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The specific expression of isoperoxidases in grapevine cells cultured in suspension in relation to vacuolar development

by

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Die spezifischen Veränderungen der Isoperoxidasen in suspensionskultivierten Rebenzellen in Beziehung zur Entwicklung der Zellvakuole

Zusammenfassung : Aus wachsenden Beeren der Rotweinsorte Gamay (*Vitis vinifera*) wurden Zellsuspensionskulturen hergestellt. Diese dienten als Modellsystem, um die spezifischen Veränderungen der Enzymaktivität von Guajakol-Peroxidase und Indolyl-3-Essigsäure-(IAA-)Oxidase während des Zellwachstums in Beziehung zur Entwicklung der Zellvakuole zu verfolgen. Der Entwicklungszustand der Vakuole wurde anhand des Verhältnisses monomere Anthocyane : Protein festgestellt. Die Untersuchungen zeigten, daß sich die Aktivität von Guajakol-Peroxidase während der exponentiellen Phase des Zellwachstums in Beziehung zum Entwicklungszustand der Vakuole veränderte. Andererseits wurde im Verlauf des Zellwachstums nie IAA-Oxidaseaktivität nachgewiesen, wahrscheinlich weil in den kultivierten Zellen höhere Konzentrationen von „Auxinprotektoren" vorhanden waren.

Dieser Befund steht im Widerspruch zu der Beobachtung, daß die Zunahme der Gesamtperoxidaseaktivität in den Zellen in erster Linie auf den Anstieg der basischen Isoperoxidasen zurückzuführen ist. Aufgrund der Tatsache, daß der IAA-Abbau hauptsächlich durch basische Isoperoxidasen bewirkt wird, wird ferner die anerkannte physiologische Rolle der basischen Isoperoxidasen in suspensionskultivierten Zellen in Frage gestellt. Eine alternative Rolle, welche die basischen Isoperoxidasen im intensiven oxidativen Stoffwechsel der Phenole bei roten Rebsorten spielen könnten, wird diskutiert.

Key words : tissue culture, cell, vacuole, growth, enzyme, peroxidase, basic isoperoxidase, IAA-oxidase, auxin protector, phenol, anthocyanin, red grapevine.

Introduction

Growth and ripening of grape berries depend on the synthesis of specific proteins which results in increased activity of several isoenzymes (DAL BELLIN PERUFFO and PALLAVICINI 1975; KOCHHAR *et al.* 1979). Peroxidase (EC 1.11.1.7, donor: hydrogen peroxide oxidoreductase) isoenzymes are widely distributed in higher plants, and they have been implicated in many metabolic changes and reactions which occur during growth and fruit ripening, including post-ripening changes such as browning reactions (BURNETTE 1977). Thus, basic peroxidase isoenzymes are apparently involved in the catabolism of indole-3-acetic acid, a plant growth regulator which controls the growth and ripening of grape fruits (COOMBE 1976), while acidic peroxidase isoenzymes seem to play a central role in cell wall biogenesis (TAIZ 1984). For this reason, the study of the cellular expression of total peroxidase activity and peroxidase isoenzymes can help to elucidate the relative importance of each isoenzyme group in the above mentioned processes.

As regards grape isoperoxidases, there is little information on the apparent changes in the isoperoxidase patterns during growth and maturation of grape berries (KOCHHAR *et al.* 1979); to date, the study of the differential expression of peroxidase isoenzymes during growth of grape berry cells in relation to vacuolar development has not been carried out.

Using suspension-cultured grape berry cells as a model system, this paper deals with the changes in both the activity and isoenzyme patterns during the cell growth cycle, in an effort to elucidate the relative importance of basic isoperoxidases in auxin catabolism during the vacuolar development of grape berry cells cultured in suspension.

Materials and methods

Source of plant material

Callus cultures derived from growing fruits of grapevines (*Vitis vinifera* L. cv. Gamay), kindly provided by Drs. J. C. Pech and A. Latché (ENSA, Toulouse, France), were used for these studies.

