Biochemical and morpho-anatomical analyses of strawberry vitroplants hyperhydric tissues affected by BA and gelling agents

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ABSTRACT

In vitro propagation has become an effective practice for large-scale production of strawberry plants. The objective of this study was to evaluate the hyperhydricity and the multiplication capacity of two strawberry varieties (*Fragaria x ananassa* Duch. 'Dover' and 'Burkley') propagated *in vitro*. Plants maintained in MS medium supplemented with 1.0 mg L^{-1} BA were individualized and transferred to the same medium solidified with Agar (6.5 g L^{-1}) or Phytagel® (2.5 g L^{-1}) and BA at different concentrations (0; 0.5; 1.0; 2.0 and 3.0 mg L^{-1}). Biochemical and anatomical analyses were carried out, as well as the   analysis of the morphological hyperhydricity characteristics. The analysis of data showed: a) the increase in cytokinin concentration increased hyperhydricity frequency in both varieties; b) at concentrations up to 2.0 mg L^{-1} BA, the replacement of Agar by Phytagel® induced a higher formation of hyperhydric shoots; and c) the addition of BA induced oxidative stress, which is characterized by increased antioxidant activity and lipid peroxidation, as well as alterations at the cellular level, such as malformation of stomata and epidermal cells. In conclusion, the culture medium containing 0.5 mg L^{-1} BA solidified with Agar provided lower hyperhydricity percentages in association with higher rates of shoot proliferation in strawberry.

Key words: oxidative stress; cytokinin; lipid peroxidation; micropropagation.

RESUMO

Análises bioquímicas e morfo-anatômicas em vitroplantas de morangueiro hiper-hídricas afetadas pelo BA e agentes geleificantes

A propagação *in vitro* tem se destacado como uma técnica efetiva na produção em larga escala de plantas sadias de morangueiro. Neste trabalho estudamos a hiperhidricidade associado a capacidade de multiplicação *in vitro* na propagação de duas variedades de morangueiro (*Fragaria x ananassa* Duch. "Dover" e "Burkley"). Plantas mantidas em meio de cultura MS, suplementadas com 1.0 mg L^{-1} de BA fora individualizadas e transferidas para o mesmo meio com Ágar (6.5 g L^{-1}) ou Phytagel® (2,5 g L^{-1}) e BA em diferentes concentrações (0; 0,5; 1,0; 2,0 e 3,0 mg L^{-1}). Foram realizadas análises bioquímica e anatômicas, além da caracterização morfológica do material hiper-hídrico. A análise

dos dados mostrou: a) o incremento na concentração de citocinina aumentou a freqüência de hiper-hidricidade para ambas as variedades; b) concentrações maiores que 2,0 mg L^{-1} de BA, com a substituição do Ágar pelo Phytagel® induziu a maior formação de ramos hiper-hídricos; e c) a adição de BA induziu o estresse oxidativo, caracterizado pelo incremento da atividade antioxidante e peroxidação de lipídeos, bem como alterações a nível celular, como má formação dos estômatos e células epidérmicas. Concluindo, o meio de cultura contendo 0,5 mg L^{-1} de BA solidificado com Ágar promoveu menor porcentagem de hiper-hidricidade associado com maiores taxas de proliferação de gemas em morangueiro.

Palavras-chave: citocininas; estresse oxidativo; peroxidação de lipídeos; micropropagação.

INTRODUCTION

Strawberry (*Fragaria* x *ananassa* Duch.) is an herbaceous, perennial and stoloniferous plant that is vegetatively propagated through runners arising from the parent plant.

In vitro propagation of strawberries is an effective practice for large-scale plant production in a controlled and disease-free environment (Calvete *et al.* 2009). During *in vitro* culture, plants are grown under special conditions, such as reduction of gas exchange, high air humidity, low light intensity and the use of sugar as an energy source. These conditions may cause increased reserves or biomass accumulation, photosynthesis inhibition, and abnormal stomata development, which can lead to the development of hyperhydricity. Hyperhydricity hinders micropropagation and acclimatization, causing significant plant losses when vitroplants are transferred to *ex vitro* conditions (Jausoro *et al.* 2010).

Hyperhydricity is a well-studied phenomenon, but it is not predictable. It occurs due to external factors, independent of explant origin and, under specific conditions, evolves to loss of regenerative capacity of the tissue (Casanova *et al.* 2008). Increased agar concentration or other gelling agents reduce the availability of water, which reduces hyperhydricity (Ivanova & van Staden 2011). However, it is often accompanied by a decrease in propagation frequency (Casanova *et al.* 2008; Gupta & Prasad 2010).

