Vitis 31, 139—147 (1992)

Gamay grapevine peroxidase: Its role in vacuolar anthocyani(di)n degradation

by

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Rebperoxidase von Gamay: Ihre Rolle beim vakuolären Abbau von Anthocyani(di)nen.

Zusammenfassung: Die Oxidation von Anthocyani(di)nen durch vakuoläre Peroxidasen der Rebsorte Gamay wurde untersucht. Die Ergebnisse zeigen, daß — im Gegensatz zu ihren Glykosiden — die Anthocyanin-Aglykone durch Peroxidasen abgebaut werden, wobei wahrscheinlich die Carbinol-Pseudobase das eigentliche Substrat ist, dessen Oxidation von H_2O_2 abhängt. Beim H_2O_2-abhängigen Abbau von Päonidin, Delphinidin und Cyanidin wurde nur eine geringe Substratabhängigkeit gefunden. Die Ergebnisse werden im Zusammenhang mit der zellulären Lokalisierung der Gamay-Peroxidase und mit ihrer möglichen Rolle beim Anthocyaninmetabolismus in der Pflanze diskutiert.

Key words: Anthocyani(di)n oxidation, carbinol pseudobase, grapevine, peroxidase, vacuole, Vitis.

Introduction

The mauve color in tenturier Gamay berries and in cell cultures derived from them originates from the presence of two main anthocyanin pigments in the vacuoles: peonidin-3-glucoside and cyanidin-3-glucoside (LOFTY *et al.* 1989; DO and CORMIER 1991). Anthocyanins are very unstable and the loss of anthocyanin-derived color in grapes can occur after thawing frozen fruits and during the processing and storage of grapevine products, such as musts and wines (BARANOWSKI and NAGEL 1983). These pigments may be lost through several mechanisms, which include: i) high pH, which promote the formation of unstable quinoidal bases, carbinol pseudobases and chalcones (GOTO 1987), and ii) degradation by enzymatic and non-enzymatic systems, such as peroxidase (EC 1.11.1.7) (MÄDER *et al.* 1977), polyphenoloxidase (EC 1.10.3.1) (PENG and MARKAKIS 1963; PIFFERI and CULTRERA 1974), and quinones formed during the non-enzymatic browning. Anthocyanin stability, on the other hand, is favoured by self-association (GOTO 1987) and, mainly, by the formation of co-pigment complexes with flavonoids (MAZZA and BROUILLARD 1990) and heavy metals (GOTO *et al.* 1986; TAKEDA *et al.* 1990).

Unlike polyphenoloxidase, an enzyme located in chloroplasts (VÁMOS-VIGYÁZÓ 1981), the presence of an active peroxidase system in the vacuoles of Gamay cell cultures (GARCÍA-FLORENCIANO *et al.* 1991) can be responsible for anthocyanin turnover in the vacuole compartment, although the fate of the anthocyanins in such a peroxidase system is unknown. In the case of polyphenol oxidase, it has been suggested that the anthocyanins are not directly degraded by the enzyme but by the quinones formed during the oxidation of other endogenous phenolic compounds, which act as primary substrates of the enzyme (PENG and MARKAKIS 1963; EBELING and MONTGOMERY 1990).

The purpose of this work was to study the substrate specificity of vacuolar Gamay peroxidases for the anthocyanins naturally found in the vacuole. The fate of anthocyanins in this oxidative model system was also explored under conditions in which

self-association (stabilization) was greatly impeded by the inclusion of an organic solvent (dioxane) in the oxidative medium.

Material and Methods

Chemicals

Anthocyani(di)ns (cyanin chloride, cyanidin chloride, delphinidin chloride and peonidin chloride) were purchased from Extrasynthèse (Genay, France). Dioxane and H_2O_2 were purchased from Merck (Darmstadt, FRG). 4-Methoxy-α-naphthol (4-MN) from Aldrich-Chemie (Steinheim, FRG). All the other chemicals used in this work were obtained from various suppliers, and were of the highest purity available.

Callus and suspension cell cultures

Grapevine (*Vitis vinifera* var. Gamay) suspension cell cultures were grown in 250-ml flasks for 16—18 d at 25 °C, and under a 14/10 h photoperiod regime of 2 $W \cdot m^{-2}$ (GARCÍA-FLORENCIANO *et al.* 1991).