Cell culture

Grapevine suspension-cultured cells were established from growing callus using a culture medium after Hawker et al. (1973), which was supplemented with 2 mg/l kinetin and 0.1 mg/l α-naphthaleneacetic acid. The culture conditions were similar to those described by Calderón et al. (1990). Cell samples for analyses were taken at various days after culture (see Results). Grapevine cells grew in clumps of up to 3 cells. Plant cell culture growth was measured as packed cell volume, and expressed as a percentage.

Homogenization and fractionation of grapevine cells

Cells were separated from the medium (total aliquot volume was 10 ml) by centrifugation (500 g_{max} for 5 min), and the packed cells were resuspended, after washing (3 times) in 10 ml of 2.5 % (w/v) sucrose in 0.1 M sodium-(2-[-N-morpholine]ethanesulfonate) buffer, pH 6.5. The resuspended cells were homogenized at 4 °C for 1 min in a Kinematica GmbH (Polytron®) mechanical blender, and cell debris were removed by centrifugation at 5000 g_{max} for 10 min.

Extraction and determination of phenolic compounds

For extraction of the phenolics (phenolic acids and anthocyanins), 1 vol. of packed cells was incubated overnight with 3 vol. MeOH at 4 °C and immediately homogenized with a mechanical blender as described above. The homogenate was filtered on filter paper and reduced to near dryness (*in vacuo*, 40 °C). The red-vine residue was dissolved in 0.05 vol. of MeOH and filtered.

The concentration of total phenolics was measured as described by Marigo (1973), using chlorogenic acid as standard, whilst the concentration of total monomeric anthocyanins was calculated as reported by Baker et al. (1986), using the spectral method described there.

Enzyme and protein assay

Peroxidase activity was measured by following the increase in absorbency at 470 nm, using guaiacol as substrate (Ros Barceló et al. 1987). Isoelectrofocusing, zymographic stain and quantification of isoperoxidases were performed as described previously (Ros Barceló 1987), using for the isoenzyme quantification benzidine as substrate and calculating isoperoxidase activities on the basis of the total peroxidase activity overlayed initially on the polyacrylamide gel (Ros Barceló 1987). For this purpose, isoperoxidase activity revealed by either benzidine or guaiacol leads to similar results.

Indol-3-acetic acid oxidase (IAA-oxidase) activity of both horseradish peroxidase (Sigma, type VI), and grapevine peroxidases was measured by following the increases in absorbency at 250 nm as described by LESCURE (1970). IAA-oxidase activity of grapevine peroxidases was measured both in the absence and in the presence (50 mg/ml) of soluble polyvinylpyrrolidone (PVP, BDH), pre-incubating in the latter case the grapevine protein fraction with PVP for 15 min at 4 °C before the assay of enzymatic activity.

Protein was determined according to BENSADOUN and WEINSTEIN (1976) using bovine serum albumin as standard.

Results and discussion

The development of polyphenol accumulation

The accumulation pattern of phenolics (monomeric anthocyanins) in relation to the growth of the grapevine suspension-cultured cells is shown in Fig. 1. The exponential phase of the growth cycle, which extends over the 10—18 d of culture, is characterized by a rapid increase in the cell protein (Fig. 1), and also in the monomeric anthocyanin/protein ratio (Fig. 1), with an abrupt termination at the end of the exponential period. Stationary phase cells do not accumulate monomeric anthocyanins and may even lose these compounds to the external medium as the cultured cells die. This phenomenon is especially apparent from day 22 of culture (data not shown).
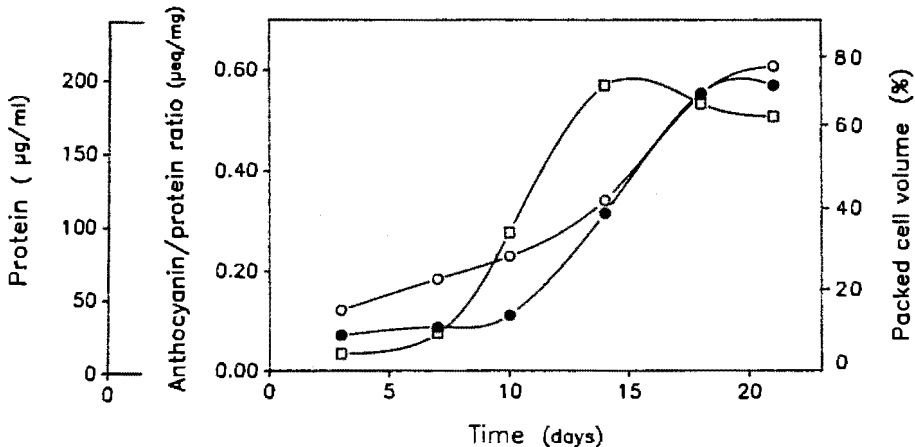

