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The effect of ancymidol on hyperhydricity, regeneration, starch and antioxidant enzymatic activities in liquid-cultured *Narcissus*

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Abstract Addition of the growth retardant ancymidol to *Narcissus* shoots and lower inner leaf sections isolated from shoots cultured in liquid medium induced hyperhydric malformations associated with morphogenetic changes. Meristematic centers initiated on the basal proximal ends appeared over the entire surface of the hyperhydric leaf sections after 6 weeks in culture. The meristematic centers which formed clusters on the leaf sections developed later into buds. In leaf sections grown in the liquid medium lacking ancymidol, hyperhydricity was not induced, and regeneration was not observed. Starch and protein levels and ascorbate peroxidase and catalase activities were examined in shoots and isolated leaf sections that were either hyperhydric or non-hyperhydric. In ancymidol-treated, hyperhydric leaf sections, ascorbate peroxidase and catalase activities were lower than in control, untreated leaf sections. The changes in starch and protein levels and in antioxidant enzymatic activities appeared to be related to the onset of meristematic-center initiation and further bud development on *Narcissus* hyperhydric leaf sections.

Keywords Ancymidol · Antioxidant enzyme activity · Hyperhydricity · Liquid culture · *Narcissus tazetta*

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Abbreviations *AC* Activated charcoal · *ANC* Ancymidol · *APX* Ascorbate peroxidase · *BA* 6-Benzylaminopurine · *CAT* Catalase · *MS* Murashige and Skoog (1962) medium · *NAA* α-Naphthaleneacetic acid

Introduction

The natural propagation rate of *Narcissus*, a bulbous plant, is very slow. Agar cultures are presently being used mainly for its micropropagation and for the establishment of new cultivars and the production of pathogen-free stock material (Seabrook 1990; Hanks 1993). The use of large-scale liquid cultures combined with mechanization (Levin et al. 1988) can greatly reduce the costs involved in the manual handling of geophyte micropropagation. This could be an efficient system for the mass propagation of *Narcissus*.

However, one of the problems encountered in liquid cultures is the phenomenon of hyperhydricity, which results in malformed plants that cannot survive transplanting ex vitro. Hyperhydricity can result from a number of stress reactions caused by abnormal environmental conditions imposed simultaneously in vitro. Franck et al. (1995) and Piqueras et al. (1998) investigated several antioxidant enzymes and found that their activities were related to hyperhydricity and tissue malformation in cultured plants. The growth retardant ancymidol, an inhibitor of gibberellin biosynthesis, has been used to control morphological and physiological disorders, to reduce shoot elongation and to induce meristematic clusters in liquid-cultured plants (Ziv 1991, 1992; Ziv et al. 1998).

Carbohydrate biosynthesis and metabolism is an important aspect of bulb growth and propagation. Starch is the major plant storage carbohydrate in several geophytes, and its concentration varies among species and the type of storage tissues (Miller 1992). Information on the regulation of starch metabolism in tissue-cultured *Narcissus* is very limited.

In the investigation reported here, we looked at bud regeneration from *Narcissus* twin-scales, the use of liquid cultures for propagation and the effects of ANC on meristematic center formation and on starch and antioxidant enzymatic activities in liquid-cultured plants.

Materials and methods

Plant material

Narcissus (*Narcissus tazetta* cv. Ziva) bulbs that had been given a hot water treatment of 43.5 °C for 3 h, with 1.5% formalin to reduce contamination, were used as the source of explants. After drying, the bulbs were kept at 30 °C in a dark chamber until the start of the experiments. Prior to explant preparation, the bulbs were subjected to a cold treatment of 15 °C in the dark for 42 days to break dormancy. They were then soaked in 5% sodium hypochlorite with two to three drops of Tween-20 for 20 min and washed with sterile distilled water three times in a laminar flow transfer hood. Twin-scales (Hanks 1993), 7× 7 mm including 2 mm of the basal plate, were isolated from aseptic bulb parts and used as the initial explants.