The capacity of the antioxidant defense system often increases under stressful conditions (Sreedhar *et al.* 2009). The artificial conditions of *in vitro* culture may predispose tissues to the damaging effects of reactive oxygen species (ROS) (Balen *et al.* 2011). Higher levels of antioxidant enzymes have been correlated with oxidative stress and hence are considered molecular markers for such conditions (Wu *et al.* 2009).

This study was carried out to characterize hyperhydricity induced by different concentrations of BA and different gelling agents in the propagation of strawberry (*Fragaria* x *ananassa*) varieties 'Dover' and 'Burkley', in association with the capacity of *in vitro* shoot proliferation.

MATERIAL AND METHODS

Plants and tissue culture

The strawberry (*Fragaria* x *ananassa*) plants, varieties 'Dover' and 'Burkley', used in the experiments was kindly provided by the Plant Tissue Culture Laboratory of the Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA Clima Temperado, Pelotas, RS, Brazil.

In vitro cultures were maintained under a 16 h photoperiod and 36 µmol m^{-2} s^{-1} light radiation provided by two fluorescent tubes (20 W, Osram, Brazil). The culture room temperature was kept at 26 ± 2 °C. Once a month, the cultures were transferred to new medium consisting of MS basal salts (Murashige & Skoog 1962) and vitamins, 30 g L^{-1} sucrose, 100 mg L^{-1} of myo-inositol, 6.5 g L^{-1} agar (Merck®, Germany) and 1.0 mg L^{-1} of BA. The pH was adjusted to 5.8 before autoclaving at 120 °C and 1.1 Pa for 20 min.

Experimental design

To determine optimal culture conditions to obtain normal and hyperhydric plants, the plant material was isolated under aseptic conditions and transferred to 240 mL glass flasks containing 30 mL of MS medium with the same concentrations of sucrose and myo-inositol. The medium was solidified with either 6.5 g L^{-1} of granulated agar (Merck®, Germany) or 2.5 g L^{-1} of Phytagel® (Sigma Chem. Co., USA) and supplemented with different concentrations of 6-benzyladenine (BA).

The treatments consisted of culture media added with 0.0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1} BA, and solidified with agar, or with Phytagel®, resulting in ten treatments. Flasks were sealed with polyvinyl chloride film (PVC) (Goodyear, Brazil) and maintained in the culture room as described above.

The experimental unit consisted of one flask with 5 shoots each. The experiment was conducted in a

completely randomized design with ten treatments and 5 replicates per treatment for each variety. Statistical analyses were performed and data were subjected to analysis of variance (ANOVA) and, when appropriate, means were compared using the Tukey's test at 5% probability.

Morphological analyses of hyperhydric plants

Hyperhydricity evaluations began one week after culture initiation and were repeated every 3 days. At the end of the experiment (35 days after culture initiation), tissues were sampled for biochemical, anatomical, pigment (chlorophyll) and ethylene analysis. The following characteristics were evaluated: number of buds per shoot and percentage of hyperhydric buds.

Anatomical and ultrastructural analyses

Anatomical and ultrastructural analyses were performed using fully expanded leaves from normal and hyperhydric plants fixed according to Karnovsky (1965). Samples prepared for photonic microscopy were submitted to a vacuum treatment for 24 h and rinsed in 0.05 M potassium phosphate buffer for 30 min. Tissues were then dehydrated in an ethylic series and embedded in metacrilate (Historesin®, Leica). The metacrilate blocks were sectioned using an automatic rotary microtome (Leica RM 2155-UK). The 5-µm thick sections were stained with Toluidine blue for 11 min and mounted in Permount® (Fisher Scientific, USA). Anatomical observations were performed using a photomicroscope (Olympus AX70TRF, Tokyo, Japan) equipped with a U-photo system (Olympus, Japan).

Samples selected for scanning electron microscopy were dehydrated in an ethylic series, evaluated and micrographed using a scanning electron microscope (LEO 1430VP, Japan).

Biochemical analysis

The enzymatic extract used to determine peroxidase, catalase and superoxide dismutase activity was obtained by homogenizing 0.5 g of leaf tissue (frozen in liquid nitrogen at -80 °C) with 4 mL of extraction solution (0.1 mM EDTA in 0.1 M potassium phosphate buffer, pH 6.8). All procedures were performed at 4 °C.

Biochemical analyses were conducted according to different authors: Peroxidase activity (POD EC1.11.1.7) (Chance & Maehley 1955); Catalase (CAT EC1.11.1.6) (Havir & McHale 1987); and Superoxide Dismutase (SOD EC1.15.1.1) (Giannopolitis & Ries 1977).

