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Differential activity of catalase and superoxide dismutase in seedlings and in vitro micropropagated oak (*Quercus robur* L.)

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Abstract Catalase (CAT) and superoxide dismutase (SOD) activity profiles were examined by native polyacrylamide gel electrophoresis in different tissues of seedlings and microcuttings of oak (*Quercus robur* L.) initiated from crown material (NL100A) and from basal epicormic shoots (NL100R), which differ in rooting ability. Two CAT isoforms were differentially active in seedlings and microcuttings; in particular, CAT-2 was activated in the basal callus of rooted microshoots. SOD isoenzymes, Mn-SOD and at least four Cu/Zn-SODs were found to be present, with Mn-SODs particularly active in microcuttings. No differences were found between the electrophoretic profiles of the two lines despite their different ontogenetic origin. The strong activity of CAT-2 in rooted microshoots indicates that this isoform is a protein specifically related to rooting.

Keywords Catalase · Micropropagation · *Quercus robur* · Rootability · Superoxide dismutase

Abbreviations *BA* Benzyladenine · *DTT* Dithiothreitol · *EDTA* Ethylenediaminetetraacetate · *IBA* Indolebutyric acid · *NBT* Nitro blue tetrazolium · *PVPP* Polyvinylpolypirrolidone

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Introduction

Micropropagation has the potential to produce large numbers of genetically identical plants at an affordable cost; however, there are some technical limitations that need to be answered before the process can be used in conventional tree improvement programmes. The cloning of mature trees is generally preferred over that of juvenile specimens as it often is not possible to determine if the latter have the genetic potential to develop the desired qualities later in their life cycle. Even though some tree species can be micropropagated from tissues collected from mature trees, for many others propagation is possible only from tissues of juvenile material, and several species, such as oak *Quercus robur* L, rapidly lose the expression of juvenility traits during early development and appear to be recalcitrant to micropropagation and the rooting of cuttings. Oak older than about 6 years cannot be propagated by classical methods except by grafting. Recently, however, the establishment and multiplication of shoot cultures derived from mature *Q. rubra* and *Q. robur* trees have been achieved using explants that originated from crown material and from basal epicormic shoots, and the requirements for in vitro rooting of the shoots were defined (Sanchez et al. 1996).

The potential of micropropagation techniques would be further enhanced by the availability of convenient and reliable markers to define the physiological state or rooting competence of the explants. Consequently, research has been directed towards identifying biochemical markers that may detect changes at a cellular level before morphological changes occur (De Klerk 1996; Greenwood 1997). After searching for indicators of development at the cellular level we focused our attention on isoenzymes. Isoesterases and isoperoxidases have been demonstrated to be useful markers of phase change in *Sequoia sempervirens* (Huang et al. 1996). The rejuvenation was correlated with both the disappearance of adult-associated esterase and peroxi-

dase isoenzymes and the appearance of isoforms characteristic of juvenile phase shoots. In contrast, peroxidases have been demonstrated to be ineffective as early markers for rooting initiation in oak. More specifically, an analysis of two lines with different rooting abilities demonstrated that rooting was associated with an increase in total peroxidase activity but not with changes in the levels of specific isoperoxidases (Welander et al. 1998).

The potential of isoenzymes as developmental markers can be further exploited using enzymes like catalase (CAT) and superoxide dismutase (SOD). Catalase (EC 1.11.1.6, $2H_2O_2 \rightarrow 2H_2O + O_2$) has been investigated extensively in the last few years because of its physiological significance in protecting cells from reactive oxygen species (ROS) and because this gene-enzyme system has been proven to be an excellent model for the study of differential gene expression under normal and stress environments (Wadsworth and Scandalios 1989; Racchi and Terragna 1993; Siminis et al. 1994; Racchi et al. 1996; Bagnoli et al. 1998). Biochemical and molecular aspects of CAT multiplicity have been studied thoroughly in maize (Scandalios 1987), cotton (Ni and Trelease 1991) and, more recently, in gymnosperms (Mullen and Gifford 1993). Recent data suggest that different classes of CATs may be discriminated on the basis of their expression profile. A nomenclature has been adopted for this classification (Willekens et al. 1995): class I, CATs expressed in photosynthetic tissues and regulated by light; class II, CATs expressed at high levels in vascular tissues; class III, CATs highly abundant in seeds and in young seedlings while almost totally absent in later developmental stages. Such a differential expression could represent a powerful tool in analysing the different phases of development.

