants produce several antioxidant enzymes that function as scavengers of ROS. In addition, nonenzymatic antioxidants, including polyamines, are involved in the prevention and alleviation of ROS effects (Tasxgin and others 2006). Among the enzymes, peroxidases (POD), ubiquitously found in higher plants with various isoforms, function as rapid detoxifiers of stress-induced H_2O_2. Superoxide dismutases (SOD) are metalloenzymes that play a key role in the plant's protective response to oxidative stress. Catalases (CAT) are tetrameric homoproteins that are among the main antioxidant defense in plants.

Polyamines (PA) affect membrane fluidity, act as ROS scavengers, and control many functions associated with DNA, RNA, and protein turnover (Tiburcio and others 1993). In a few cases involving experiments with herbicides, oxidative injury occurring in hyperhydric tissues was directly related to changes in PA levels (Zheleva and others 1993; Ye and others 1997). The possible role of PAs in HHS is rarely studied, although some reports establish similarities between the symptoms of hyperhydricity and other physiologic situations that enhance PA synthesis (Piqueras and others 2002).

Vanilla planifolia is a member of Orchidaceae, and vanilla is popular for its natural flavor prepared from the extract of carefully cured vanilla beans. Although vanilla plants are propagated by cuttings, there is a large demand for elite planting material produced via the micropropagation technique. While developing a protocol for micropropagation of vanilla, George and Ravishankar (1997) studied the effects of various growth regulators and different culture conditions, where a maximum number of shoots was formed in liquid medium and a higher number of shoots than in solid medium occurred in a two-phase culture system. We reported long-term culturing of shoots in vitro and the use of genetic markers for testing the clonal nature of plantlets thus produced (Sreedhar and others 2007a). For scale-up of shoot cultures in a bioreactor, our initial trials in fully submerged cultivation of vanilla shoots in an air-lift bioreactor (2-l capacity) resulted in rapid death and leaching of white exudates into the medium. Therefore, a thorough investigation was considered, with the goal of acclimatizing the shoot cultures to liquid medium. The present study focuses on unraveling the major structural and biochemical changes that occur in vanilla shoots grown in liquid medium. The present study also records the changes in kinetics of nutrient utilization from the medium, measured in terms of pH, osmolarity, and conductance.

Materials and Methods

Plant Material and In Vitro Culturing Conditions

A vanilla clone collected from the Burliar plantation, Nilgiris, India, served as the mother plant from which shoot cultures were established under conditions described earlier (George and Ravishankar 1997). These shoots were routinely multiplied (Sreedhar and others 2007a) in gelled Murashige and Skoog (MS) (1962) culture medium supplemented with 8.87 μM BAP and 2.69 μM NAA and maintained at 25 ± 2°C under a 16-h photoperiod with illumination of 37.5 μmol photons m^{-2} s^{-1} provided by fluorescent lamps.

Transferring the shoots to liquid medium involved the culturing of shoots in Erlenmeyer flasks (150 ml) containing 40 ml MS-based medium supplemented with 8.87 μM BAP and 2.69 μM NAA. Approximately 2.5 g of the plant material was used as initial inoculum, which included one to two cluster(s) having five to six shoot buds (of length 0.5-1 cm) each. The culture vessels were maintained at 25 ± 2°C under a 16-h photoperiod, with illumination of 37.5 μmol photons m^{-2} s^{-1} on a gyratory shaker set at 90 rpm throughout the culturing period of 5 weeks. The cultures thus established were continuously bathed in liquid medium and called the continuous immersion system. The shoots cultured on a solid medium with the same composition as above but gelled with 7.2 g l^{-1} agar (HiMedia, India) served as the control. Morphologic and biochemical changes were observed at various stages of HHS for 5 weeks.

Morphologic Changes

Although the shoot multiplication was better in the complete immersion system (CIS) compared to the solid system (Fig. 1), the shoots in CIS displayed HHS toward the end of the culture period of 5 weeks. The four stages toward HHS are shown in Fig. 2. The other drastic morphologic changes that occur while progressing toward HHS were recorded by light microscopy, followed by scanning electron microscopy (SEM). Leaf morphology and changes at the tissue level were observed. For the latter, freehand sections about 0.5–1 mm thick were made using a new stainless steel razor blade. The sections were mounted on a slide and observed under an inverted light microscope (Leitz, LABOVERT, Ernst Leitz GmbH, Wetzlar, Germany) at 320× magnification and the responses were documented through photomicrographs. For scanning electron microscopy (SEM), the normal and hyperhydric cultures were processed according to Fowke and others (1994). The samples were fixed in 2% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) for 6 h, dried in an

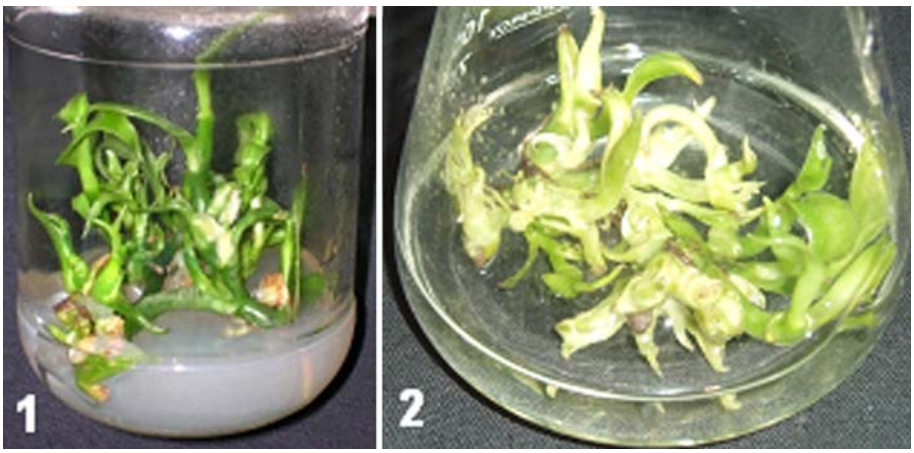

Fig. 1 Shoot cultures of *Vanilla planifolia* cultured on (1) solid and (2) complete immersion system (CIS) (liquid media) after 5 weeks of culture