Lipid peroxidation

Lipid peroxidation was evaluated through the formation of malonaldehyde (MDA), a final product of lipid

peroxidation, using the thiobarbituric acid test (TBA), according to Buege & Aust (1978). The amount of the complex MDA-TBA was determined using the molar extinction coefficient of 1.56×10^{-5} mol^{-1} cm^{-1} (Dhindsa *et al.* 1981).

Chlorophyll analyses

The chlorophyll content analysis was performed using hyperhydric and normal strawberry plants. Samples consisting of 100 mg of fresh leaves were ground in a mortar with 80% acetone (w/w) and washed sand, and filtered with filter paper. After the first filtration the paper was washed in 80% acetone (w/w) to remove debris, and the filtered solution was poured into a volumetric flask. The flask was filled with 25 mL of solution and absorbance at 645 and 663 çm was determined (Hendry & Price 1993).

RESULTS AND DISCUSSION

BA induced both shoot proliferation and hyperhydric morphology

Hyperhydricity is an undesirable physiological state and represents a potential barrier to the exploitation of *in vitro* propagation of several *in vitro* cultured plants. It causes a decrease in survival rate when plants are transferred to *ex vitro* conditions, resulting in considerable commercial losses (Rojas-Martinez *et al.* 2010).

In our study, for both evaluated varieties ('Dover' and 'Burkley'), the BA-free treatments resulted in plants without hyperhydricity. However, the rate of shoot proliferation was very low in BA-free media when compared to BA-supplemented media. According to Jain *et al.* (2001), growth regulator-free medium produces a high frequency of normal plants, while BA-supplemented medium leads to the development of hyperhydricity.

The first hyperhydricity symptoms were visualized at 9 and 12 days after subculture of the 'Burkley' and 'Dover' varieties, respectively. The hyperhydricity symptoms were characterized by the appearance of translucent and fragile leaves, with shorter shoots (Figure 1). The first new shoots showed abnormal development resulting in a rosette-like structure at the explant base that was largely immersed in the medium (Figure 1B), with explants characterized by thickened and slightly elongated internodes (Figure 1C). In both varieties, the rates of formation of new axillary shoots were significantly increased in BA-supplemented medium and, in both varieties, the formation of new shoots was higher in treatments using Phytagel® as the gelling agent, with or without BA-supplement (Figure 2). The same efficiency for shoot proliferation was observed by Kumar *et al.* (2010).

In treatments using agar as the gelling agent, the formation of new axillary shoots was increased with 1.0

and 2.0 mg L^{-1} BA, and did not differ significantly from the treatment with 0.5 mg L^{-1} BA for 'Burkley' (Figure 2). For 'Dover', the treatments supplemented with BA did not differ significantly (Figure 2).

Plants (both varieties) showing normal characteristics, such as long internodes, expanded leaves and root differentiation in the first 10 days of culture were observed in 100% of the cultures when BA was omitted (data not shown). The BA-free treatments exhibited significantly lower shoot proliferation than the other treatments for both strawberry varieties (Figure 2).

Hyperhydricity evolution occurred during the development of the strawberry plants in different concentrations of BA, using either Agar or Phytagel® as a gelling agent (Table 1). No statistical differences were detected between varieties in response to both gelling agents. However, hyperhydricity did increase as BA concentration increased in the culture medium. At the lowest BA concentration, in Phytagel®-supplemented culture medium, there was a tendency to induce hyperhydricity, although this was not significant at 5% probability (Table 1). Likewise, the replacement of agar by Phytagel® induced

hyperhydricity in shoots of *Prunus avium* (Franck *et al.* 1997). Conversely, for apple, agar supported the shoot proliferation associated with the lowest percentages of hyperhydricity (Dobránszki *et al.* 2011).

Alterations in anatomical and ultrastructural of leaves increase with BA increased concentrations

Anatomical and ultrastructural studies of diverse species that compared hyperhydric and normal *in vitro*-grown plants have been reported (Picoli *et al.* 2008; Jausoro *et al.* 2010).

