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Role of wall peroxidases in oat growth inhibition by DIMBOA

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Abstract

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), an effective growth-inhibitor of oat coleoptiles, enhanced 1524 and 246 times the rate of NADH oxidation by ionically and covalently bound oat cell wall peroxidases, a reaction that generates H_2O_2. This effect was similar to that of *p*-coumaric acid, while ferulic acid was a poor activator of this reaction. Coniferyl alcohol did not activate NADH oxidation. DIMBOA was poorly utilized by ionic or covalent oat wall peroxidases in the presence of H_2O_2. With horseradish peroxidase, DIMBOA efficiently promoted compound III breakdown with a decomposition rate constant of 4.88×10^{-2} s^{-1}. This effect was 14 times higher than that obtained with *p*-coumaric acid that gave a decomposition rate constant of 0.35×10^{-2} s^{-1}. The diferulate and lignin levels in the cell walls from oat coleoptiles increased by 56 and 30%, respectively, upon DIMBOA treatment. The effect of DIMBOA on oat wall peroxidases would thus contribute to its growth inhibitory effect by promoting H_2O_2 synthesis and phenol coupling at the cell wall level.

Keywords: DIMBOA; Hydroxamic acids; *Avena sativa*; Gramineae; Peroxidases; Diferulic acid; Lignin

1. Introduction

Peroxidases (E.C.1.11.1.7) are involved in the modulation of plant cell growth both by promoting cell wall rigidity through lignin synthesis and oxidative cross-linking of polysaccharide components (Fry, 1986; Iiyama, Lam, & Stone, 1994; Ros Barceló, 1997) as well as by generating H_2O_2 needed for these reactions through the oxidation of NADH with molecular oxygen (Gross, Janse, & Elstner, 1977; Halliwell, 1978). In the former reactions, cell wall peroxidases in the presence of H_2O_2, oxidize phenolic lignin precursors and polysaccharide-bound ferulate residues to phenoxy radicals ($A^{\cdot}$ in reactions 2–3 described below), which polymerize to form lignin and diferulate (DFA) bridges between cell wall polysaccharides, as represented by the following reactions:

$$\text{ferric peroxidase} + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

$$\text{compound I} + AH \rightarrow \text{compound II} + A\cdot \quad (2)$$

$$\text{compound II} + AH \rightarrow \text{ferric peroxidase} + A\cdot + H_2O \quad (3)$$

The reactions that constitute the peroxidative cycle of the enzyme, include the formation of oxidized forms of peroxidase, compounds I and II (reactions 1–3). Besides, in the presence of O_2 and a suitable reductor like NADH, peroxidase is converted mainly into the catalytically inactive compound III (CoIII) (reactions 4–5) (Yokota & Yamazaki, 1965; Halliwell, 1978). In this oxidative cycle of peroxidases, the slow breakdown of CoIII can be enhanced by some phenolics to render the native enzyme and $O_2^{\cdot -}$ radicals, which in the presence of Mn^{+2} and NADH generates the H_2O_2 required in the peroxidative cycle of the enzyme (reactions 6–7) (Halliwell, 1978; Yokota & Yamazaki, 1965).

$$\text{ferric enzyme} + \text{NADH} \rightarrow \text{ferrous enzyme} + \text{NAD}^{\cdot} \quad (4)$$

$$\text{ferrous enzyme} + O_2 \rightarrow \text{compound III} \quad (5)$$

$$\text{compound III} + \text{phenol} \rightarrow \text{ferric enzyme} + \text{phenol} + O_2^{\cdot -} \quad (6)$$

$$\text{NADH} + O_2^{\cdot -} + H^+ \xrightarrow{Mn^{+2}} \text{NAD}\cdot + H_2O_2 \quad (7)$$

In addition, NADH can act as reductant of compound I and compound II in the peroxidative cycle of the enzyme to produce $\text{NAD}^{\cdot}$ radicals (reactions 2–3), which react rapidly with O_2 to form $O_2^{\cdot -}$ and then H_2O_2 by reaction (7) (Halliwell, 1978).