Fig. 2 Shoots cultivated in normal and liquid medium showing various degrees of hyperhydricity. (1) Shoots cultivated on solid medium. (2) Shoots from CIS 1 week after inoculation (H1 stage). (3) Shoots from CIS 3 weeks after inoculation (H2 stage). (4) Shoots from CIS 5 weeks after inoculation (H3 stage)

alcohol series up to 100% (v/v), sputter-coated with gold, and examined in a LEO Scanning Electron Microscope 435 VP (Leo Electron Microscopy Ltd., Cambridge, UK).

Measurement of pH, Conductance, and Osmolarity

The pH of the medium was adjusted to 5.8 prior to autoclaving, and the changes in the pH after autoclaving and after the placement of shoot bud inoculum were monitored using a digital pH meter (Control Dynamics, India) calibrated with buffer standards.

Osmolarity was measured to determine the level of total solutes, both charged and neutral compounds in the medium, by using an automatic cryoscopic osmometer (Osmomat 030-D Gonotech GmbH, Berlin, Germany). Calibration of the instrument was done using triple-distilled water and sodium chloride standard. The osmolarity was expressed as osmol kg^{-1}. The depletion of nutrients from the medium was examined by measuring the electrical conductance using a conductance meter (Wiss-teelm-werkstalten model LF-54, Wielhalm, Germany) that was precalibrated with triple-distilled water. The conductance was expressed in the units of mS (milliSiemens). The pH, osmolarity, and conductance of the media were recorded before inoculation, just after inoculation, and every week during the 5 weeks of culture.

Chlorophyll Content

The chlorophyll content was calculated using Lichtenthaler equations (Lichtenthaler 1987) after measuring the absorbance of the acetone extracts at 645 and 661.5 nm.

Carbohydrate Content

Samples were repeatedly crushed in absolute ethanol and the alcohol solubles and insolubles were separated by filtration and vacuum evaporation of alcohol. Total carbohydrate in each fraction was estimated by the phenol sulfuric acid method (Dubois and others 1956), reducing sugars by the dinitrosalicylic acid method (Miller 1959), and nonreducing sugars were quantified by subtracting the reducing sugar values from the total sugar values. The alcohol insoluble material was dried, and after recording the weight, a known weight was hydrolyzed using 72% (v/v) H_2SO_4, keeping the samples in an ice bath. The mixture was appropriately diluted and used for estimating reducing or nonreducing sugars as above.

Analysis of Polyamines

The extraction of polyamines and their HPLC analyses were conducted according to the method of Flores and

Galston (1982). Authentic standards of putrescine (*Put*), spermidine (*Spd*), and spermine (*Spm*) (Sigma, St. Louis, MO) were benzoylated before HPLC analysis. Free polyamines were extracted by homogenizing the plant materials (1 g of tissue) in 10 ml of 5% (v/v) ice-cold perchloric acid using a pestle and mortar. The homogenate was then centrifuged for 30 min at 20,000*g*. Free polyamines in the supernatant were benzoylated and analyzed by HPLC (Shimadzu LC6A, Tokyo, Japan). The elution system consisted of a methanol:water (64:36) solvent, running isocratically with a flow rate of 1.0 ml min^{-1}. The benzoylpolyamines were eluted through a C_{18} column (300 mm × 4.6 mm i.d., pore size = 5 μM); an SLC-6A system controller and a CR4A data processor were used. Eluted compounds were detected using the UV detector SPD-AV set at a sensitivity of 0.04 AUFC and absorbance at 254 nm. A relative calibration procedure was used to determine the polyamines in the samples, using data of the standards—*Put*, *Spd*, and *Spm*—by comparing peak areas and retention times. Results were expressed as nanomoles per gram of fresh weight. Extractions from three different samples were made independently and each extract was quantified in triplicate.

Protein Content and Antioxidant Enzyme Activity

Protein content of the plant material was estimated by Lowry's method (Lowry and others 1951). The plant material (1 g each) for enzyme assay was extracted by crushing with pestle and mortar with 10 ml of respective buffer. After homogenization, the homogenate was centrifuged at 12,000 rpm twice and the supernatant was used.

POD enzyme was estimated following the method explained previously (Sreedhar and others 2007b). The material was extracted in sodium phosphate buffer (pH 6.0) containing 1 mM dithiothreitol and 0.1 mM phenyl methyl sulfonyl fluoride. *SOD* activity was measured spectrophotometrically based on the reduction of nitroblue tetrazolium (NBT) to water-insoluble blue formazan as described by Murthy and others (2002). Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT to 50%. The specific activity was expressed in terms of units per mg protein. *CAT* activity was determined by adding sample extract in 50 mM phosphate buffer (pH 7) containing 18 mM H_2O_2 in a total volume of 3 ml. The consumption of H_2O_2 by CAT was measured by the decrease in absorbance at 240 nm (ε = 39.4 mM/cm) at 25°C (Beers and Sizer 1952).

Chlorophyll, carbohydrates, free polyamines, and protein contents along with enzyme activities were recorded initially and on the first, third, and fifth week after inoculation in both normal (solid) and liquid cultures at respective moisture contents.