Agar cultures

Twin-scales explants were established in 9.5× 2.5-cm test tubes on solidified (0.65% agar, type A, Sigma A-4550) MS media supplemented with 2.5 m*M* sodium phosphate, 0.8 m*M* adenine sulfate, 5 μ*M* NAA, 10 μ*M* BA and without (control) or with (treatment) 5 g/l activated charcoal. Adventitious buds were induced on twin-scales.

Agar medium in culture boxes (Rahan Meristem, Israel) was also used for growing shoots obtained from liquid cultures. Normal shoots started to develop from hyperhydric meristematic leaf section clusters after 6 weeks of subculture on the medium described above but with 0.8% agar.

Liquid cultures

The 4-week-old adventitious buds induced from twin-scales were subcultured in liquid media, similar in composition to agar cultures but without agar and AC, in the absence (control) and presence (treatment) of 9.75 μ*M* ANC. The buds were cultured in 250-ml Erlenmeyer flasks containing 70 ml medium for 4 weeks. The 10-mm-long lower inner leaf sections (Fig. 1) were isolated from the shoots which developed from the adventitious buds in liquid media without and with ANC and subcultured further in the same liquid media. The sequence of the stages of subcultures and the culture period is presented in the flow chart in Fig. 2.

The induction of hyperhydric meristematic leaf section clusters and proliferation were carried out in ANC liquid medium in flasks and disposable presterilized plastic bioreactors (for details, see Ziv et al. 1998).

Culture conditions

Both agar and liquid cultures in flasks and bioreactors were kept in a growth room at 25°/22 °C (day/night) under a 16-h day length with light supplied by cool-white fluorescent lamps (70 μmol m^{-2} s^{-1}). The flasks were rotated on a gyratory shaker at 70 rpm.

Sample preparation

Samples of shoots and leaf sections cultured in liquid media were collected, rinsed three times with distilled water and dried on filter paper. The first sample was collected before culture initiation.

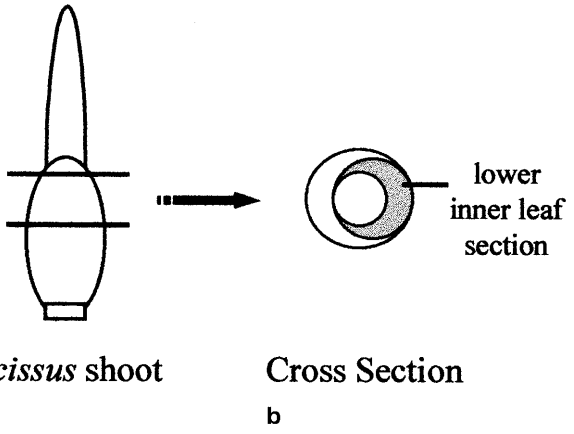

Fig. 1 A shoot (**a**) and cross section (**b**) showing the lower inner leaf section of an explant isolated from the shoot

Starch assay

Fresh plant material was dried in an oven at 55°–60 °C for 48 h, then macerated with a pestle and mortar to a fine powder. Dried powder (0.5 g) of each plant sample was used for starch determination. This was performed colorimetrically after the hydrolysis of the starch to glucose according to Thivend et al. (1972) in a UVIKON 810 (Kontron Instruments, Switzerland) spectrophotometer at 570 nm.

Enzyme assays

Samples (0.2 g fresh weight) from shoots and leaf sections were cut vertically starting at the basal part, macerated and extracted with 2 ml 50 m*M* potassium phosphate buffer (pH 7.8). Extracts were centrifuged at 4 °C, 10,000 rpm for 10 min. Enzymatic activities were determined by following the continuous changes in optical absorbance in a UVIKON 810 spectrophotometer.

Ascorbate peroxidase was determined according to Asada (1984) by measuring the decrease in absorbance at 290 nm as ascorbate is oxidized. The reaction mixture (1 ml) contained 50 m*M* potassium phosphate at pH .0, 0.5 m*M* ascorbate, 0.1 m*M* H_2O_2 and the enzyme extract.