SODs (EC 1.15.1.1, $2O_2^- + 2H^+ \; H_2O_2 + O_2$) are a family of metallo-enzymes whose presence has been demonstrated in both the cytosol and different cell organelles. Three types of SODs have been classified on the basis of the metal present at the catalytic site: Cu/Zn-SOD, Mn-SOD, and Fe-SOD (Fridovich 1989). Plants generally contain Cu/Zn-SOD in the cytosol and chloroplasts, Fe-SOD in chloroplasts and Mn-SOD in the mitochondrial matrix and peroxisomes (Palma et al. 1986; Salin 1988; Asada 1992; Bowler et al. 1994).

Because of the distinct tissue and sub-cellular distribution of CATs and SODs along with their sensitivity to stress conditions, the present study was focused on an evaluation of their suitability as biochemical markers of the developmental state in two lines of oak differing with respect to ontogenetic age and rootability. Accordingly, we investigated the CAT and SOD electrophoretic profiles in different tissues of the two lines during shoot multiplication and root induction with the aim of finding isoforms which were active in a phase-related manner.

Materials and methods

Plant material and sampling

In *Quercus robur* seed development lacks the terminal period of rapid desiccation that is generally present in orthodox seeds. Because of their high moisture content at shedding, oak seeds have the potential to germinate immediately and are highly desiccation sensitive (Finch-Savage and Blake 1994). Consequently, after the harvest, acorns were put on a moist, heat-sterilised paper held in plastic trays covered with transparent propagator lids; these were placed under fluorescent light (Osram daylight tubes; photon flux density of 60 μmol $m^{-2} s^{-1}$) at 20° ± 1 °C. Radicles, epicotyls and leaves were sampled from seedlings 8–10 cm in height. All samples were frozen in liquid nitrogen and stored at –80 °C until used.

Shoot culture of *Q. robur* material consisted of two lines which were established from the crown and from the root collar region of a 100-year-old tree, designated NL100A and NL100R, respectively (Evers et al. 1993), kindly provided by M. Welander (Department of Horticulture, University of Agriculture Science, Alnarp, Sweden). In both lines clonal shoot multiplication was achieved by subculturing decapitated shoot on Woody Plant Medium (WPM: Lloyd and McCown 1980) containing 20 g/l sucrose, 0.2 mg/l BA and 6 g/l agar (Bacto agar, Difco). Six to seven shoots were cultured per jar in 300-ml glass jars under a 16 h (light)/8 h (dark) photoperiod (fluorescent lighting supplied by Osram daylight tubes; photon flux density of 60 μmol $m^{-2} s^{-1}$), at 21 ± 1 °C; subculturing was monthly.

Root initiation

For root initiation, shoots 25 mm long were excised from proliferating culture and their basal halves stripped of leaves. The rooting medium consisted of 1/3 medium WPM supplemented with 2 mg/l IBA. The shoots were cultured, six per jar, in 300-ml glass jars containing 60 ml of rooting medium with or without IBA. The shoots were kept in the rooting medium with IBA for a period ranging from 1 to 5 days and then transferred onto WPM hormone-free medium and kept under a 16 h (light)/8 h (dark) photoperiod (fluorescent light supplied by daylight Osram tubes; photon flux density of 60 μmol $m^{-2} s^{-1}$), at 21° ± 1 °C. The rooting percentage was scored after 4 weeks. For each treatment and clone there were ten replicate jars with six shoots per jar. The activities of the enzymes were assayed after 3 days of culture in rooting medium with IBA and after 4 weeks on WPM hormone-free medium following IBA treatment.

Preparations of crude extracts

Each shoot was cut into stem and leaves, shoot basal end, callus and radicle; all samples were frozen in liquid nitrogen and stored at –80 °C until used. Samples were powdered with liquid nitrogen and then extracted in a proportion of 1/10 (w/v) in extraction buffer. The extraction buffer used to determine SOD activity was composed of 0.1 *M* potassium phosphate buffer, pH 7.8, 2 m*M* EDTA, 8 m*M* $MgCl_2$, 4 m*M* DTT, 0.1% (v/v) Triton X-100 and 2% (w/v) PVPP; for CAT activity measurement the extraction buffer was the same but had a pH of 7.2 and 1% (v/v) Triton X-100. Homogenates were centrifuged at 4 °C at 14,000 *g* for 10 min, and the supernatant was subjected to dialysis overnight at 4 °C against 1,000 volumes of 0.1 *M* potassium phosphate buffer, pH 7.8 or pH 7.2, respectively, containing 2 m*M* EDTA (Bagnoli et al. 1998). The samples were used for activity assays and for native PAGE (polyacrylamide gel electrophoresis)

Enzyme assays

CAT activity was determined by measuring the initial rate of H_2O_2 decomposition at 240 nm (: 0.0036 mM^{-1} cm^{-1}) (Havir and McHale 1987) in a reaction mixture consisting of 50 m*M* potassium phosphate, pH 7.0 and 12.5 m*M* H_2O_2.