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corp., Redmond, WA), and *post-hoc* mean separations were performed by Duncan's multiple-range test at $P \leq 0.05$ (Harter 1960) by using the statistical program SPASS7.0.

Isozymic Analysis of POD

For analyzing the isozymes of POD from the normal and hyperhydric stage (stage H2), a standard protocol was used. A zymogram was prepared by polyacrylamide gel electrophoresis (7.2% w/v) (PAGE) carried out at 120 V for 4 h using a 12 × 14 × 0.5-cm piece of gel without SDS. The gel was stained for POD activity with 100 ml sodium phosphate buffer solution containing 10 ml of 0.25% (w/v) orthodianisidine dihydrochloride and 10 ml of 1% (v/v) hydrogen peroxide and immediately photographed (Thimmaraju and others 2007).

Results

Morphologic and Ultrastructural Changes

Normal and HHS shoots of vanilla displayed significant differences when leaves and stem portions were observed by optical microscopy and SEM. Normal vanilla shoots cultured on solid medium displayed a xerophytic morphology with succulent waxy leaves. The moisture content was 88.8% in normal shoot cultures, 90.3% in H1 stage cultures, 93.7% in H2 stage, and 96.4% in H3 stage cultures. Light microscopy observations of stem sections of H2 cultures revealed distinct degradation of vascular tissues and lesser degradation of cortical tissues, a reduction in the size of the cortical cells, and hypertrophy of tissues inside the vascular cylinder. Degradation of the endodermal cells was noted in the hyperhydric shoots (Fig. 3a), whereas the leaf sections showed higher degradation of vascular bundles, loss of compactness of the palisade parenchyma with abnormal enlargement, and more intercellular space (Fig. 3b). The SEM of the stem surface revealed uniform cuticular ridges in normal cultures and scattering in hyperhydric cultures (Fig. 4a). Vascular tissue was severely collapsed in hyperhydric shoot cultures (Fig. 4b, c). Stomata showed regular structure in normal leaves and lacked closure mechanisms in hyperhydric leaves (Fig. 4d).

Changes in Medium Kinetics in CIS

The pattern of variation in key media characteristics like pH, osmolarity, and conductance during the course of HHS

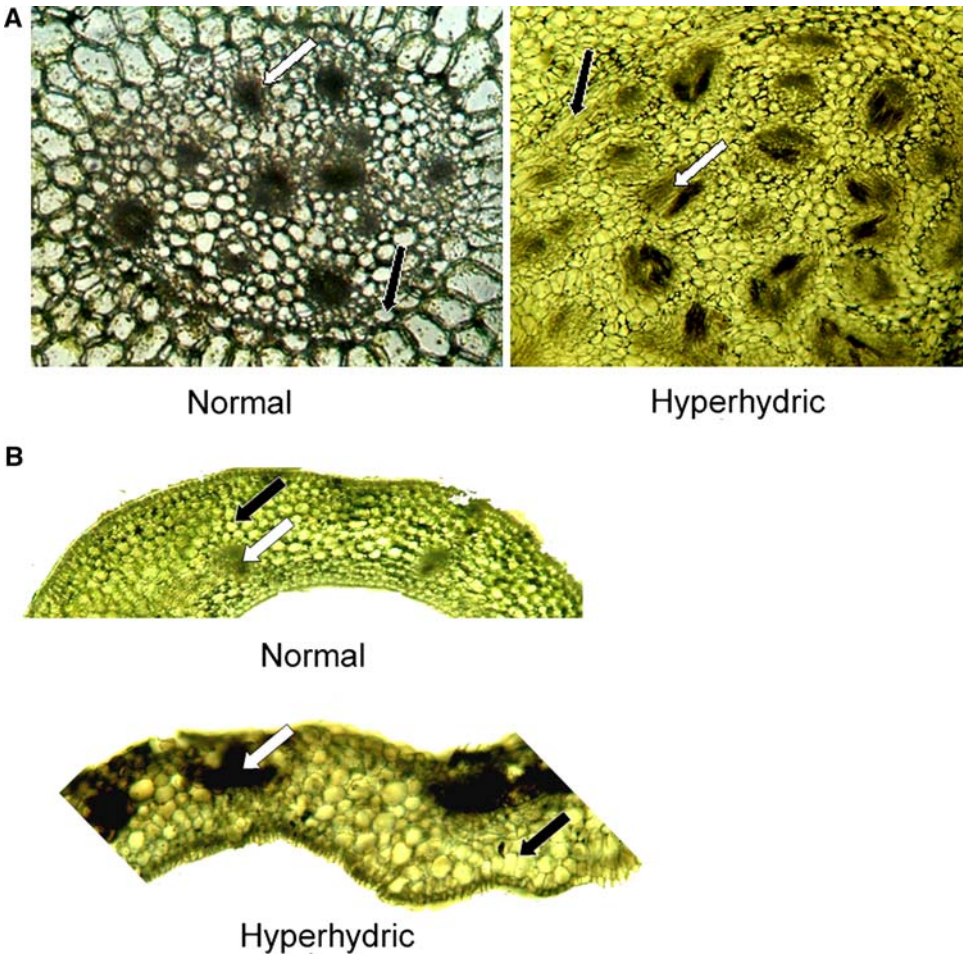

Fig. 3 Cross-sections of stem (**a**) and leaf (**b**) in normal and hyperhydric (stage H2) shoot cultures of vanilla. **a** Vascular tissue (*white arrows*) and endodermis (*black arrows*) of stem. **b** Vascular tissue (*white arrows*) and palisade parenchyma (*black arrows*) of leaf

development is presented in Fig. 5. A sudden fall in the pH of the medium from 5.7 to 4.3 was observed just after inoculation (JAI) of the shoot cultures; it stabilized within a week, attaining an equilibrium pH of 4.7 throughout the culturing period of 5 weeks (Fig. 5). Osmolarity of the medium, which was initially 0.225 osmol kg^{-1}, showed a steady decline to a level of 0.069 osmol kg^{-1} by the end of the culturing period with a steep fall between the second and third weeks (Fig. 5). Medium conductance, which reflects the electrolyte concentration, had an initial value of 6.4 mS. There was a steady decline to a level of 4.05 mS by the end of the culturing period, indicating a slow and continuous uptake of nutrients by the shoot cultures (Fig. 5).