Fig. 1: Changes in the packed cell volume (●), cell protein (○) and monomeric anthocyanin/protein ratio (□) during the growth cycle of grapevine cells cultured in suspension. Typical results from five independent experiments are shown.

Veränderungen während des Wachstums suspensionskultivierter Rebenzellen: Volumen der sedimentierten Zellen (●), Zellprotein (○), Verhältnis monomere Anthocyane : Protein (□). Es werden die typischen Ergebnisse aus fünf unabhängigen Versuchen gezeigt.

Due to the fact that most of the protoplast protein in plant cells is confined to cytosol (MARTY et al. 1980), the monomeric anthocyanin/protein ratio can be considered as a suitable parameter to quantify vacuolar development. Thus, it is established from Fig. 1 that maximum vacuolar development is reached for these grapevine suspension-cultured cells at day 15 of culture.

Although in an earlier report (see DAVIES 1972) attention was drawn to an apparent correlation between the initiation of polyphenol accumulation and the cessation of net

protein synthesis in Paul's Scarlet rose cell cultures grown in media containing higher levels of auxin, in cultures grown at lower auxin concentrations no such correlation could be observed (Davies 1972; Lofty et al. 1989); in fact, polyphenol (and/or anthocyanin) accumulation began some time before net protein synthesis was terminated. These observations are confirmed by us (Fig. 1) in grapevine suspension cell cultures, which require lower auxin concentrations for optimal growth (Hawker et al. 1973).

Time-course of the expression of guaiacol-peroxidase and IAA-oxidase activities during growth

Changes in guaiacol-peroxidase activity during the growth cycle of grapevine cells cultured in suspension are shown in Fig. 2. From this figure, it can be concluded that during the exponential growth phase the total guaiacol-peroxidase activity present in the cells increased gradually and reached maximum values at the end of the exponential growth phase. Subsequently, the total guaiacol-peroxidase activity present in the cells decreased. At day 18 of culture, the increase in peroxidase activity (18-fold, Fig. 2) was greater than the increase found for the protein (4.5-fold, Fig. 2), so the increase in total peroxidase activity on a protein basis (specific activity) was 4.0-fold during this culture period. This observation suggests that the peroxidase activity is expressed differentially during this growth phase, and its expression coincides with the increase in the index (monomeric anthocyanins/protein ratio) of vacuolar development (Fig. 1), but it is out of phase with regard to the maximal relative growth rate of the grapevine cells cultured in suspension (Fig. 2).