Catalase (CAT) was determined using a modified method of Klapheck et al. (1990) by measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption. The reaction mixture (1 ml) contained 50 m*M* potassium phosphate buffer at pH 7.0, 0.036% (w/w) H_2O_2 and the enzyme extract.

Protein levels were determined spectrophotometrically at 595 nm according to the Bradford method (1976).

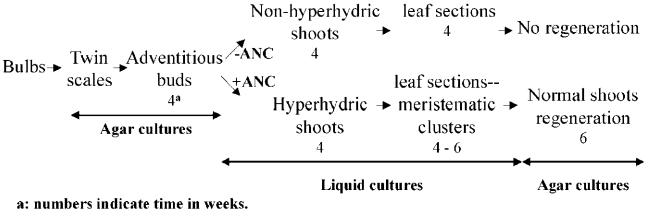

Fig. 2 Flow chart showing the sequence of the stages of subcultures and the culture period

Statistical methods

Results were analyzed weekly over a period of 4 weeks with 15 replications each; the experiments were repeated at least three times. One-way analysis of variance was carried out and the results are presented as the mean ± standard error (SE).

Results and discussion

Adventitious bud regeneration

Adventitious buds regenerated from twin-scales cultured on agar-solidified MS media without and with AC after 4 weeks. The presence of AC significantly increased the bud regeneration potential of *Narcissus* bulb explants and is probably related to the removal of unidentified inhibitory substances and tissues exudates from the culture medium. The outer twin-scales had a significantly higher regeneration potential than the inner ones when cultured on the medium with AC. In subsequent experiments, only the outer scales were isolated from the bulbs for twin-scales explants. An average of 12 adventitious buds was induced from each outer twin-scales on the medium with AC. On the medium with AC, the number of adventitious buds induced from both outer and inner twin-scales, their fresh weight, diameter and length were significantly higher than those obtained on the medium without AC, as can be seen in Table 1.

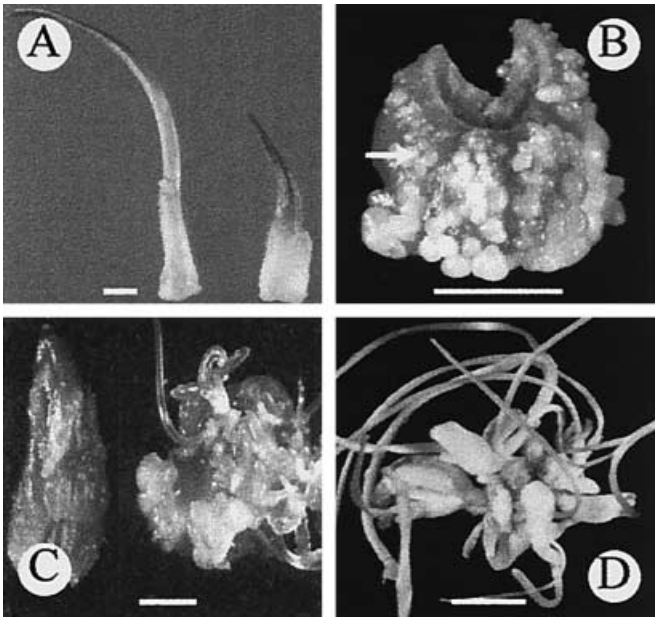

Fig. 3 **A** Non-hyperhydric (*left*) and hyperhydric (*right*) shoots induced in liquid media in the absence (control) and presence (treatment) of ANC. **B** A hyperhydric leaf section cultured in ANC medium showing a cluster with numerous meristematic centers after 6 weeks. *Arrow* Meristematic center. **C** Leaf sections after 8 weeks of culture in liquid medium. *Left* Without ANC, no regeneration of buds; *right* with ANC, high regeneration. **D** Shoot and bulblet development from a meristematic cluster on agar medium after 12 weeks. *Bar* **(A–D)**: 1 cm

Meristematic cluster formation and shoot regeneration

Buds induced from the outer twin-scales explants on agar-solidified medium containing AC were subcultured in liquid media in the absence and presence of ANC. After 4 weeks in culture, non-hyperhydric shoots elongated in the liquid medium lacking ANC (Fig. 3A, left), whereas hyperhydric malformed shoots were observed in the liquid medium with ANC (Fig. 3A, right; Table 2). The lower inner leaf sections were isolated from the non-hyperhydric and hyperhydric shoots and subcultured in the same liquid media in which the shoots were initially induced.