Changes in Chlorophyll and Carbohydrate Contents

At the H1 stage, shoot cultures exhibited less variation in the levels of total chlorophyll and chlorophyll-b compared to normal shoots (Table 1). However, at subsequent periods there was a significant loss of chlorophyll at the H2 and H3 stages. The loss of chlorophyll-a was about 72.5% and the loss of chlorophyll-b was over 64% from normal to the H2 stage, with a loss of 70% of total chlorophyll. The H3 stage had lost the largest amount of chlorophyll, which is evident in the Fig. 2.

An estimation of total carbohydrates, taking into account the reducing and nonreducing sugars from both soluble and insoluble fractions, was performed for the normal and the hyperhydric shoot cultures (Table 1). Total soluble sugar was highest in normal shoots and total insoluble sugar was highest in both normal and stage H1 cultures. Hyperhydric shoot cultures had significantly lower concentrations of soluble reducing sugars compared to the normal shoots. Total soluble and soluble reducing sugars were very low in stage H3 cultures. The greatest amount of reducing sugars was found in the insoluble fraction in the H1 stage. However, normal cultures showed the highest content of nonreducing sugars in the insoluble fraction. For nonreducing sugars in the insoluble fractions, there was a steady decline in their levels from normal to the H2 stage, with an abnormally high level at the H3 stage.

Polyamines Content

The change in the content of free polyamines is presented in Table 2. A significantly sharp increase in the

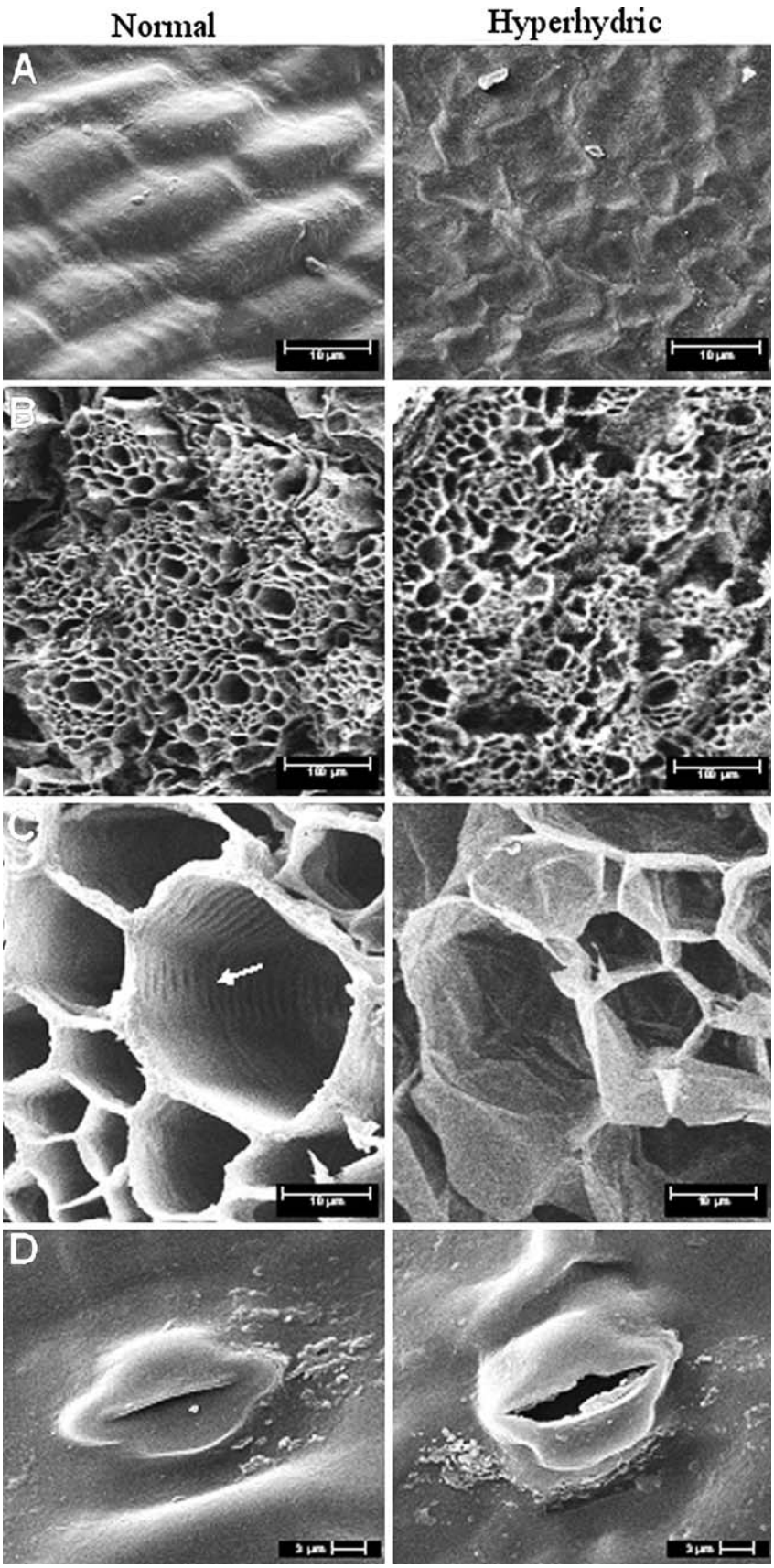

Fig. 4 Scanning electron micrographs of normal and hyperhydric shoot cultures (stage H2) of vanilla. **a** Stem surface. **b** Vascular tissue. **c** An enlarged view of **b** showing secondary wall thickening (*arrow*) of the vascular tissue. **d** Stomatal structure