The normal plants showed a well-defined dorsiventral mesophyll (Figure 3A and C) with a single layer of palisade parenchyma occupying approximately half of mesophyll width. The spongy parenchyma contained two to three cell layers with few intercellular spaces. When the BA concentration increased, the delimitation of the spongy and palisade parenchyma gradually disappeared, and round and hypertrophied cells were observed with an apparent increment in leaf lamina width and reduction of its length (Figure 3B and D). The cells located below of
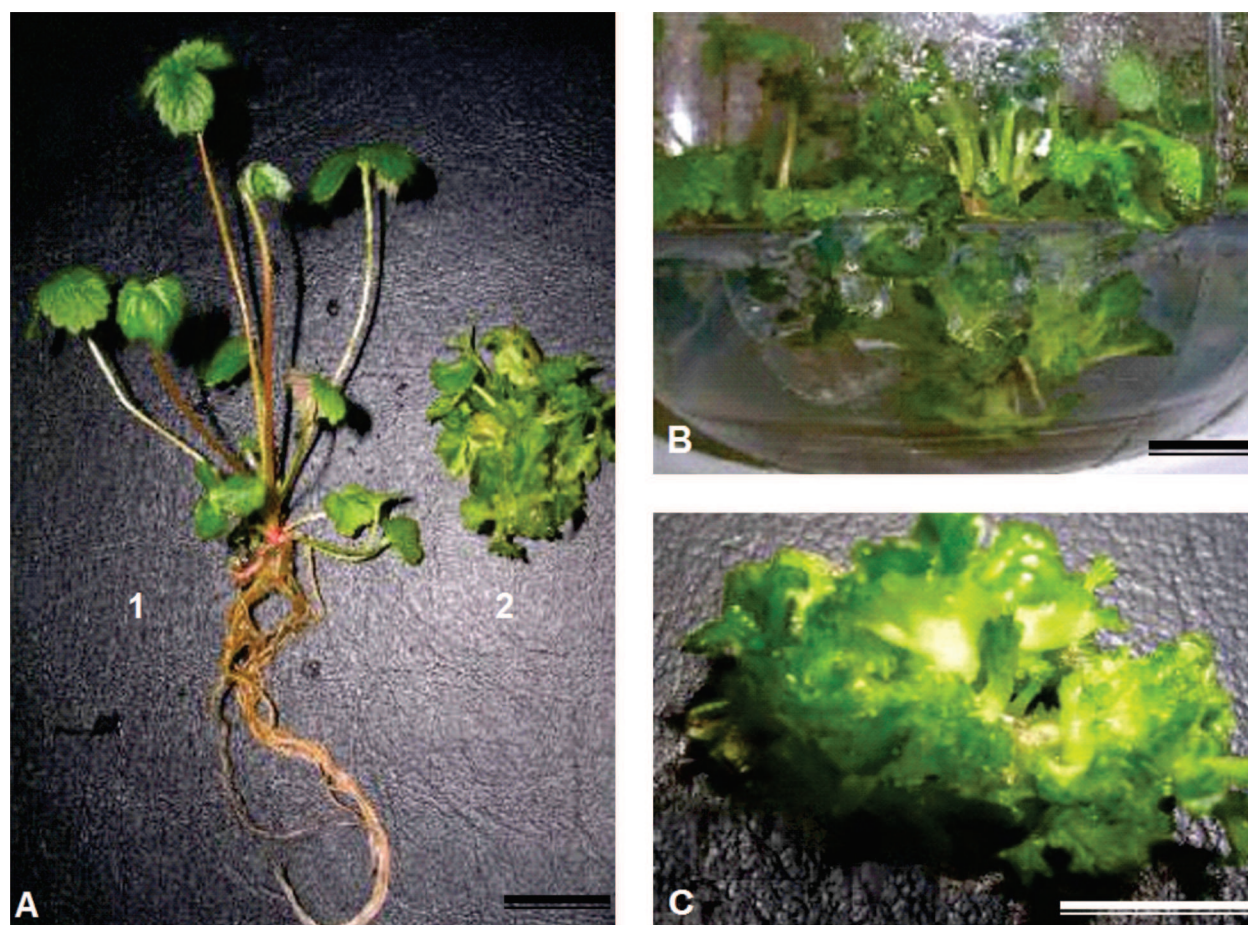
Figure 1. Strawberry shoots (*Fragaria* x *ananassa*). (**A**) Comparison of a normal plant (1) and hyperhydric plant (2) after 35 days of cultivation. (**B**) Detail of a hyperhydric plant of the variety 'Burkley' on MS medium. (**C**) Shoots of the variety 'Dover' with typical hyperhydric symptoms.
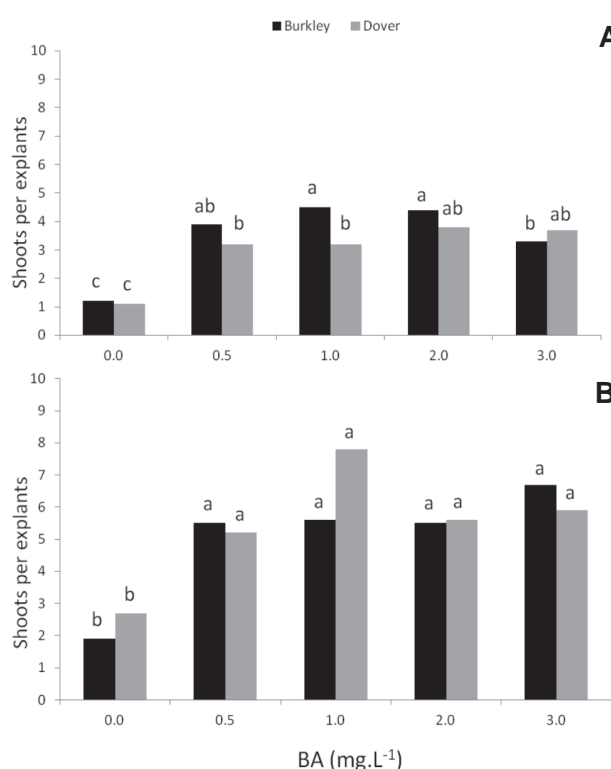
Figure 2. Mean number of shoots per explant of the varieties 'Burkley' (black bars) and 'Dover'(gray bars) under different BA concentrations in MS medium solidified with either agar **(A)** or Phytagel® **(B)**. Bars with different letters are significantly different by the Tukey test at 5% probability.