SOD activity was determined by the ferricytochrome-*c* assay method using xanthine/xanthine oxidase as the source of superoxide radicals, according to McCord and Fridovich (1969).

Protein content was determined by the method of Lowry (Lowry et al. 1951) as modified by Peterson (1977), using bovine serum albumin as the standard. Experiments were repeated, and the values reported in the table are the means ± standard errors (SE) of at least three independent experiments with ten replicates per line. Means were grouped according to Gabriel's simultaneous test procedure.

Electrophoresis

SOD and CAT isoenzyme patterns were identified by native PAGE on 15% and 7.5% acrylamide gels, respectively. Defined amounts of protein, 20 μg for CAT and 100 μg for SOD, were loaded in each lane, and electrophoresis was run in 0.03 *M* Tris-glycine buffer pH 8.8 at 4 °C using a Mini-Protean system Bio-Rad. SOD activity was visualised by the NBT staining method according to Beauchamp and Fridovich (1971). The different isoenzymes of SOD were identified by staining parallel gels previously incubated at 25 °C for 20 min in either 50 m*M* potassium phosphate buffer pH 7.8 or in buffer containing 2 m*M* KCN or 5 m*M* H_2O_2. Cu/Zn-SODs are inhibited by KCN and H_2O_2 and Fe-SODs are inactivated by H_2O_2, whereas Mn-SODs are resistant to both inhibitors (Fridovich 1989).

CAT activity staining was performed according to Chandlee and Scandalios (1983) after pre-treating the gels in 0.01% (v/v) H_2O_2 for 10 min. The stain mixture contained 1% (w/v) $FeCl_3$ and 1% (w/v) $K_3Fe(CN)_6$ in distilled water.

Results and discussion

The best rooting responses, in both the lines, were obtained with 3 days of IBA treatment (Fig. 1). The rooting percentage was significantly higher for NL100R than for NL100A. Moreover NL100R showed a higher rooting percentage and a faster response to IBA. In fact, after 1 and 2 days of treatment the rooting percentage of NL100R was double that of NL100A. These data are in agreement with previous results obtained by Welander and Nystrom (1997).

The NL100R shoot culture line, developed from the basal sprout of the tree, had a higher rooting rate than the crown-derived culture, NL100A. The different ontogenic origin of the shoots, due to the location of the original material on the tree and reflecting a gradient of increasing maturity from bottom to the top, may account for this result.

In general, CAT and SOD activities in the micropropagated shoots were substantially similar between the two lines (Table 1). The specific activities of the enzymes, particularly catalase, were high in the leaves and declined in the stems.

At day 3 after IBA treatment (Table 2) no appreciable changes in the specific activity of the enzymes were observed in the shoot basal ends in comparison to T_0. After 4 weeks of culture in rooting medium the CAT activity of basal callus developed at the basal end of unrooted shoots showed a significant increase, while no differences were observed in the basal callus of rooted shoots. On the other hand, SOD activity in the same experiment did not vary significantly, and no remarkable differences were observed between the two lines (Table 2). CAT and SOD specific activities measured in microshoot tissues (Table 1) were twofold higher than those of the growing seedlings (Table 3).

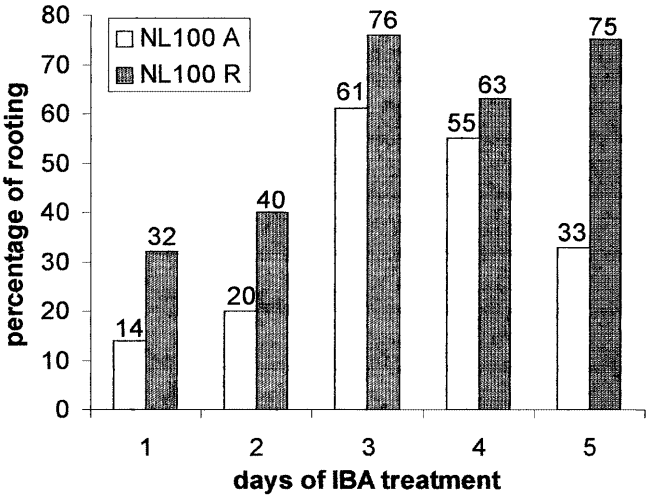

Fig. 1 Rooting percentage of micropropagated *Quercus robur* shoots originating from two parts of an adult oak tree after 4 weeks of culture on rooting medium. *NL100A* epicormic shoots from the main trunk, *NL100R* stem sprouts from the root collar