For the above reasons, cell wall peroxidases are likely targets of plant growth regulators that could alter their

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levels or activities. Particularly, natural hydroxamic acids, like 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and their benzoxazolinone decomposition products, that have been described as plant growth inhibitors (Venis & Watson, 1978; Pérez, 1990) could alter cell wall peroxidase activities. DIMBOA belongs to a family of benzoxazinones present in several cereals (Niemeyer, 1988) that have been associated with allelopathic interactions (Barnes, Putnam, Burke, & Aasen, 1987; Pérez, 1990). Recently we have reported that DIMBOA effectively enhances NADH oxidation catalyzed by horseradish peroxidase (HRP) (Rojas, Pérez, & González, 1997). Enhancement of NADH oxidation by DIMBOA at the cell wall level could contribute to its plant growth inhibitory effect by generating the H_2O_2 required for diferulate and lignin synthesis, which are peroxidase-catalyzed reactions that promote cell wall rigidity.

In this paper we report the correlation between growth inhibition of oat (*Avena sativa* L.) coleoptiles by DIMBOA and the effect of this hydroxamic acid on oat cell wall peroxidase reactions, as an attempt to contribute to the knowledge of the biochemical basis of its growth inhibitory effect.

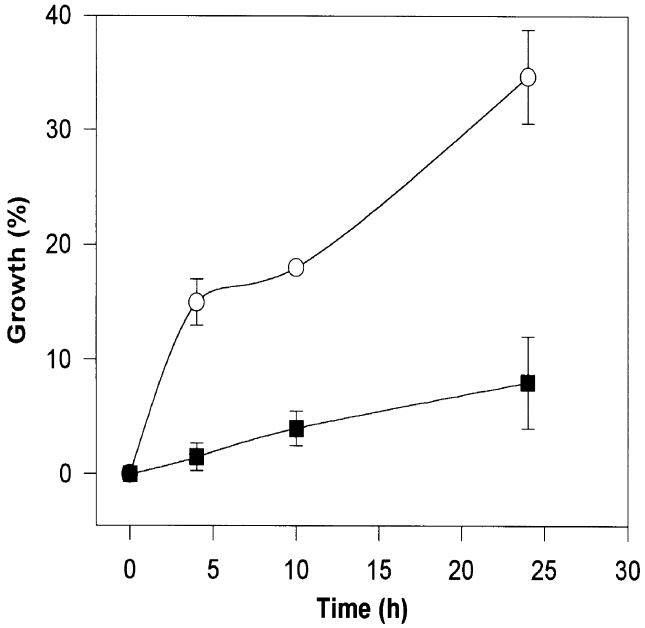

Fig. 1. Effect of DIMBOA in the growth of oat coleoptile segments. Segments of 1–1.5 cm were incubated in the dark in 5 mM malate buffer pH 5.5 (○) or in the same buffer containing 75 μM DIMBOA (■). Growth is expressed as percent of initial fresh weight.

2. Results and discussion.

2.1. Growth inhibition of oat coleoptiles by DIMBOA

DIMBOA, a naturally occurring hydroxamic acid in maize, effectively inhibited the growth of oat coleoptile segments (Fig. 1). The inhibitory effect was observed at concentrations over 50 μM in the incubation buffer; at 75 μM, a growth inhibition of 84% was obtained after 4 h of incubation. The effect was due to the hydroxamic acid and not to its decomposition product, MBOA, as the half life of decomposition of DIMBOA at pH 5 is 27 h (Niemeyer, Bravo, Peña, & Corcuera, 1982) and in the experiment depicted in Fig. 1 the DIMBOA solution was replaced every 6 h of incubation. DIMBOA and the benzoxazolinone MBOA, have already been reported to inhibit root growth in *Avena fatua* (Pérez, 1990) while the benzoxazolinones DMBOA and MBOA have been found to inhibit auxin induced growth of oat coleoptile segments (Venis & Watson, 1978; Hasegawa et al., 1992).