Meristematic centers began forming on the ANC-treated, lower peripheral tissues of leaf section explants after 3 weeks in culture. After 6 weeks, the meristematic centers covered the entire surface of ANC-treated hyperhydric leaf sections, forming meristematic clusters (Fig. 3B). In comparison, no meristematic centers or regeneration were observed on the leaf sections cultured in the liquid medium lacking ANC (Fig. 3C, left). A strong regeneration response, with an average of 35 buds per leaf section meristematic cluster, was observed after 8 weeks culture in ANC liquid medium (Fig. 3C, right). Whole meristematic clusters were subcultured for further proliferation in flasks and in disposable presterilized plastic bioreactors containing ANC liquid medium in which the meristematic clusters were originally induced. The biomass increase of the meristematic clusters in the bioreactors

Table 1 The number of buds regenerated from the outer and inner twin-scales, their weight, diameter and length after 30 days of culture in semi-solid agar media (mean ± SE)

Treatments	Number of buds		Weight (mg)		Diameter (mm)		Length (mm)	
	Outer	Inner	Outer	Inner	Outer	Inner	Outer	Inner
Without AC (control)	1.3 ± 0.5	1.0 ± 0.3	3.6 ± 1.2	3.9 ± 1.4	0.7 ± 0.2	0.8 ± 0.2	1.5 ± 0.5	1.8 ± 0.4
With AC	11.9 ± 0.6	4.2 ± 0.6	21.6 ± 2.5	27.8 ± 7.1	2.2 ± 0.6	2.2 ± 0.3	5.9 ± 0.6	5.6 ± 0.7
t-test	***	***	***	**	**	***	***	***

,* Significant at $P < 0.01$ and 0.001, respectively, when with AC and without AC are compared

was fivefold that in the flasks (data not shown). An average of 19 normal shoots developed per cluster upon subculture on medium similar to that used for bud induction on twin-scales but with the addition of 0.8% agar (Fig. 3D).

Starch and protein levels

Starch levels increased significantly during the 3rd and 4th weeks in leaf sections cultured in the medium with ANC and were significantly higher than in those leaf sections cultured in the medium lacking ANC (Fig. 4).

Protein levels

Total soluble protein level decreased after 2 weeks in shoots cultured in both media but increased slightly during the 4th week. The temporal increase in protein levels of the lower inner leaf sections cultured in media without and with ANC reached a maximum after 3 weeks. During this period, meristematic centers started to form at the basal parts of the leaf sections cultured in the medium with ANC.

Protein levels were higher during the 3rd and 4th weeks in both shoots and leaf sections cultured in the medium lacking ANC vs. their counterparts in ANC-containing medium (Fig. 5).

Changes in antioxidant enzymatic activities in shoots and leaf sections

Ascorbate peroxidase activity

APX activity decreased after 2 weeks in shoots cultured in both media; during that time, hyperhydricity and shoot malformation were observed in ANC medium (Fig. 6, shoots). APX activity was slightly higher in the shoots cultured in ANC medium than in those in the medium without ANC during the 4th week, when hyperhydric shoots were clearly visible. In the leaf sections, APX activity increased during the 1st and 2nd weeks in both media; this increase was significant during the 2nd week, when it reached its highest level. Between the 2nd and 3rd weeks, APX activity decreased and reached a relatively stable level. APX activity was significantly higher during the 4th week in leaf sections cultured in medium without ANC than in

Table 2 Growth and length of non-hyperhydric and hyperhydric shoots and leaf sections induced in liquid media after 4 weeks in culture (mean ± SE). Growth value = (final fresh weight–initial fresh weight)/initial fresh weight