These results suggest the need for an additional defence against oxidative stress in the cultured tissues and, in the case of CAT, particularly in the callus developed at the basal part of unrooted shoots.

SOD converts the oxygen superoxide anion and generates H_2O_2, which is eliminated through the increase in the activity of the defence enzymes, such as catalase. Intense SOD and CAT activities have been reported in vitro during horse chestnut somatic embryogenesis (Bagnoli et al. 1998) and during protoplast division (Siminis et al. 1994; de Marco and Roubelakis-Angelakis 1996), thereby supporting the occurrence of oxidative stress during the culture. The in vitro culture definitely imposes abnormal environmental conditions – for example, a high level of hormones, high ammonium content of the culture medium, high relative humidity in the flask atmosphere – that contribute in modifying the metabolism of cells. In the case of oak, all of the cultured in vitro tissues showed a high level of activity of the two enzymes irrespective of the ontogenetic origin of the line; moreover, a significant increase was found in the callus developed at the basal part of unrooted shoots. On the whole, our data indicate the occurrence of a change in cell metabolism that does not seem to be related to the origin of the explants. In fact, both lines NL100A and NL100R, despite being ontogenetically different (NL100R considered to be juvenile and NL100A to be more mature), showed the same increase in CAT and SOD activities in all the tissues analysed. These results cannot be linked to differences in the genetic back-

Table 1 CAT and SOD specific activities in microshoot tissues of NL 100 *Quercus robur* clones after 4 weeks of subculture on WPM. Values are expressed as the mean ± SE of at least three experiments with ten replicates per line. All differences between lines within tissues are not significant

	Catalase (U/mg protein)		Superoxide dismutase (U/mg protein)	
	NL100A	NL100R	NL100A	NL100R
Shoot basal end	27.2 ± 3.1a,b*	21.6 ± 2.8a	49.0 ± 3.2a	48.3 ± 3.9a
Stem	19.1 ± 2.9a	16.0 ± 1.7a	36.6 ± 2.8b	35.8 ± 3.2a
Leaf	38.4 ± 3.4b	40.4 ± 4.2b	51.0 ± 3.5a	45.8 ± 4.2a

* Mean values of tissues with the same letter within each line are not significantly different at $P = 0.05$ as determined by the Gabriel range test

Table 2 CAT and SOD specific activities in microshoot tissues of *Q. robur* at the beginning of the experiment and after 4 weeks of culture on hormone-free medium following IBA treatment. Values are expressed as the mean ± SE of at least three experiments with ten replicates per line. All differences between lines within tissues are not significant

	Catalase (U/mg protein)		Superoxide dismutase (U/mg protein)	
	NL100A	NL100R	NL100A	NL100R
Shoot basal end (T_o)	29.2 ± 3.1a*	23.9 ± 3.2a,b	46.0 ± 3.6a	46.8 ± 3.1a
Callus – rooted	32.9 ± 3.9a	29.9 ± 2.9b	44.7 ± 2.4a	47.2 ± 3.8a
Callus – unrooted	59.4 ± 2.4b	57.3 ± 4.3c	45.5 ± 3.2a	48.7 ± 4.6a
Radicle	10.6 ± 3.1c	15.3 ± 2.9a	16.7 ± 3.4b	17.3 ± 3.6b

T_o refers to the beginning of culture following the IBA treatment
* Mean values of tissues with the same letter within each line are not significantly different at $P = 0.05$ as determined by the Gabriel range test

Table 3 CAT and SOD specific activities in seed tissues of *Q. robur* during germination. Values are expressed as the mean ± SE of at least three independent experiments with five replicates per tissue

Developmental stage	Catalase (U/mg protein)	Superoxide dismutase (U/mg protein)
Radicle	8.7 ± 1.5a*	13.5 ± 1.6a
Epicotyl	8.0 ± 0.7a	14.2 ± 1.3a
Leaf	10.3 ± 1.1a	11.0 ± 0.9a