In contrast to the guaiacol-peroxidase activity, IAA-oxidase activity of grape peroxidases is not expressed during the entire culture period (Fig. 2). This enzymatic activity cannot be measured even after pre-incubation of the protein extract with soluble PVP in order to remove endogenous phenolic compounds, which can act as powerful inhibitors of this enzymatic activity and generally possess a polyphenolic nature (Stonier 1972).
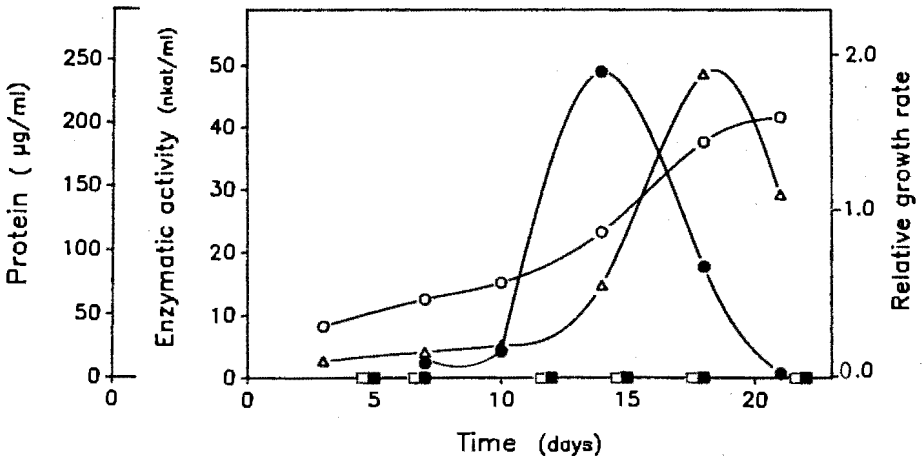

Fig. 2: Changes in the peroxidase activity (△), IAA-oxidase activity measured either in the presence (■) or absence (□) of polyvinylpyrrolidone, protein (○), and relative growth rate (●) during the growth cycle of grapevine cells cultured in suspension. Typical results from five independent experiments are shown.

Veränderungen während des Wachstums suspensionskultivierter Rebenzellen: Peroxidaseaktivität (△), IAA-Oxidaseaktivität in Gegenwart (■) oder bei Abwesenheit (□) von Polyvinylpyrrolidon, Protein (○) sowie relative Wachstumsrate (●). Es werden die typischen Ergebnisse aus fünf unabhängigen Versuchen gezeigt.

In order to test the presence of these possible inhibitors in the grape cell homogenates, such as cinnamic acids and both non-acylated and acylated anthocyanins, which
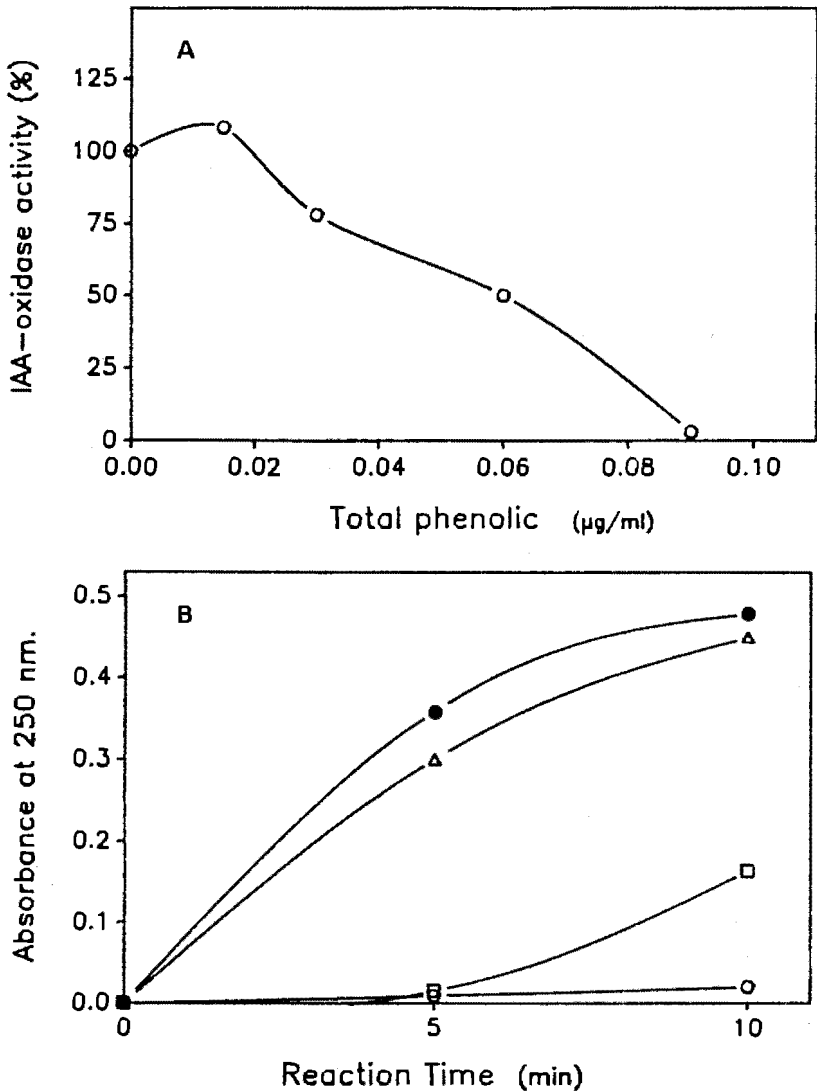