Treatments	Growth value		Average length (cm)
	Shoots	Leaf sections[a]	Shoots
Non-hyperhydric (−ANC)	48.2 ± 4.4	8.5 ± 3.7	10.1 ± 0.2
Hyperhydric (+ANC)	33.5 ± 6.6	4.9 ± 0.9	4.6 ± 0.1
t-test	**	**	***

,* Significant at $P < 0.01$ and 0.001, respectively, when with ANC and without ANC are compared

[a] Lower inner leaf sections – 10 mm long

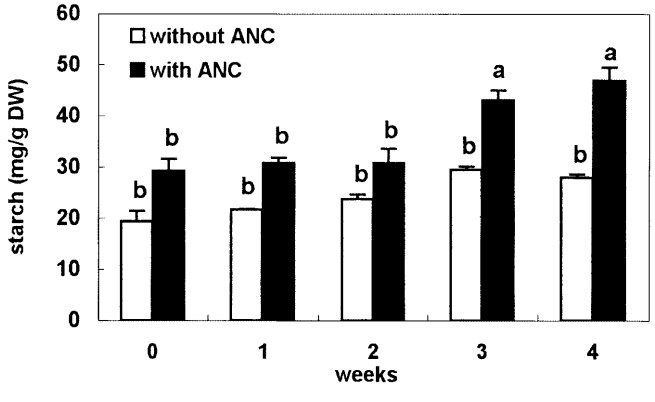

Fig. 4 Starch accumulation in leaf sections cultured for 4 weeks in liquid media in the absence (control) and presence of ANC. *Bars* with *different letters* are significantly different at $P = 0.05$

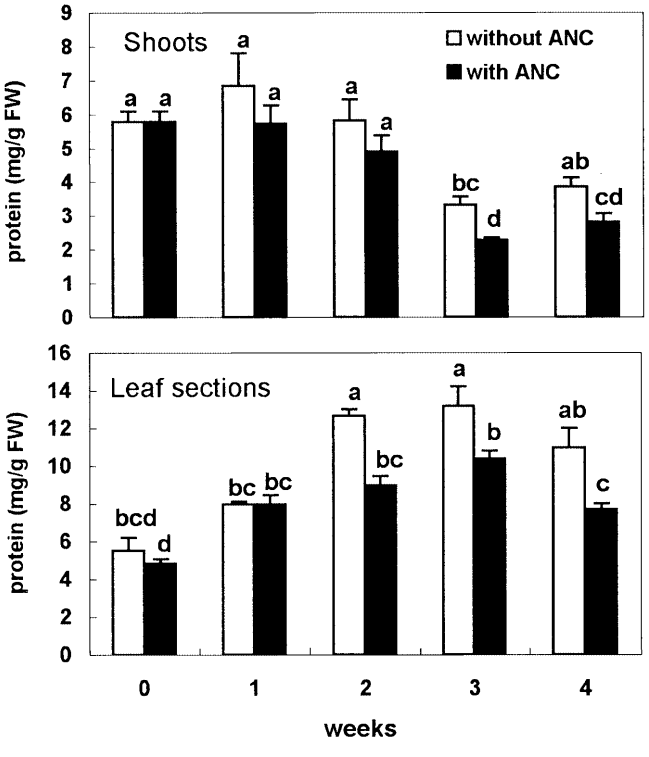

Fig. 5 Protein level changes in shoots and leaf sections cultured for 4 weeks in liquid media in the absence and presence of ANC (shoots were in ± ANC media for 0–4 weeks; leaf sections were then isolated from the shoots and subcultured in ± ANC media for another 0–4 weeks). *Bars* with *different letters* are significantly different at $P = 0.05$