* Mean values with the same letter are not significant different by at $P = 0.05$ as determined by the Gabriel range test

ground of the cultured shoots and the seedlings. Similar data have been obtained from the analysis of several genotypes, and it is quite likely that these changes have to be related in general to stress conditions imposed by in vitro culture. Relationships between changes in peroxidase and isoperoxidase activity and different aspects of rooting have been investigated in different herbaceous and woody species (Berthon et al. 1987; Gaspar et al. 1992; De Klerk 1997). These markers have also been utilised in oak (Welander et al. 1998), but quantitative or qualitative changes in peroxidase activity are not suitable as markers of rooting; in fact, they often do not show the appropriate variations.

Quantitative change in the activity of an enzyme is by itself inadequate to describe the complex changes occurring at the cell level; accordingly, isoenzyme patterns of CAT and SOD activity were analysed in tissues of both microcuttings and seedlings.

The electrophoretic analysis revealed remarkable differences in CAT activity profiles in tissues of developing seedlings. A fast-migrating activity band and a slower one, subsequently referred to as CAT-1 and CAT-2, were active, although not to the same extent, in the leaf and in the radicle, respectively. In the epicotyl, the activity is prevalently associated with CAT-2 (Fig. 2A, lane 2), but a CAT-1 band is also detectable.

The electrophoretic pattern of SODs in the seedling tissues showed several bands of activity, which were identified as Cu/Zn-SODs, since they were inhibited by KCN and H_2O_2. The SOD enzyme profiles changed slightly according to the tissues: three Cu/Zn-SODs were clearly visible in the leaf, and a number of faint bands were visible in the epicotyl and radicle (Fig. 2B). The Mn-SODs were barely detectable in the seedling tissues, in which no Fe-SODs were observed; hence, the enzyme activity is mainly associated with the Cu/Zn-SOD isoforms.

In microcuttings, different patterns of catalase isoforms activity were found among the shoot basal end, stem and leaf of the NL100R line (Fig. 3A, lanes 1–3). Catalase activity at the basal end was distributed among CAT-2, CAT-1 and three intermediate bands, whose presence strongly suggests the tetrameric structure for the oak catalase. Although the protein structure in oak is unknown, data available in the literature on other plant species indicate the occurrence of heterotetramers between CAT-1 and CAT-2 (Scan-

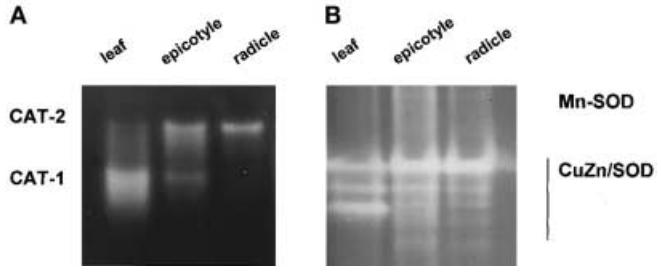

Fig. 2A, B CAT and SOD protein profiles on native PAGE of different seedling tissues of *Q. robur* during post-germination growth. **A** 20-μg aliquots of crude soluble proteins were loaded onto a 7.5% acrylamide gel (CAT). **B** 100-μg aliquots of crude soluble proteins were loaded onto a 15% acrylamide gel (SOD)

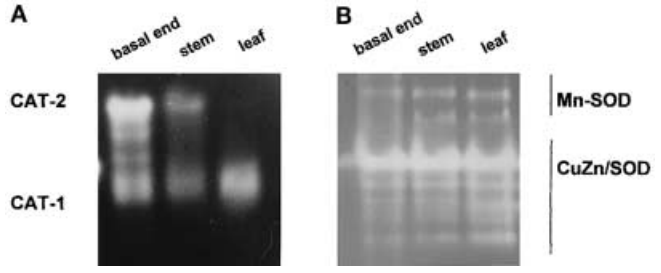

Fig. 3A, B CAT and SOD protein profiles on native PAGE of different NL100 *Q. robur* microshoot tissues during propagation. **A** 20-μg aliquots of crude soluble proteins were loaded onto a 7.5% acrylamide gel (CAT). **B** 100-μg aliquots of crude soluble proteins were loaded onto a 15% acrylamide gel (SOD)