the smaller size vascular bundles were hypertrophied, especially the epidermal cells, giving the dermal tissue of hyperhydric plants an undulated appearance.

The abaxial and adaxial surfaces of normal and hyperhydric leaves of the two strawberry varieties were analyzed using scanning electron microscopy. This analysis showed epidermal cells with plasmolysed aspect (Figure 3F and HB) and lower deposition of epicuticular wax in hyperhydric plants than in the normal plants (Figure 3E and G). Structural differences were also observed between the two varieties, such as

a higher deposition of epicuticular wax in the variety 'Burkley' (Figure 3F and H).

The strawberry leaf is amphistomatic, with more stomata on the abaxial surface (Figure 3). Abnormal stomata were observed in leaves of plants grown in medium with hyperhydricity inducing factors (Figure 3E and G). These stomata were characterized by hypertrophied and deformed guard cells, unlike the typical elliptical-shaped cells found in non-hyperhydric leaves (Figure 3E and G). Thus, the presence of hyperhydric guard cells was higher in hyperhydric leaves when compared to non-hyperhydric leaves, and these cells were deformed because the cell wall that delimits the stomatal pore was protracted and often disrupted (Figure 3F and H), hence the stomata could not close properly. In addition, the irregular guard cells of the hyperhydric leaves were more round than elongated, which was probably due to a reduction in elasticity or changes in the pattern of cellulose microfibril deposition. Similar results were found in hyperhydric leaves of many plants (Picoli *et al.* 2008; Gupta & Prasad 2010) with abnormalities in the guard cell morphology.

Chlorophyll content in hyperhydric plants was approximately 50% lower than the levels found in normal plants

Throughout the development of shoots, we observed a decrease in the intensity of the green color in hyperhydric shoots when compared with the non-hyperhydric ones. Chlorophyll *a*, *b* and total contents in hyperhydric plants were respectively 50, 60 and 51% lower than the content found in normal plants (Figure 4). Similar results were reported by Sreedhar *et al.* (2009) in hyperhydric tissues of vanilla. The peroxidases seem to play an important role in the reduction of chlorophyll content. In spinach leaves, the chlorophyllases seem to have little involvement in the degradation of chlorophyll and peroxidases are the main enzymes involved in this process (Yamauchi & Watada 1991).

Table 1. Hyperhydricity frequencies (%) in strawberry plants cultured with different BA concentrations in MS medium, solidified with either Agar or Phytagel®

BA (mg L⁻¹)	Hyperhydricity (%)			
	'Burkley'		'Dover'	
	Agar	Phytagel®	Agar	Phytagel®
0.0	0 e A	0 c A	0 d A	0 c A
0.5	36 d A	55 b A	35 c A	60 b A
1.0	52 c A	64 b A	56 b A	60 b A
2.0	80 b A	90 a A	90 a A	76 b A
3.0	100 a A	80 a A	100 a A	100 a A

[1] Means with the same small letters in the columns or capital letters in the rows, for each variety and each BA concentration, are not significantly different by the Tukey test at 5% probability.

Antioxidant metabolism increased in hyperhydric tissues

As noted by Saher *et al.* (2004), the excess water in hyperhydric tissues can lead to saturation levels causing hypoxia. Under this condition of stress, some metabolic activities in H_2O_2-generating plants can be increased, producing toxic levels of H_2O_2 and generating oxidative stress. The existence of oxidative injury in hyperhydric tissues was confirmed in this study, with the increase in the antioxidant enzyme activity and in the MDA content found in hyperhydric leaves when compared with leaves of non-hyperhydric (normal) plants.