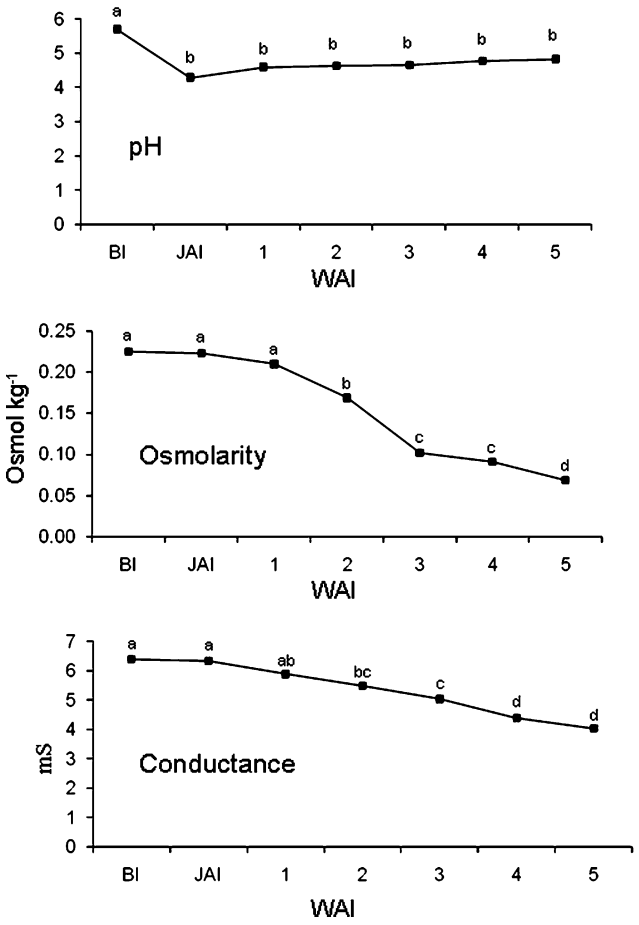

Fig. 5 Kinetic parameters of the medium used for culture of vanilla shoots. pH, osmolarity, and conductance in complete immersion system (CIS). BI before inoculation, JAI just after inoculation, WAI, weeks after inoculation. Data presented as mean of five replicates. Means with different letters are significantly different at $P \leq 0.05$, according to Duncan's multiple-range test

concentration of all three PAs was noted in stage H2 cultures. A sudden increase in the concentration of *Put* was found in cultures of stage H2 from base levels in normal and H1 stage cultures. However, it showed a pitfall in H3 stage cultures. Almost a 1.8-fold increase in *Spd* content was observed in the H2 stage compared to normal and the H1 stage, and it dropped in the H3 stage. *Spm* was high in the normal cultures, with a significant decline at the H1 stage and steeply increased at the H2 stage. The increase in *Spm* concentration was 1.6-fold in H2 stage cultures compared to H1 stage shoots. It again declined to nearly half of its concentration from the H2 to the H3 stage.

Protein Content and Activities of Antioxidant Enzymes

Vanilla shoot cultures displayed wide variations in total protein concentrations, with a significant loss with the progression toward HHS (Fig. 6a). The lowest protein content of 8.14 mg g^{-1} FW was found in the H3 stage, which was nearly 77% less than that in normal shoots.

POD High POD activity was maintained in normal, H1, and H2 stage cultures (Fig. 6b). The highest enzyme activity (356.93 U mg^{-1} protein) was noted in the H1 stage, which was on par with the activity in H2 stage cultures (350.35 U mg^{-1} protein). However, HHS stage H3 showed a significant loss in enzyme activity (59.93 U mg^{-1} protein) (Fig. 6b). In the zymogram (Fig. 7), POD activity showed three isoforms in both normal and HH shoot cultures with a significant difference between the two, that is, the bands of H2 stage cultures showed high intensity indicative of high activity whereas those of normal shoots (N) showed faint activity.

SOD SOD activity was highest in normal and H1 stage cultures, with a significant loss in H2 stage cultures. A substantial drop in SOD activity was noted (nearly sixfold) (6.01 U mg^{-1} protein) in H3 stage cultures compared to normal and H1 stage cultures (Fig. 6c).

CAT It was noted that there was significantly higher CAT activity in H2 stage cultures compared to H1 stage and normal cultures. The enzyme displayed the highest activity of 82.75 U mg^{-1} protein in H2 stage cultures, with a more than 1.4-fold increase in activity compared to that in the H1 stage (58.04 U mg^{-1} protein). However, there was a steep decline (more than twofold) in CAT activity in H3 stage cultures (38.48 U mg^{-1} protein) compared to H2 stage cultures (Fig. 6d).