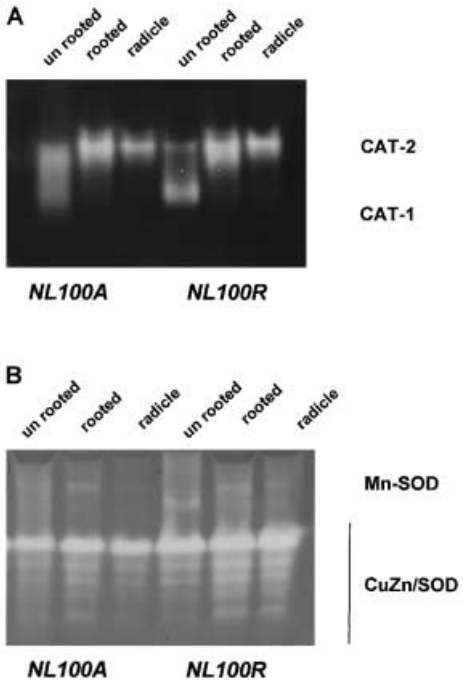

Fig. 4A, B CAT and SOD protein profiles on native PAGE of *Q. robur* callus developed at the shoot basal end and in the radicle after 4 weeks of culture in rooting medium. **A** 20-μg aliquots of crude soluble proteins were loaded onto a 7.5% acrylamide gel (CAT). **B** 100-μg aliquots of crude soluble proteins were loaded onto a 15% acrylamide gel (SOD)

dalios 1987). When all the data were taken into account, the pattern of catalase activity did not reveal substantial differences between the tissues cultured in vitro and those of seedlings. In contrast, the isoenzyme patterns of SODs in the microcuttings differed greatly from those in the seedlings. In fact, in all in vitro tissues, at least four Cu-Zn-SODs and two bands identified as Mn-SODs on the basis of resistance to KCN and H_2O_2 were observed (Fig. 3B, lanes 1–3). The presence of Mn-SODs in in vitro cultured tissues that were absent in the seedlings supports the existence of oxidative stress conditions in the microcuttings.

Mn-SOD has been demonstrated to increase dramatically in plant cells under stress as a specific defence against oxidative stress generated in mitochondria. An increase in peroxisomal Mn-SOD activity has also been reported to occur under stress conditions (Palma et al. 1987, 1991). Furthermore, a strong induction of Mn-SOD has been demonstrated to arise during somatic embryogenesis of horse chestnut, suggesting the occurrence of oxidative stress conditions during in vitro culture that could account for the difficulties observed in the development of the somatic embryo into a plantlet (Bagnoli et al. 1998).

An analysis performed on the NL100A line (data not shown) did not reveal differences from NL100R with respect to the electrophoretic pattern. These results indicate that the different ontogenetic origin and the rooting efficiency of the lines cannot be proven by the electrophoretic profiles of CATs and SODs.

An electrophoretic analysis performed on microshoots after 4 weeks of culture on rooting medium added some interesting data (Fig. 4). Irrespective of the line, the patterns of catalase activity in the basal callus were different in rooted (Fig. 4A, lanes 2 and 5) and unrooted microshoots (Fig. 4A, lanes 1 and 4). In rooted microshoots, CAT-2 was strongly active in both the basal callus and the root (Fig. 4A, lanes 3 and 6), while in unrooted microshoots both CAT-1 and CAT-2 were active. This shift from CAT-1 to CAT-2 isoforms that was observed in the callus at the basal end of the rooted microshoots monitored a change in the physiological state of the cuttings in relation to the formation of the root primordia, suggesting the possibility of using this protein as a marker. On the other hand, the CAT-2 isoform was equally active in the two lines, NL100A and NL100R, despite their different rooting capacity, therefore excluding the possibility of using this catalase isoform to predict the rootability of a cutting. Further investigations by means of immunolocalisation and in situ hybridisation should be conducted to gain a better insight into the different CAT isoform distribution and physiological function during root formation.

The isoenzyme pattern of SODs after 4 weeks of culture on rooting medium did not reveal noteworthy differences between the basal callus of rooted and

unrooted shoots, though small differences were visible between the lines. The activity pattern displayed by NL100R is more intense because of the level of activity found in the line was slightly higher.

In conclusion, CAT and SOD isoenzymes in oak seem to be ineffective in defining the developmental state of a cutting, while they are sensitive indicators of stress conditions during micropropagation. Nevertheless, the activity of the CAT-2 isoform associated with the basal tissues of the rooted microshoots deserves to be investigated further as a protein specifically related to rooting.

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