Fig. 3: A) Effect of the concentration of MeOH soluble phenolic compounds extracted from exponentially growing grapevine cells on the IAA-oxidase activity of horseradish peroxidase. — B) Time-course of the oxidation of IAA by horseradish peroxidase in the presence of 0.03 µg/ml (△), 0.06 µg/ml (□), and 0.09 µg/ml (○) of total phenolic compounds extracted from exponentially growing grapevine cells. (●) Control in the absence of added phenolics.

A) Einfluß von MeOH-löslichen Phenolverbindungen, die während der exponentiellen Wachstumsphase aus Rebenzellen extrahiert wurden, auf die IAA-Oxidaseaktivität von Meerrettich-Peroxidase. — B) Zeitlicher Ablauf der Oxidation von IAA durch Meerrettich-Peroxidase bei unterschiedlichen Konzentrationen der Gesamtphenole: 0,03 µg/ml (△), 0,06 µg/ml (□) und 0,09 µg/ml (○); diese wurden während der exponentiellen Wachstumsphase aus Rebenzellen extrahiert. Kontrolle ohne Phenolzusatz (●).

are widely distributed in red grapes cell suspension cultures (LOFTY et al. 1989), the extraction of the MeOH-soluble polyphenolic compounds was carried out from exponentially growing grapevine cells. Subsequently, these compounds were characterized as possible inhibitors of the IAA-oxidase activity of horseradish peroxidase. The results are shown in Fig. 3 A; it can be established that a concentration as low as 0.06 µg/ml in the reaction media is capable of reducing this enzymatic activity in the order of 50 %.

The action of these compounds induces a delay in the oxidation of IAA (Fig. 3 B), characteristic of the well-known compounds called 'auxin protectors' (STONIER 1972), which appear to play an important role in the control of the catabolism of auxins during the growth and maturation of fruits (HARTMANN et al. 1987).

Furthermore, these results suggest that the auxin catabolism pathway is probably almost totally inhibited in cells of red grapes cultured in suspension, since both red grape peroxidase (E. GARCÍA-FLORENCIANO, M. A. PEDREÑO, R. MUÑOZ and A. ROS BARCELÓ, manuscript in preparation) and anthocyanins (HOPP and SEITZ 1987) are compartmentalized, in their totality, in the cell grape vacuole.
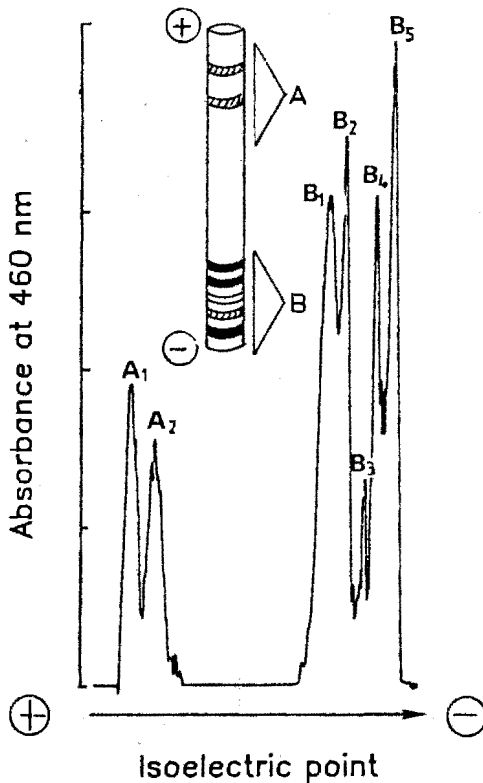