Discussion

Vanilla planifolia normally displays xerophytic characteristics and, therefore, one can expect difficulty in growing this plant in submerged (hydrophytic) conditions. However, as in most of the other shoot cultures that easily adapt to agitated medium, vanilla shoots were also expected to grow and multiply better in CIS. Although shoot number increased in CIS as expected (Fig. 1), the shoots underwent severe hyperhydricity conditions leading to HHS (Fig. 2), where progressive accumulation of water was evident. In other plants where hyperhydricity conditions are reported, anatomically there was hypertrophy of the cortex and pith and the parenchyma displayed large intercellular spaces (Hazarika 2006). Even in the case of vanilla with HHS, there was a general loss of cellular integrity and pronounced degradation of vascular tissues. Severe structural damage was observed at the surface of the leaves. The endodermis, palisade, and vascular tissues were severely collapsed (Fig. 3). The conducting vessels, particularly the xylem and tracheids, in normal plants displayed a beautiful architecture of secondary walls in xylary tissues, whereas the vasculature of HHS plants appeared decomposed

Table 1 Changes in the levels of chlorophyll and carbohydrates in normal (N) (shoots cultivated on solid medium) and hyperhydric shoot cultures of vanilla

Stage	Chlorophyll (% FW)			Soluble sugars (mg g^{-1} FW)			Insoluble sugars (mg g^{-1} FW)		
	Total	Chlorophyll-a	Chlorophyll-b	Total	Reducing sugars	Nonreducing sugars	Total	Reducing sugars	Nonreducing sugars
N[†]	0.0057 ± 0.0005^{a}	0.0040 ± 0.0003^{a}	0.0017 ± 0.0002^{a}	13.45 ± 1.24^{a}	8.86 ± 0.34^{a}	4.58 ± 0.13^{a}	4.55 ± 0.2^{a}	2.94 ± 0.18^{b}	1.6 ± 0.04^{a}
H1	0.0051 ± 0.0005^{a}	0.0034 ± 0.0003^{b}	0.0018 ± 0.0002^{a}	10.04 ± 0.5^{b}	6.01 ± 0.22^{b}	4.04 ± 0.3^{a}	4.84 ± 0.21^{a}	4.11 ± 0.2^{a}	0.73 ± 0.07^{b}
H2	0.0017 ± 0.0017^{b}	0.0011 ± 0.0001^{c}	0.0006 ± 0^{b}	6.92 ± 0.34^{c}	3.79 ± 0.17^{c}	3.13 ± 0.18^{b}	2.63 ± 0.12^{b}	2.54 ± 0.11^{b}	0.09 ± 0^{d}
H3	0.0003 ± 0^{c}	0.0002 ± 0^{d}	0.0001 ± 0^{c}	2.78 ± 0.21^{d}	2.15 ± 0.15^{d}	0.63 ± 0.06^{c}	1.46 ± 0.08^{c}	1.1 ± 0.1^{c}	0.37 ± 0.02^{c}

Hyperhydric shoots: 1 week after inoculation (H1), 3 weeks after inoculation (H2), and 5 weeks after inoculation (H3)

Data presented as mean ± SD of five replicates. Means superscribed with different alphabets are significantly different at $P \leq 0.05$, according to Duncan's multiple-range test

[†] No significant differences in the chlorophyll and carbohydrate contents were noticed among solid-medium cultivated shoot cultures of initial, first, third, and fifth weeks of culturing

(Figs. 3 and 4). Vascular bundles lacked the typical arrangement found in normal shoots, indicative of a lack of lignin synthesis and degradation of existing secondary wall structures (George 1996). It has been a general observation that most stomata of in vitro plantlets do not have a closure mechanism, a situation linked to water loss and death of plantlets during acclimatization under low relative humidity (Hazarika 2006). On the other hand, vanilla plants in vitro displayed structurally normal stomata (Fig. 4d). Regarding the induction of the HHS stage, significant changes in carbohydrates indicate structural degradation of cell walls.

With respect to the medium's kinetics, the drop in pH upon introduction of cultures into the medium is due to the acidic nature of the plant material (Escalona and others 1999). Although vanilla shoots were obtained from previous cultures, the trimming of unwanted tissues creating cut ends probably resulted in leaching of sap. This would lead to a sudden drop in the pH of the medium to 4.3, and its improvement and stabilization later at 4.7 suggests that the ionic status is maintained and hence there could not be a catastrophic effect of the medium's pH on the development of HHS. The rapid drop in the osmolarity of the medium noted between the first and third weeks of culture indicates high uptake of minerals and sugars, and the steady decline in conductance reflects the progressive uptake of ions (minerals). These trends inversely correlate with the increase in shoot growth and accumulation of biomass (data not shown). The decreased rate of change in osmolarity and conductance from the third week onward indicates poor utilization of nutrients. Another possibility could be saturation of the water/nutrient uptake system.

Polyamines (PAs), chemically nonprotein straight-chain aliphatic amines, are known to play an important role in various cellular processes (Bais and Ravishankar 2002). PAs have been implicated in direct scavenging of free radicals, thereby reducing oxidative effects. They may also act indirectly by elevating the levels of antioxidants. *Put* is known to suppress the level of superoxide and H_2O_2 in leaves of stressed plants. Chilling-induced H_2O_2 production was inhibited in cucumber seedlings after *Spd* pretreatment (Shen and others 2000). *Spm* (a tetramine), *Spd* (a triamine), and their precursor *Put* (a diamine) are known to play a major role in assisting plants and their cells/tissues in adapting to stressful conditions by acting as antioxidants and protecting biological membranes against peroxidation (Verma and Mishra 2005). In vanilla, the *Spm* level was far higher than *Spd*, although both are derived from *Put*, showing a particular dynamic of free PAs. Such a stress-dependent increase in the levels of *Spm* has also been observed in other studies (Kumar and Rajam 2004; Silveira and others 2006). A similar trend was observed even in the case of banana cultures with progressive subcultures

Table 2 Levels of free polyamines [putrescine (*Put*), spermidine (*Spd*), and spermine (*Spm*)] in solid-medium and CIS-cultured shoots