Peroxidase and anthocyanin fractions

Peroxidase fractions were obtained by homogenization of the cells in 2.5 % (w/v) sucrose in 0.1 M Na-Mes buffer (pH 6.5) as described by GARCÍA-FLORENCIANO *et al.* (1991). They were purified by chromatography on insoluble polyvinylpolypyrrolidone (PVPP, Sigma, St. Louis, MO, USA), and dialyzed overnight against buffered activated charcoal (GARCÍA-FLORENCIANO *et al.* 1991).

Anthocyanins were extracted from the cells with methanol containing 0.01 % HCl, and purified by adsorption onto PVPP columns (WROLSTAD and STRUTHERS 1971). Finally, they were crystallized from methanol-diethyl ether mixtures.

Determination of enzymatic activities and anthocyanins

The assay of peroxidase activity using 4-MN was carried out as described by FERRER *et al.* (1990). The amount of peroxidase activity was expressed in nkat, and this was calculated using $\varepsilon_{593} = 2.1 \times 10^4\ M^{-1} \cdot cm^{-1}$ for the dye product.

Anthocyanins were measured as described by GARCÍA-FLORENCIANO *et al.* (1990).

Oxidation of anthocyani(di)ns by peroxidase

Oxidation of the anthocyani(di)ns by Gamay peroxidase was followed by a decrease in absorbance at 535 nm at 25 °C in a reaction medium of 50 µM of anthocyani(di)ns, 5 mM H_2O_2, and 0.75 nkat of Gamay peroxidase, in 28.5 mM Na-phosphate, 28.5 mM Na-borate, 28.5 mM Na-citrate (PBC) buffer, pH 4.0. Oxidation rates were determined from the slope at $t = 0$ of the time course decay within 2 min, and after initiating the reaction by addition of enzyme. Initial absorbance values of anthocyani(di)n solutions were taken as the 0.000 unit of absorbance, and so absorbance decay was expressed in negative values.

Results

The oxidation of anthocyani(di)ns by peroxidase was studied at pH 4.0 as a compromise between the optimum actuation pH of Gamay peroxidases, established around

4.0—5.0, and the pH of the grapevine vacuolar sap, calculated by MOSKOWITZ and HRAZDINA (1981) to be around 3.0.

In the case of the anthocyani(di)ns tested, the anhydrobase form is regarded as the dominant species initially present at pH 4.0. This conclusion was reached on the basis of the λ_{max} in the 530—550 nm spectral range for all the buffered anthocyani(di)n solutions (JURD and ASEN 1966). At this pH, aqueous solutions of anthocyani(di)ns are virtually colorless after 10—20 min of incubation, probably because the anhydrobases are almost totally and immediately converted by hydration of the flavylium salts into their colorless carbinol pseudobases (GOTO 1987).

The decay of the cyanidin anhydrobase at this pH was followed by a decrease in absorbance at 535 nm (Fig. 1 A). This shows that even at pH 4.0 the anhydrobase is highly unstable and decays almost totally after 10 min of incubation.

When anthocyanins are dissolved at fairly high concentration in aqueous buffers, self-association produces a noticeable hypsochromic shift in visible absorption, as well as a large Cotton effect, suggesting that the anhydrobase chromophores stack in a helical form (GOTO *et al.* 1986). The driving force of this stacking appears to be hydrophobic interaction between the aromatic nuclei because the addition of an organic solvent strongly destroys this stacking (GOTO *et al.* 1986). Under such conditions (the inclusion of an organic solvent in the reaction medium), the study of the nature of the conversion of the anhydrobase into the corresponding carbinol pseudobase seems to be possible.

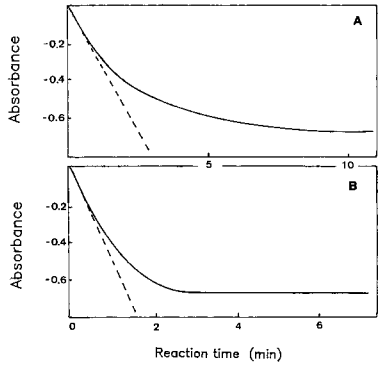

Fig. 1: Time course of the decay of the cyanidin anhydrobase (decrease in absorbance at 535 nm) in reaction media containing (A) 50 µM of cyanidin, and (B) 50 µM cyanidin, 5 mM H_2O_2 and 0.75 nkat of Gamay grapevine peroxidase.