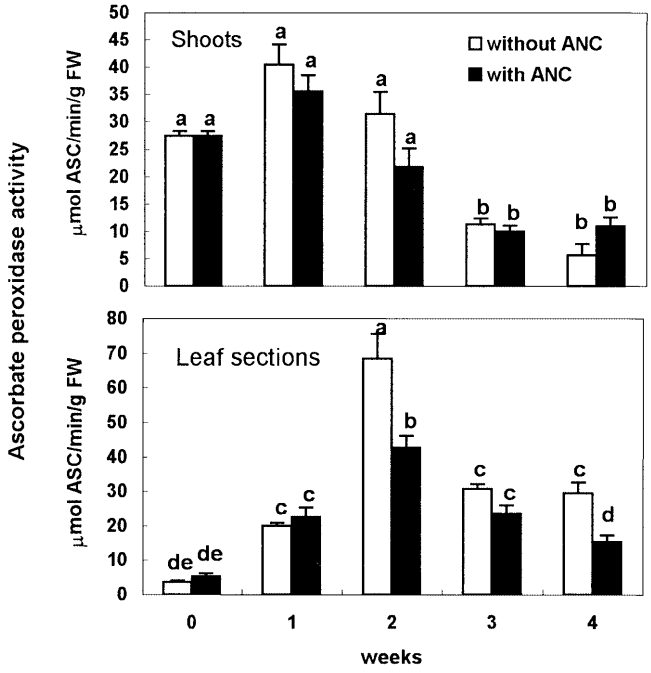

Fig. 6 Ascorbate peroxidase activity in shoots and leaf sections cultured for 4 weeks in liquid media in the absence and presence of ANC. *Bars* with *different letters* are significantly different at $P=0.05$

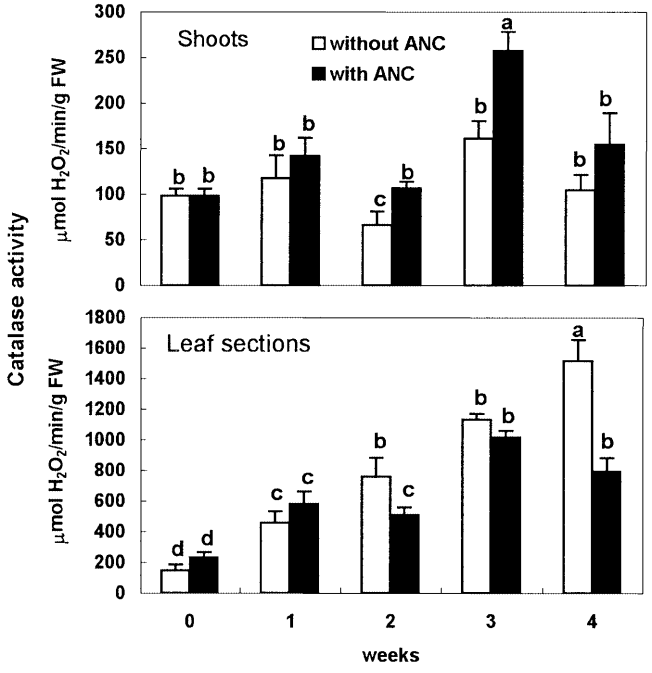

Fig. 7 Catalase activity in shoots and leaf sections cultured for 4 weeks in liquid media in the absence and presence of ANC. *Bars* with *different letters* are significantly different at $P=0.05$

those cultured in medium with ANC (Fig. 6, leaf sections). In general, APX activity was lower in ANC-treated leaf sections during the 2nd to 4th weeks.

Catalase activity

CAT activity was significantly higher after 3 weeks in the shoots cultured in medium with ANC than in those cultured without ANC, when shoot hyperhydricity was observed (Fig. 7, shoots). In the leaf sections cultured in the medium lacking ANC, CAT activity increased constantly, reaching its highest level during the 4th week. In the leaf sections treated with ANC, CAT activity increased after 2 weeks of culture, then decreased slightly during the 4th week. CAT activity was significantly lower in the leaf sections cultured in medium with ANC than in those without ANC during the 4th week (Fig. 7, leaf sections). At that time meristematic centers started to appear all over the ANC-treated leaf sections; these subsequently formed meristematic clusters.