	Put (nmol g^{-1} FW)		*Spd* (nmol g^{-1} FW)		*Spm* (nmol g^{-1} FW)	
	Solid	CIS	Solid	CIS	Solid	CIS
1	$12.92 \pm 1.09^{a,b}$	12.92 ± 0.09^{b}	18.1 ± 0.81^{a}	18.1 ± 0.81^{b}	2074.44 ± 199.12^{a}	2074.44 ± 199.12^{a}
2	16.9 ± 1.91^{a}	9.05 ± 0.91^{b}	17.93 ± 0.99^{a}	18.64 ± 1.76^{b}	1985.4 ± 89.12^{a}	1291.77 ± 122.34^{b}
3	16.34 ± 2.03^{a}	595.07 ± 57.95^{a}	19.96 ± 0.12^{a}	32.00 ± 3.01^{a}	2100.3 ± 34.19^{a}	2121.27 ± 211.1^{a}
4	14.68 ± 1.17^{a}	64.5 ± 6.22^{b}	20.1 ± 1.99^{a}	8.48 ± 0.88^{c}	1947.36 ± 34.88^{a}	1087.63 ± 104.7^{b}

Solid-medium cultured shoots: (1) 0 weeks after inoculation (N), (2) 1 week after inoculation, (3) 3 weeks after inoculation, and (4) 5 weeks after inoculation. CIS cultured shoots: (1) 0 weeks after inoculation (N), (2) 1 week after inoculation (H1 stage), (3) 3 weeks after inoculation (H2 stage), and (4) 5 weeks after inoculation (H3 stage)

Data presented as mean ± SD of triplicates. Means superscribed with different alphabets are significantly different at $P \leq 0.05$, according to Duncan's multiple-range test

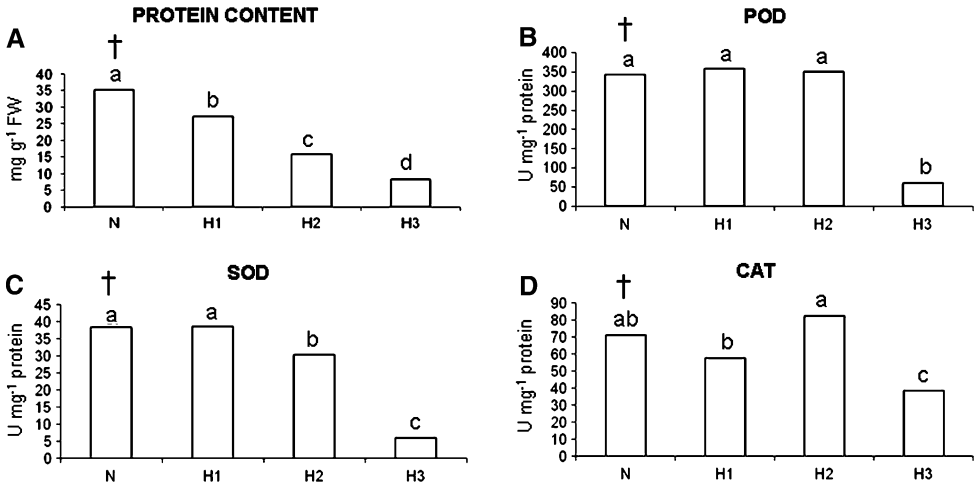

Fig. 6 Protein content and activity of antioxidant enzymes in normal (shoots cultured in solid medium) and hyperhydric shoot cultures: shoots from CIS 1 week after inoculation (H1 stage), shoots from CIS 3 weeks after inoculation (H2 stage), and shoots from CIS 5 weeks after inoculation (H3 stage). Data presented as mean of five replicates. Means with different letters are significantly different at $P \leq 0.05$, according to Duncan's multiple-range test. †No significant differences in the protein content and activity of antioxidant enzymes were noticed among solid-medium cultivated shoot cultures of initial, first, third, and fifth week of culturing

(Venkatachalam and Bhagyalakshmi 2008). The control vanilla shoot cultures (in solid medium) did not show significant variations in the contents of PAs during the 5-week culture; on the other hand, our study with banana cultures showed a slow accumulation of free PAs over a 12-week period (Venkatachalam and Bhagyalakshmi 2008). In the present study, the cultures in CIS displayed the accumulation of free PAs, *Spm*, *Put*, and *Spd*, increasing substantially during HHS progression. The H3 stage, where shoots had attained HHS, the levels of *Spd* and *Put* decreased and most of the proteins and activities of antioxidant enzymes also decreased possibly due to proteolysis. Pea leaves treated with the herbicide atrazine, which induces HHS in plants, showed higher accumulation of *Put*, *Spd*, and *Spm* (Piqueras and others 2002), indicating that even in the case of vanilla shoots, the increase in all three PAs was possibly associated with HHS. The polycationic nature of PAs can help them bind strongly to nucleic acids, proteins, and phospholipids. They also interact with membrane phospholipids to stabilize them under stress conditions. *Spm* is known to be prominently involved in free radical scavenging. Whereas *Put* was enhanced by cold stress, *Spd* was enhanced by water stress in chickpea, although the accumulation of PAs was short-lived in the presence of both stresses together (Nayyar and Chander 2004). Nevertheless, the oxidative injury to stressed tissue could be reduced by exogenous application of respective PAs leading to the concomitant increase in the endogenous levels of respective PAs, suggesting their direct or indirect role as antioxidants.