Zeitverlauf des Abbaus der Cyanidin-Anhydrobase (gemessen als Abnahme der Absorption bei 535 nm) in Reaktionsmedien mit (A) 50 µM Cyanidin und (B) 50 µM Cyanidin + 5 mM H_2O_2 + 0,75 nkat Gamay Rebperoxidase.

Spectrophotometric studies of the conversion of peonidin and delphinidin anhydrobases to the corresponding carbinol pseudobases in PBC buffer, pH 4.0, containing 15 % dioxane are shown in Fig. 2. These illustrate the existence of a rapid interconversion of the anhydrobase to the carbinol pseudobase, these two species seeming to be the only ones present in dissolution judging from the isosbestic points in the set of spectral changes (arrowheads, Figs. 2 A and B).

The addition of Gamay peroxidase and H_2O_2 to these reaction media accelerated the decay of the corresponding anhydrobases, as illustrated in Figs. 2 C and D in the case of the cyanidin anhydrobase. This effect was particularly apparent when the initial rate of the anhydrobase decay was compared (Fig. 1 B). The addition of peroxidase and H_2O_2 increased the cyanidin anhydrobase decay by 79 %.

The peroxidase-mediated stimulation of the decay of the cyanidin anhydrobase takes place without major modifications in the nature of the spectral changes in reaction medium (Figs. 2 C and D). In fact, the position of the isosbestic points, putative indicators of the anhydrobase-carbinol pseudobase transformation, are maintained in the reaction media containing Gamay peroxidase (Figs. 2 C and D). This suggests, in the first instance, that peroxidase probably oxidizes the carbinol pseudobase (Fig. 3, II). Such a reaction mechanism could explain the peroxidase-mediated acceleration of the anhydrobase decay, since this species is in equilibrium with the carbinol pseudobase (GOTO 1987).

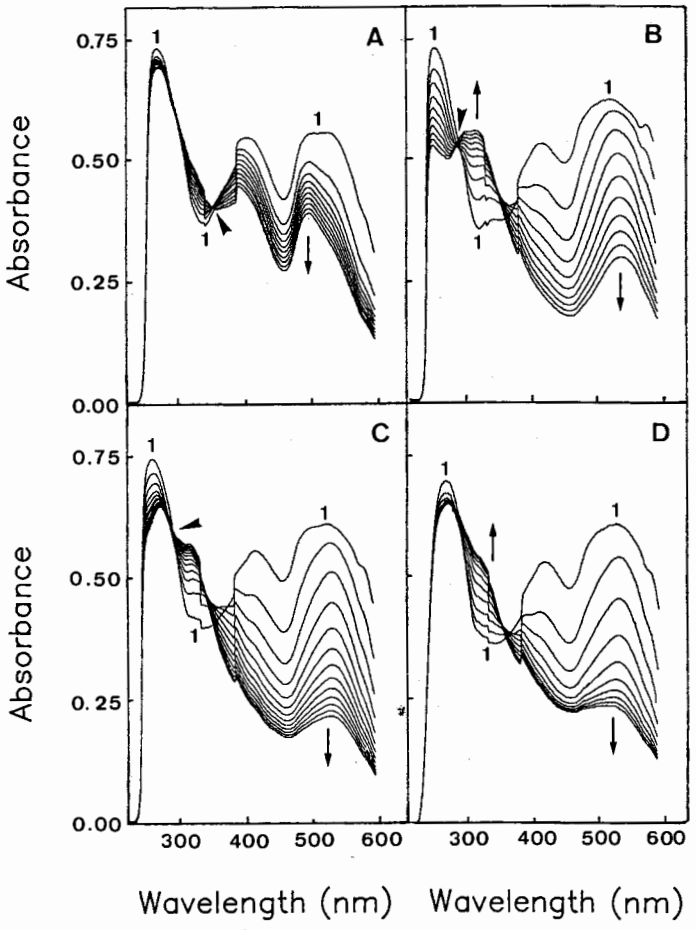

Fig. 2: Spectrophotometric changes in reaction media composed of 190 µM peonidin (A), 190 µM delphinidin (B), and 190 µM cyanidin (C) in PBC buffer, pH 4.0, containing 15 % dioxane. In D, the reaction medium containing 190 µM cyanidin was supplemented with 5 mM H_2O_2 and 0.80 nkat of grapevine peroxidase. Scans were at 1.5 min intervals. Line 1: 0.5 min after the addition of the anthocyani(di)n. The arrow indicates the direction of the spectral changes and the arrowheads the presence of isosbestic points.