There was an increase in the activity of the antioxidant enzymes peroxidase and catalase directly proportional to the BA concentration in the culture medium (Tables 2 and 3). In the variety 'Burkley', the peroxidase (POD) activity increased with the addition of BA either with agar or Phytagel®. In addition, independent of the presence or absence of BA, no significant difference was detected between these gelling agents (Table 2). The results were similar for the variety 'Dover', except for treatments supplemented with agar, in which the peroxidase activity increased up to 1.0 mg L^{-1} BA (Table 3).

The catalase (CAT) activity in both varieties also increased with the BA supplementation to the medium. In media with Phytagel®, the CAT activity in the variety 'Dover' (Table 3) was higher with 2.0 mg L^{-1} BA, while in 'Burkley' (Table 2) it was higher in both 0.5 and 1.0 mg L^{-1}
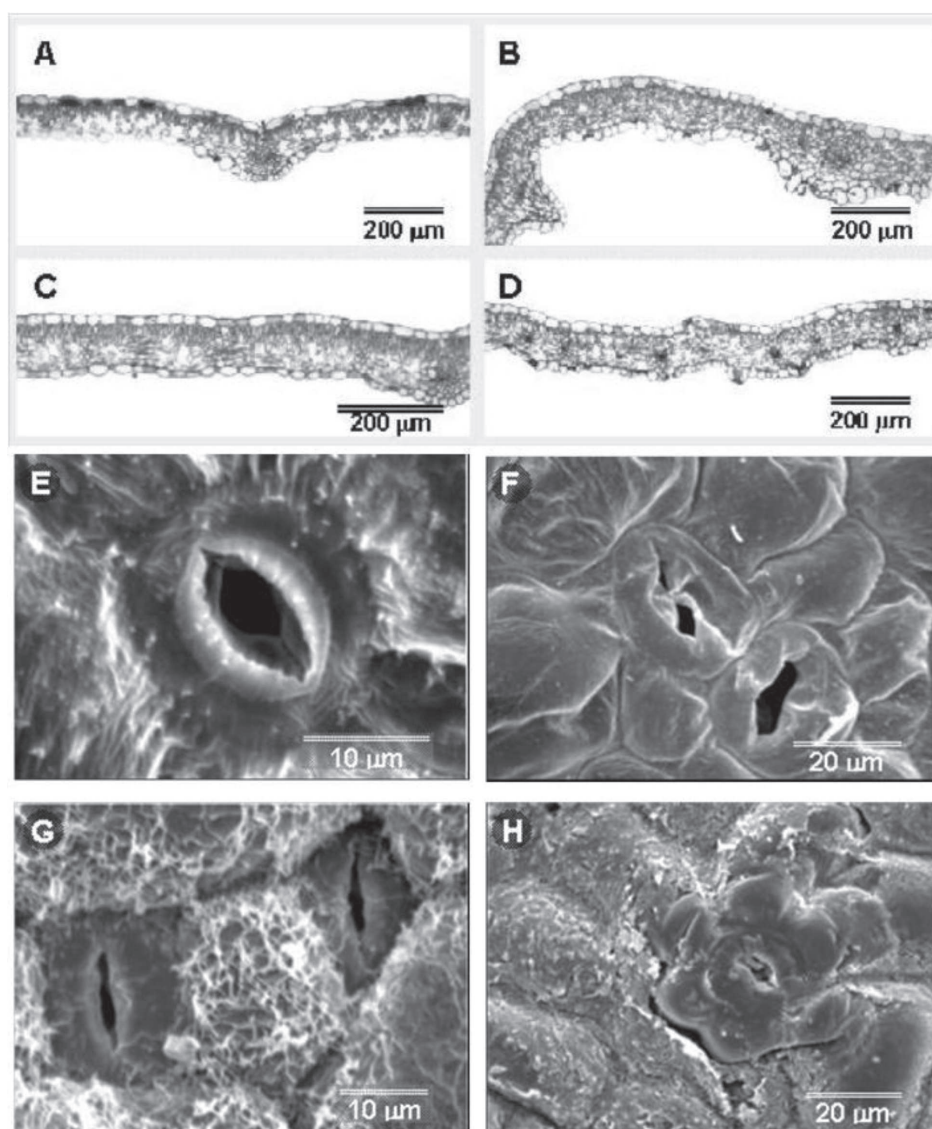
Figure 3. Toluidine Blue-stained transversal sections of strawberry (*Fragaria* x *ananassa* Duch.) leaves: normal (**A** and **C**, *in vitro* plants propagated in medium without growth regulators, and hyperhydric (**B** and **D**) propagated *in vitro* plants in culture medium with BA. Scanning electron microscopy micrographs of the abaxial surface of normal strawberry (*Fragaria* x *annanassa* Duch.) leaves (**E** and **G**) propagated *in vitro* in medium without plant growth regulators, and hyperhydric leaves (**F** and **H**) that were propagated *in vitro* in medium with 3 mg L^{-1} BA.