Fig. 4: Isoenzyme pattern of the peroxidase activity present in grapevine cells after 18 d of culture. Zymographic stain of peroxidase isoenzymes was achieved using benzidine as substrate.

Isoenzymmuster der Peroxidaseaktivität nach 18tägiger Kultur von Rebenzellen. Die Anfärbung des Enzymogramms erfolgte unter Verwendung von Benzidin als Substrat.

Time course of the expression of peroxidase isoen-
zymes

The isoenzyme pattern of the peroxidase activity present in the cells after 18 d of
culture is shown in Fig. 4. It shows mainly two peroxidase isoenzyme groups, named
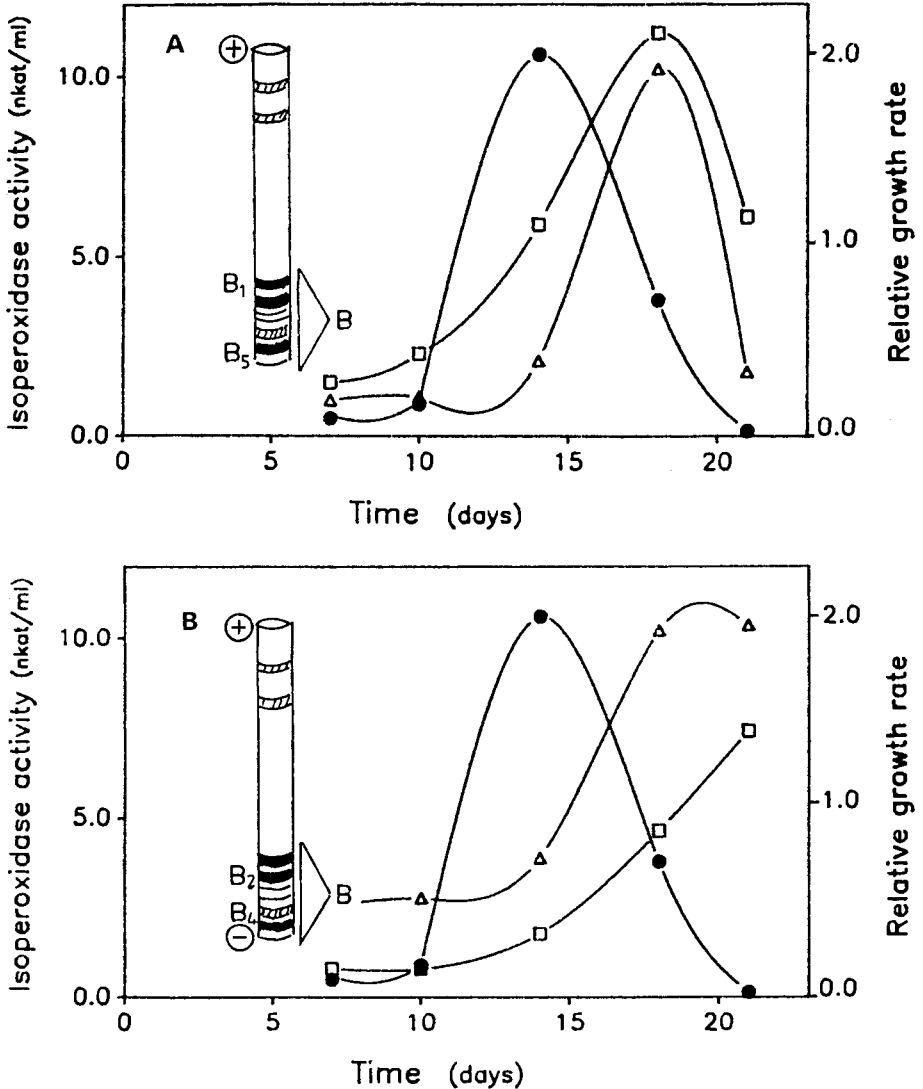