Spektrophotometrische Änderungen in PBC-Puffermedien pH 4,0 mit 15 % Dioxan und (A) 190 µM Päonidin, (B) 190 µM Delphinidin, (C) 190 µM Cyanidin. (D) wie (C) aber mit 5 mM H_2O_2 und 0,80 nkat Peroxidase. Scan-Abstände 1,5 min, Linie 1: 0,5 min nach Zugabe des Anthocyan(id)ins. Der Pfeil indiziert die Richtung der Spektraländerungen und Pfeilspitzen isobestische Punkte.

Following this reasoning, the oxidation rate of anthocyanins by Gamay peroxidase was measured from the initial rate of decay of the corresponding anhydrobases. The oxidation rates were calculated from the time-course recording of the decreases in absorbance at 535 nm of the reaction media. Due to the instability of the corresponding anhydrobases (Figs. 2 A—C), the oxidation of anthocyanins by peroxidase was expressed as the activation percentage of the anhydrobase decay in the absence of peroxidase and H_2O_2.

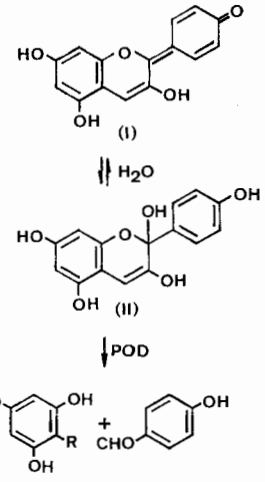

Fig. 3: Structural transformation of the anthocyanin anhydrobase (I) into the carbinol pseudobase (II), and degradation of the latter by a peroxidase-mediated reaction.

Strukturumwandlung der Anthocyanin-Anhydrobase (I) in die Carbinol-Pseudobase (II) und deren peroxidativer Abbau.

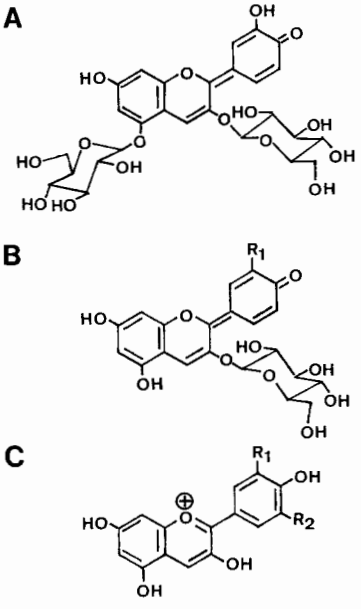

Fig. 4: Structures of the anthocyani(di)ns tested. (A) Cyanin. (B) Cyanidin- (R_1 = OH) and peonidin- (R_1 = OCH_3)-3-glucoside. (C) Cyanidin (R_1 = OH, R_2 = H), peonidin (R_1 = OCH_3, R_2 = H) and delphinidin (R_1 = R_2 = OH).

Strukturen der untersuchten Anthocyan(id)ine. (A) Cyanin-, (B) Cyanidin- (R_1 = OH) und Päonidin- (R_1 = OCH_3) 3-Glucosid. (C) Cyanidin (R_1 = OH), Päonidin (R_1 = OCH_3, R_2 = H) und Delphinidin (R_{1+2} = OH).

The results shown in Table illustrate that although peonidin, delphinidin and cyanidin were substrates of Gamay peroxidases, the carbinol pseudobase derived from cyanin (the 3,5-diglucoside of cyanidin, Fig. 4 A) was not oxidizable. As with cyanin, Gamay anthocyanins, mainly composed of peonidin (Fig. 4 B, R_1 = OCH_3) and cyanidin-3-glucosides (Fig. 4 B, R_1 = OH) (Lofty *et al.* 1989; Do and Cormier 1991), were also non substrates of Gamay peroxidase (Table).

In the case of the three aglycones studied (peonidin, delphinidin and cyanidin), H_2O_2 alone did not appreciably affect the rate of the spectral changes (Table).