BA supplemented medium. On the other hand, when Agar was used as the gelling agent, higher catalase activities were observed in the medium with 2.0 mg L^{-1} and 3.0 mg L^{-1} BA, respectively, in both varieties.

The SOD activity was significantly reduced with the addition of BA to the culture medium of the variety 'Burkley'. Conversely, for 'Dover', the SOD activity increased with the 2.0 and 0.5 to 1.0 mg L^{-1} BA treatments, respectively, using agar and Phytagel® as gelling agents.
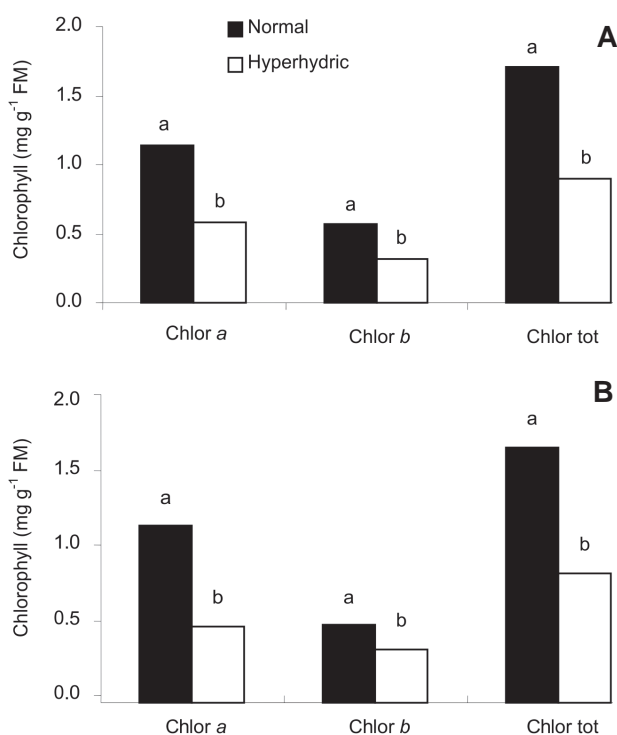
Figure 4. Chlorophyll content in normal and hyperhydric strawberry plants after 35 days in culture, varieties 'Dover' (**A**) 'Burkley' (**B**). Values represent the mean. Bars with different letters for each chlorophyll set differed significantly by the Tukey's test at 5% probability.

The higher peroxidase activity associated with increases in BA concentration and hyperhydricity showed a clear relationship with antioxidant enzyme activity. Among the eleven species tested, only two showed reductions in peroxidase activity, 66 and 42%, when hyperhydric plants were compared with normal plants (Kevers *et al.* 1984). The others showed higher activity in hyperhydric plants in relation to the normal plants.

The varieties 'Dover' and 'Burkley' showed differences in response to the hyperhydricity induction. The latter was more sensitive, with less shoot proliferation compared with the former, showing hyperhydric characteristics in lower BA concentrations. In the variety 'Burkley', the antioxidant mechanisms were more active than in the variety 'Dover', with POD and CAT activities increased with 0.5 mg.L^{-1} BA and basal levels of SOD showing a three-fold increase.

The atmospheric conditions in sealed cultures are analogous to those found in association with waterlogged soils and are associated with reduced transpiration and excessive water uptake, which can result in near hypoxic tissue oxygen concentrations and interference with respiration at the level of electron transport (Rojas-Martinez *et al.* 2010).

Lipid peroxidation was influenced by the BA and gelling agents, added to the culture medium (Table 4). The BA significantly increased the formation of the MDA-TBA complex and, consequently, the lipid peroxidation in both varieties. In the absence of BA, there was no significant difference between the gelling agents used in relation to lipid peroxidation.

The concentration of the MDA-TBA complex in the variety 'Burkley' was higher in the treatment with 0.5 mg L^{-1} BA, regardless of the gelling agent (Table 4). When agar was used in the medium, the addition of 1.0 to 3.0 mg L^{-1} of BA did not differ significantly in the concentration of the MDA-TBA complex, although in the absence of

Table 2. Effect of BA concentrations and gelling agents on the enzyme activity in shoot tissues of strawberry variety 'Burkley' cultured *in vitro*.