Fig. 5: A) Changes in the level of B_1 ($\triangle$) and B_5 ($\square$) basic isoperoxidases, and B) changes in the level
of B_2 ($\triangle$) and B_4 ($\square$) basic isoperoxidases, and relative growth rate ($\bullet$) during the growth cycle of
grapevine cells cultured in suspension. Typical isoenzyme pattern of cellular peroxidase at day 18 of
culture (inset of figures). Typical results from five independent experiments are shown.

Konzentrationsänderungen basischer Isoperoxidasen während des Wachstums suspensionskulti-
vierter Rebenzellen: A) Isoperoxidasen B_1 ($\triangle$) und B_5 ($\square$). B) Isoperoxidasen B_2 ($\triangle$) und B_4 ($\square$)
sowie relative Wachstumsrate. Der Einsatz zeigt jeweils das typische Isoenzymmuster am 18. Kul-
turtag. Es werden die typischen Ergebnisse aus fünf unabhängigen Versuchen gezeigt.

A_{1-2} and B_{1-5}, which changed both qualitatively and quantitatively during the growth cycle. The most striking changes in the peroxidase isoenzyme pattern took place during the exponential (10—18 d of culture) and post-exponential (18—22 d of culture) growth phases (Figs. 5 and 6).
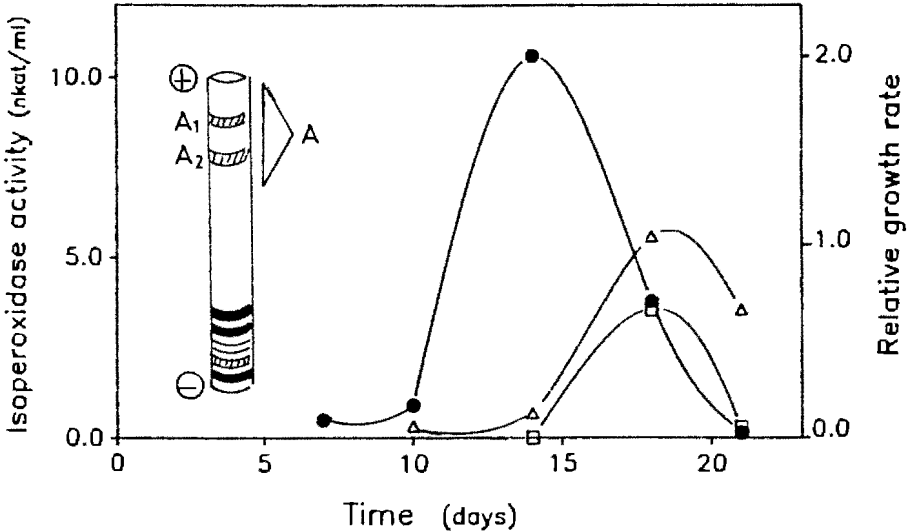

Fig. 6: Changes in the level of A_1 (△) and A_2 (□) acidic isoperoxidases and the relative growth rate (●) during the growth cycle of grapevine cells cultured in suspension. Typical isoenzyme pattern of cellular peroxidase at day 18 of culture (inset of figure). Typical results from five independent experiments are shown.

Konzentrationsänderungen der sauren Isoperoxidasen A_1 (△) und A_2 (□) sowie relative Wachstumsrate (●) während des Wachstums suspensionskultivierter Rebenzellen. Der Einsatz zeigt das typische Isoenzymmuster am 18. Kulturtag. Es werden die typischen Ergebnisse aus fünf unabhängigen Versuchen gezeigt.