Substrate specificity of Gamay peroxidases for the three anthocyanidins tested was weak, although peonidin (Fig. 4 C, R_1 = OCH_3, R_2 = H) and delphinidin (Fig. 4 C, R_1 = R_2 = OH), were apparently better substrates than cyanidin (Table).

On the other hand, the inability of the glycosides to react in this peroxidase system could not have been due to self-association and chiral stacking, since the inclusion of 15 % dioxane in the reaction medium did not appreciably modify the rate of decay of the corresponding anhydrobases with respect to the control.

This differential reactivity of the aglycones compared with the glycosides has also been observed in studies using flavonols as peroxidase substrates, in which a free hydroxyl group in position C_3 and H_2O_2 were necessary (SCHRÖDER and BARZ 1979).

Table

Effect of H_2O_2 and Gamay grapevine peroxidases (GPO) on the rate of decay of the anthocyani(di)n anhydrobases in PBC buffer, pH 4.0, at 25 °C. The decay was estimated from the decreases in absorbance at 535 nm per min (control without H_2O_2 and enzyme = 100 %). Gamay anthocyanins were tested in 0.1 M Tris-acetate buffer, pH 4.0.

Wirkung von H_2O_2 und Gamay-Peroxidase (GPO) auf die Abbaurate der Anthocyan(id)in-Anhydrobasen in PBC-Puffer (Gamay-Anthocyanine in 0.1 M Tris-Acetatpuffer) bei pH 4,0 und 25 °C. Die Abbaurate wurde nach der Abnahme der Extinktion bei 535 nm pro min geschätzt (Kontrolle ohne H_2O_2 und Enzym = 100 %).

	Anthocyani(di)ns				
	Peonidin (50 µM)	Delphinidin (50 µM)	Cyanidin (50 µM)	Cyanin (50 µM)	Gamay anthocyanins (65 µM)
Control	100 ± 10	100 ± 3	100 ± 5	100 ± 2	100 ± 0
H_2O_2	105 ± 12	107 ± 2	103 ± 1	100 ± 7	101 ± 1
H_2O_2 + GPO	205 ± 18	206 ± 3	179 ± 3	109 ± 3	102 ± 3

Discussion

The formation, accumulation and further metabolism (turnover) of anthocyanins play an important role in the differentiation programmes and development in grapes (STRACK and WRAY 1989). Turnover and degradation are thus seen as the last steps in the full development of anthocyanin metabolism.

Whilst considerable progress has been made in work on the biosynthesis of the main classes of anthocyanins, the enzymology of the last steps in anthocyanin turnover is still incomplete. In the pivotal step of flavonoid biosynthesis, p-coumaroyl-CoA enters a condensation reaction with three molecules of malonyl-CoA to form a C_{15} chalcone intermediate, the tetrahydroxychalcone (STRACK and WRAY 1989). In the following step, the C_{15} skeleton is formed and produces the actual precursor for anthocyanin formation, i.e. the flavan-3,4-cis-diol, which then appears to be converted into the anthocyanidin flavylium cation by a hydroxylation step at C-2 followed by two dehydrations (STRACK and WRAY 1989). Finally, the molecule is stabilized by glycosilation of the O-heterocycle. Further anthocyanin modifications include additional hydroxylations, methylations of free hydroxyl groups, glycosilations and acylations, which lead to the vast array of anthocyanins present in grapes (HEBRERO *et al.* 1988).

Once they are synthesized on endoplasmic reticulum, anthocyanins are mainly stored in vacuoles of the sub-epidermal cell layers of grape berries (MOSKOWITZ and HRAZDINA 1981). Previous of this, anthocyanins are packed into small cytoplasmic vesicles (anthocyanoplasts), which later fuse with tonoplasts and discharge their anthocyanin content (DOKE and VAUGHN 1982; GARCÍA-FLORENCIANO *et al.* 1992).

A few enzymes apparently associated with anthocyanin catabolic pathways are known at present: β-glycosidases, peroxidases and polyphenoloxidases.

Polyphenoloxidase is located in the chloroplast in healthy higher plant cells (VÁMOS-VIGYÁZÓ 1981). It is synthesized in the cytoplasm under nuclear control (LAX *et al.* 1984), the end localization of polyphenoloxidase being the photosystem II of thylakoid membranes (LAX and VAUGHN 1991). Since nothing is known about the vacuolar efflux of anthocyanins to chloroplasts, it is improbable a role for polyphenoloxidase in anthocyanin degradation *in planta*.