| BA(mg L^{-1}) | Enzyme Activity[1] | | | | | |
| | POD[2] | | CAT[3] | | SOD[4] | |
	Agar	Phytagel®	Agar	Phytagel®	Agar	Phytagel®
0.0	2.15 b A	2.63 b A	0.09 c A	0.07 c A	1948.15 a A	1486.81 a A
0.5	20.23 ab A	21.39 ab A	0.46 c B	4.07 a A	261.66 c B	320.43 b A
1.0	24.36 ab A	26.72 ab A	2.21 b A	3.39 a B	537.08 b A	287.08 b B
2.0	34.72 a A	34.44 a A	3.31 a A	2.37 b B	151.90 c A	93.80 c B
3.0	43.80 a A	45.63 a A	2.46 ab A	2.46 b A	157.86 c B	372.93 b A

[1] Means with the same small letters in the columns or capital letters in the rows are not significantly different by the Tukey test at 5% probability.

[2] POD - Peroxidase (mmoles min^{-1} mg^{-1} Fresh Mass - FM).

[3] CAT - Catalase (mmoles min^{-1} mg^{-1} FM).

[4] SOD – Superoxide dismutase (SOD units min^{-1} g^{-1} FM).

Table 3. Effect of BA concentrations and gelling agents on the enzyme activity in shoot tissues of strawberry variety 'Dover' cultured *in vitro*

| BA(mg L⁻¹) | Enzyme Activity[1] | | | | | |
| | POD[2] | | CAT[3] | | SOD[4] | |
	Agar	Phytagel®	Agar	Phytagel®	Agar	Phytagel®
0.0	3.50 d A	3.64 e A	0.06 c A	0.11 c A	585.99 b A	587.51 b A
0.5	32.14 c A	42.90 d A	0.19 c A	0.51 c B	599.84 b B	816.49 a A
1.0	106.97 a A	56.28 c B	0.30 bc A	0.31 c A	383.28 c B	879.07 a A
2.0	93.71 b A	78.81 b A	0.91 b A	1.86 b A	1204.83 a A	364.46 c B
3.0	101.64 ab A	108.49 a B	3.78 a A	3.69 a A	643.29 b A	489.84 bc B

[1] Means with the same small letters in the columns or capital letters in the rows are not significantly different by the Tukey test at 5% probability.

[2] POD - Peroxidase (mmoles min⁻¹ mg⁻¹ Fresh Mass - FM).

[3] CAT - Catalase (mmoles min⁻¹ mg⁻¹ FM).

[4] SOD – Superoxide dismutase (SOD units min⁻¹ g⁻¹ FM).

Table 4. Effect of BA concentrations and gelling agents on the lipid peroxidation in shoot tissues of strawberry varieties 'Burkley' and 'Dover' cultured *in vitro*

| BA (mg L⁻¹) | Lipid peroxidation (nmoles de MDA-TBA g⁻¹ FM) | | | |
| | 'Burkley' | | 'Dover' | |
	Agar	Phytagel®	Agar	Phytagel®
0.0	3.94 c A	4.43 b A	6.41 c A	7.85 c A
0.5	44.81 a A	27.18 a A	12.82 b B	37.66 a A
1.0	18.21 b A	26.70 a A	15.16 b A	12.16 c A
2.0	17.40 b A	18.69 a A	36.70 a A	15.87 bc B
3.0	17.63 b B	23.33 a A	11.11 b A	24.04 b A

[1] Means followed by same small letters in the columns or capital letters in the rows for each variety and for each BA concentration, are not significantly different by the Tukey test at 5% probability.

BA, reduction in the concentration of this complex could be seen. When using Phytagel®, the absence of BA reduced the formation of the MDA-TBA complex. This result was significant in relation to treatments containing BA, but did not differ among the BA concentrations. Although the treatment with 0.5 mg L⁻¹ BA showed higher levels of the MDA-TBA complex, it did not differ significantly from other treatments with BA.

In the variety 'Dover' (Table 4), the concentration of the MDA-TBA complex was higher in the presence of 0.5 mg L⁻¹ BA, when Phytagel® was the gelling agent, and differed significantly from the other treatments. However, the use of agar resulted in an increase, but this was only significant in the presence of 2.0 mg L⁻¹ BA, when compared with the other treatments with and without BA.

CONCLUSIONS

In conclusion, the present study has provided insights into the morphological and biochemical changes that occur during the progression of hyperhydricity in strawberry propagated *in vitro*; mainly in increased cytokinin concentration and when agar was replaced by Phytagel®. The rapid loss of chlorophyll and the concurrent increase in antioxidant enzyme activity are useful biochemical indicators of hyperhydricity in strawberry. The culture medium containing 0.5 mg L⁻¹ BA and solidified with Agar promoted lower hyperhydricity percentages in relation to the higher rates of shoot proliferation and should be applied for efficient strawberry *in vitro* propagation.

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