Thus, during the exponential growth phase the peroxidase isoenzymes of the B group increased in activity, especially B_1 and B_5, reaching maximum values at day 18 of culture (Fig. 5 A). Subsequently, they decreased again to the level observed at the beginning of the exponential growth phase. However, the peroxidase isoenzymes B_2 and B_4 increased continuously during these growth phases, reaching maximal values at the onset of the stationary phase (Fig. 5 B). Isoperoxidase B_3 was not quantified due to its extremely low level throughout the growth cycle.

On the other hand and in contrast to the B_2 and B_4 basic isoperoxidases, peroxidase isoenzymes of the A group, i.e. A_1 and A_2, show a time course expression (Fig. 6) similar to B_1 and B_5 isoperoxidases (Fig. 5 A), reaching maximal values at the end of the exponential growth phase (18 d of culture).

Because the main destination of acidic peroxidase isoenzymes is the cell wall and intercellular (free) spaces (Mäder and Walter 1986; Ros Barceló et al. 1988), it is plausible to think that the increase in isoenzyme activity of the A_1 and A_2 isoperoxidases that takes place in the cells at the end of the exponential growth phase (Fig. 6) can be the consequence of the processes of synthesis and package of these extracellular isoenzymes, previous to the secretion to the extracellular space. Furthermore, this will be in accordance with the strong increase in acidic extracellular peroxidases occurring at the onset of the stationary phase in suspension-cultured plant cells (Cuenca et al. 1989) and

seems to be related with the stiffening of the cell wall, which takes place during the growth cessation of the cultured cells (see CUENCA *et al.* 1989).

A detailed reflexion points to the strong increase of basic isoperoxidases, which takes place in the cells at the end of the exponential growth phase (Figs. 5 A and B). Thus, the increase in basic isoperoxidases is the main factor responsible for the increase in the total peroxidase activity present in the cells (Fig. 2), an increase that coincides with the vacuolar development of grapevine cultured cells (Fig. 1).

In cultured cells of *Nicotiana tabacum*, basic isoperoxidases have been associated with the central vacuole (SCHLOSS *et al.* 1987), and this seems to be the case with grapevine basic isoperoxidases (E. GARCÍA-FLORENCIANO, M. A. PEDREÑO, R. MUÑOZ and A. ROS BARCELÓ, manuscript in preparation). These isoenzymes, moreover, are the strongest candidates for effecting the oxidation of IAA (MAZZA *et al.* 1970). Due to the fact that auxin catabolism is apparently almost completely inhibited by auxin protectors in grapevine cells cultured in suspension (Figs. 2 and 3), cell basic isoperoxidases could have another distinct function. For example, a central role in the oxidative phenol metabolism, including the oxidative coupling of stilbene moieties to give stress metabolites of the viniferin family (HOOS and BLAICH 1988; CALDERÓN *et al.* 1990), cannot be discarded.

The study of the nature and importance of this latter constitutive metabolic pathway, in which basic isoperoxidases could play a central role, is under way in our laboratory.

Summary

The establishment of suspension cell cultures from red grapes has been carried out in order to be used as a model system in the study of the specific expression of both guaiacol-peroxidase and indole-3-acetic acid (IAA)-oxidase activities during the growth of suspension-cultured cells in relation to vacuolar development. From these studies it was found that, although guaiacol-peroxidase activity is expressed differentially during the exponential growth phase, along with the vacuolar development indexed as the cell monomeric anthocyanin/protein ratio, IAA-oxidase is never expressed during the growth cycle, probably due to the presence of higher levels of auxin protectors in the cultured cells.

This fact is in contrast with the observation that the increase in basic isoperoxidases is the main factor responsible for the increase in total peroxidase activity present in the cells. Furthermore, due to the fact that IAA catabolism is mainly mediated by basic isoperoxidases, the well-established physiological role of basic isoperoxidases in suspension-cultured cells is questioned. A plausible role for basic isoperoxidases in the intense phenolic oxidative metabolism which occurs in red grapes during growth is discussed.

Acknowledgements

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