Plant growth retardants have been reported to affect cell division and cell enlargement, probably by interfering with gibberellin and sterol biosynthesis (Dicks 1980), as well as to alter protein biosynthesis and membrane permeability (Grossmann et al. 1986). Ziv (1991) applied the growth retardants ANC and paclobutrazol to liquid-cultured plants to reduce or inhibit shoot malformation and hyperhydricity. In the present experiments, it appears that ANC actually enhanced hyperhydricity in *Narcissus* shoots and leaf sections cultured in liquid medium and significantly inhibited shoot elongation. At a later stage, ANC enhanced the formation of meristematic centers on the hyperhydric leaf sections and the formation of clusters. Preliminary results indicate that there is a significantly lower level of gibberellin, as much as twenty-fold, in ANC-treated leaf sections, which may explain the inhibition of shoot elongation (Chen, unpublished).

In liquid-culture systems, the tissue is constantly submerged in the medium, a condition which may cause oxygen deprivation and aeration stress. Ziv (1991) reported that the conditions in liquid cultures can affect enzyme activity and result in various changes in plant morphogenetic and metabolic processes. High levels of ethylene evolution (Kevers et al. 1984; Ziv and Arie 1991) have been observed in hyperhydric tissue cultured *Dianthus* leaves. Franck et al. (1995) found higher superoxide dismutase activity and lower CAT, APX and glutathione reductase activities in hyperhydric shoots of *Prunus avium* L. Piqueras et al. (1998) found that the activities of superoxide dismutase and CAT were highest in hyperhydric shoots of *Nicotiana tabacum* L. in vitro. In *Narcissus* shoots and leaf sections cultured in liquid media, a concentration of 9.75 μM of the N-bearing heterocyclic growth retardant ANC affected antioxidant enzymatic activities relative to control shoots and leaf sections from a liquid medium without ANC. APX activity was lower in ANC-treated shoots during the 1st and 2nd weeks before the shoots became hyperhydric. During the 4th

week, when hyperhydric shoots had already formed, APX activity was higher, which is different from the situation observed in *Prunus avium* L. CAT activity was higher in ANC-treated shoots during the 4-week culture period and increased significantly during the 2nd and 3rd weeks when hyperhydricity was observed in the shoots; this situation is different and similar to the results observed in hyperhydric shoots of *Prunus avium* L. and *Nicotiana tabacum* L. respectively.

Narcissus shoots cultured in liquid media consisted of leaves, scales, the basal plate, axillary buds and meristems. The results obtained from shoots appear to be complex, probably because of the correlative relation of the various organs. The use of isolated leaf sections overcame this complexity. Starch was found to be the dominant storage carbohydrate in *Nerine* leaf scales (Vishnevetsky et al. 2000). In ANC-treated *Narcissus*, higher amount of starch accumulated in hyperhydric leaf sections, presumably serving as a source of energy for the formation of meristematic centers and clusters, as reported also for *Nicotiana* bud regeneration (Murashige and Nakano 1968). APX and CAT activities were significantly lower in the leaf sections cultured in ANC medium than in those cultured in medium without ANC. The lower activities of these enzymes could be correlated to meristematic center initiation and the development on hyperhydric leaf sections during the 2nd and 4th weeks. Significant differences in APX and CAT activities were observed shortly before or after the shoots became hyperhydric and when meristematic centers began forming on the leaf sections. It is possible that hyperhydric malformation is the cause for the increase in APX and CAT activities in the shoots. The formations of meristematic centers may have triggered the decrease in APX and CAT activities in the leaf sections. Our results suggest that hyperhydric shoots and the formation of meristematic clusters in liquid-cultured *Narcissus* are closely related with changes in antioxidant enzymatic activities as well as with changes in protein and starch levels.

Conclusion

ANC-enhanced hyperhydricity appears to be an initial requirement for the formation of meristematic centers on *Narcissus* leaf sections. Starch and protein levels, as well as antioxidant enzyme activities, were correlated with hyperhydricity and with the formation of meristematic clusters. These clusters may constitute a suitable plant system for further proliferation and biomass production in liquid media in flasks and bioreactors. The significant increase of biomass in the bioreactors was probably due to better aeration. Normal shoots can be regenerated from the clusters after subculture to MS semi-solid agar medium. The use of this system is suggested for large-scale micropropagation of *Narcissus*.

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