Unlike polyphenoloxidase, both β-glycosidases (MARTY *et al.* 1980) and peroxidases (GARCÍA-FLORENCIANO *et al.* 1991) are located in vacuoles. For this reason, and starting from the view point of subcellular compartmentalization analysis, the participation of both enzymatic systems in anthocyanin degradation *in planta* is plausible.

Secondary phenol catabolism pathways in plants generally start from the action of β-glycosidases that yield the corresponding aglycones of the primary compounds (BARZ and KOSTER 1981). Further, the aglycon released by the enzymatic hydrolysis would undergo an enzymatic oxidation catalyzed by peroxidases and strictly dependent on the supply of H_2O_2 (BARZ and KOSTER 1981). This would be the case for Gamay anthocyanins, since the glycosides are not substrates of peroxidases, whereas the corresponding aglycones are (Table).

Thus, it can be assumed that degradation of anthocyanins in grapes is initiated by a glycosidase-catalyzed removal of sugar moieties, the released aglycones and carbohydrates being channeled separately into the catabolic process. In this general pathway, the corresponding anhydrobase aglycones would suffer a rapid decay, favoured by the slight acidic pH of the vacuolar sap (MOSKOWITZ and HRAZDINA 1981), to the corresponding carbinol pseudobases (see Fig. 3).

Intensive enzymatic studies on the catabolism of flavonoids by peroxidase in plants have revealed that, after removal of the various glycosidic moieties, not only flavonols (SCHRÖDER and BARZ 1979; SCHREIER and MILLER 1985), but also chalcones (RATHMELL and BENDALL 1972; WONG 1989), and flavanones (PATZLAFF and BARZ 1978), can be oxidized by peroxidases. According to SCHRÖDER and BARZ (1979), flavonols are degraded via 2,3-dihydroxyflavanones, and for this enzymatic oxidation, the hydroxyl function in positions 3 and 4' must be free. Furthermore, these 2,3-dihydroxy structures can suffer a later decay by extensive peroxidation to resorcinol-type compounds and substituted p-hydroxybenzaldehydes.

From the structural analogy between the carbinol pseudobases derived from the anthocyanidin anhydrobases (Fig. 3) and the 2,3-dihydroxyflavanones, it can be assumed that the carbinol pseudobases are the true substrates for the enzyme. This would explain the absence of reactivity of cyanin in this peroxidase/H_2O_2 system, as compared to cyanidin (Table), since in the former the OH group in C_3 is substituted by a glycoside group. Such reasoning would explain the failure of Gamay anthocyanins to react (Table), since they are mainly composed of peonidin- and cyanidin-3-glycosides (LOFTY *et al.* 1989; DO and CORMIER 1991).

In conclusion, since Gamay vacuolar peroxidases are not apparently involved in other peroxidase-mediated metabolic reactions (GARCÍA-FLORENCIANO *et al.* 1990 and 1991), due to the previously reported strong antioxidative activity of anthocyanins (IGARASHI *et al.* 1989), one of their possible functions would appear to be anthocyanin turnover through anthocyanidin degradation. For this oxidative reaction, the presence of H_2O_2 and a free hydroxyl group in positions C_3 and C_4, in the initial anhydrobase are necessary. This is probably due to the fact that the true peroxidase substrate is the carbinol pseudobase, the 2,3-dihydroxy structures being necessary for the oxidative degradation to take place.

Summary

The oxidation of anthocyani(di)ns by Gamay grapevine vacuolar peroxidases was studied. The results suggest that, unlike their glycosides, Gamay anthocyanins aglycones are degraded by peroxidases, the carbinol pseudobase probably being the true substrate, the oxidation of which was strictly dependent on H_2O_2. Only a weak substrate specificity was found in the H_2O_2-dependent degradation of peonidin, delphinidin and cyanidin. These results are discussed in the light of the subcellular localization of Gamay peroxidase, and of the possible involvement of this enzyme in anthocyanin turnover and degradation *in planta*.

Acknowledgements

The authors thank Dr. J.C. PECH and Dr. A. LATCHÉ for providing the plant material used in this study. This work has been partially supported by a grant from the CICYT (Spain), project number 296/89. A. A. CALDERÓN holds a fellowship (BFPI) from the MEC (Spain).

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Received, 17. 10. 1991

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