Reduction in the chlorophyll content was one of the earliest symptoms observed with the onset of HHS (Fig. 2), which probably serves as an early marker for HHS. The changes in the levels of reducing and nonreducing sugars are expected to occur due to hydrolysis of polysaccharides and interconversions of soluble sugars as happens in ripening fruits with higher water activity. The decrease in soluble sugars at the H3 stage may be due to their further

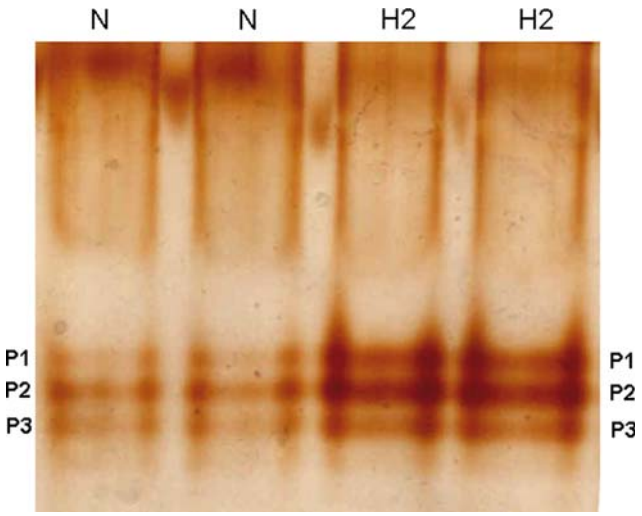

Fig. 7 Zymogram on denaturing gel of PODs after activity staining. N: of normal shoots and H2: of hyperhydric (stage H2, shoots from complete immersion system 3 weeks after inoculation) shoot cultures. P1, P2, and P3 represent various isoforms of PODs

degradation which forms uronides. However, because there is no drastic change in the pH of the medium, its conductance, or osmolarity, the degradative products may be held within the tissues without leaching into the medium.

The capacity of the antioxidant defense system often increases under stress conditions (Gressel and Galun 1994). Saher and others (2004) observed that hyperhydric leaves of carnation suffering from oxidative stress accumulate H_2O_2 and the tissues try to reduce the damage via induction of subcellular antioxidant systems by increasing the activity of detoxifying enzymes like CAT and PODs. In other studies, higher levels of antioxidant enzymes have been correlated with oxidative stress and hence are considered as molecular markers for such situations (Mishra and others 2006; Arbona and others 2008). Therefore, the significant increase in the activity of CAT and maintenance of higher POD activity associated with the progression of HHS is indicative of oxidative stress in vanilla shoot cultures. Among the three enzymes, SOD appears foremost in the defense against ROS injury, catalyzing the dismutation of O_2^- to H_2O_2 and molecular oxygen. The H_2O_2 produced is then scavenged by several classes of PODs, the activity of which is also high at the H1 stage. PODs are homoproteins present as multiple isozymes in plant tissues and are distributed throughout the cell functioning as catalysts of the reduction of H_2O_2 to H_2O. They belong to a large family of enzymes that have the ability to oxidize/reduce different substrates in the presence of H_2O_2 and thus maintain the redox status of the cell. Although the activity of POD was highest in vanilla cultures, the simultaneous increase in the activity of this enzyme and of CAT in the hyperhydric cultures at the H3 stage indicates their orchestrated effects in scavenging H_2O_2 as well as other free radicals. It is also known that PODs exist as a large group of isoenzymes with an extreme range of isoelectric points serving a multitude of functions. Each group is thought to have a different function in the cell. Acidic (anodic) isoenzymes of POD are known for their involvement in growth and differentiation of cells; the basic forms are assumed to provide H_2O_2 for other PODs (Gulen and Eris 2004). In the present study, the zymograms of POD from normal and HH shoots showed no new isoform formation in response to HH stress. However, higher-intensity bands are indicative of an increase in their activities.

Earlier studies have presented both physical and chemical methods for the reversal and control of hyperhydricity. Among the physical methods, measures like flushing out ethylene, CO_2, and other volatile components accumulated in the headspaces of culture vessels appear essential to contain HHS (Lai and others 2005). Avoiding the complete submergence of shoots has also been suggested for which the use of temporary immersion systems providing intermittent contact between the plant material and the liquid culture medium have been proposed (Etienne and Berthouly 2002). In the case of vanilla, our earlier studies have shown that despite 10 years of maintenance in solid medium (Sreedhar and others 2007a) or any increase or decrease of cytokinin concentration in solid medium (George and Ravishankar 1997), the induction of HHS did not occur. Our present study shows that liquid medium in particular induces HHS, indicating that water potential plays an important role in the induction of HHS. However, in the case of *Vitis vinifera* (cv. Albarino), although the increase in cytokinin level enhanced the HHS condition, the addition of a liquid phase to the solid medium further increased HHS (Couselo and others 2006). It is worth noting that our present study does not involve any change in growth regulator, where the level of cytokinin is constant in both the liquid and the solid medium. Agar at a concentration of 0.8% was found to be the best gelling agent for avoiding hyperhydricity as lower concentrations clearly induced hyperhydricity. A double-phase culture (using a liquid and gelling agent solidified culture system) was also proposed to obtain higher biomass and shoot number compared to solid medium in Japanese pear (Kadota and others 2001). However, as mentioned above, this aggravated the HHS condition in *Vitis vinifera* (cv. Albarino) (Couselo and others 2006). As for chemicals, activated charcoal, phloridzin, and paclobutrazol have been used for specific shoot cultures (George 1996). These treatments have been considered for further work on genetic regulation of hyperhydricity in the authors' lab.

In conclusion, the present study has provided insight into the morphologic and biochemical changes that occur during the progression of HHS in vanilla shoots. One of the

main consequences of HHS appears to be oxidative stress, which is evident from the increase in the levels of activities of antioxidant enzymes. The rapid loss of chlorophyll and the concurrent increase in the contents of free PAs are useful biochemical indicators of HHS in *V. planifolia.*

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