2.2. DIMBOA utilization by oat cell wall peroxidases

DIMBOA was utilized by oat cell wall peroxidases (ionically and covalently wall-bound fractions) both in the oxidative and in the peroxidative cycle, in agreement with our previous results with HRP isoenzyme C (Rojas et al., 1997). In the oxidative cycle, DIMBOA resulted to be an efficient stimulator of NADH oxidation (Table 1) and this effect was dependent on DIMBOA concentration with saturating kinetics, i.e. rate increased with increasing DIMBOA and levelled off at concentrations higher than 100–150 μM. The reaction accumulated H_2O_2 as evidenced by the release of O_2 upon catalase addition at different times into the reaction mixture (Fig. 2). The amount of O_2 uptake during NADH oxidation correlated linearly with the amount of O_2 released after catalase addition (Fig. 2, insert). The slope of the straight line in the insert was 0.6, close to the stoichiometric relation of 0.5 expected between O_2 generated from H_2O_2 upon catalase addition and O_2 uptake during NADH oxidation by peroxidases. The natural phenolic compounds *E*-ferulic acid (*E*-FA), *p*-coumaric acid (*p*-CA) and coniferyl alcohol (CA) were also tested as activators of NADH oxidation and showed marked differences in their effectiveness. Maximum rates (V_{max}) and semisaturating effector concentrations ($E_{0.5}$) were calculated from the respective hyperbolic curves and are shown in Table 1. Stimulation factors were also calculated for each compound from the ratio between V_{max} and the rate in the absence of effectors. *p*-CA was the most effective phenolic activator, with stimulation factors of 1375 and 300 for the ionic and the covalent fractions, respectively. The effect of DIMBOA was similar to that of *p*-CA, giving stimulation factors of 1524 and 246 for the ionic and the covalent peroxidase cell wall fractions. *E*-FA was a poor activator while CA showed no stimulatory effect over NADH oxidation catalized by oat wall peroxidases (Table 1). *p*-CA has been reported as an effective stimulator of NADH oxidation by HRP (Halliwell, 1978; Rojas et al.,

Table 1
Effect of DIMBOA and of natural phenolics on NADH oxidation by oat cell wall fractions

	Ionic wall fraction			Covalent wall fraction		
Effector	V_{max} (mol/s × 10^{11})	$E_{0.5}$[a] (μM)	stimulation factor[b]	V_{max} (mol/s × 10^{11})	$E_{0.5}$ (μM)	stimulation factor
None	0.2±0.03	–	1	0.8±0.06	–	1
DIMBOA	304.7±25	95.5±4.5	1524	209.5±13.3	139.2±4.0	246
Ferulic acid	5.7±0.3	4.1±0.6	29	2.3±0.14	0.7±0.2	2.7
p-Coumaric acid	275.0±12	210.1±14	1375	255.2±8.5	268.2±10	300
Coniferyl alcohol	0.2±0.03	–	1	0.8±0.06	–	1

Rate values are normalized per gram of fresh weight tissue.
[a] Effector concentration that gives half of the maximum rate.
[b] Ratio between the V_{max} for each effector and the rate of NADH oxidation in the absence of effectors.

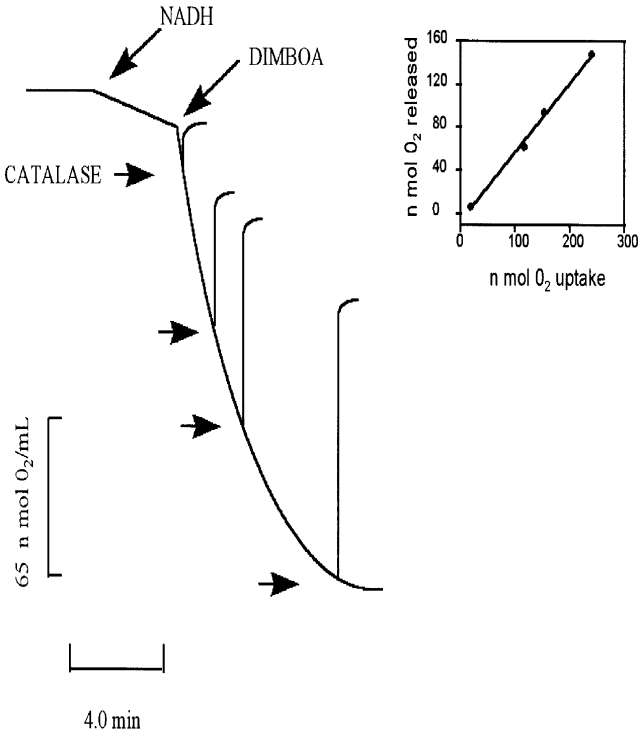

Fig. 2. NADH oxidation by ionic wall peroxidases in the presence of DIMBOA, monitored by O_2 uptake. At different reaction times indicated by the arrows, 40 μg of catalase (from *Aspergillus niger*, Sigma) were added to the reaction mixture containing 0.3 mM NADH, 50 μM $MnSO_4$, 75 μM DIMBOA and ionic peroxidase fraction in K–Pi buffer (0.1M, pH 6.8). Insert: O_2 uptake and O_2 release before and after catalase addition, respectively, were plotted during the progress of the reaction.

1997), while *E*-FA is a poor activator of this reaction (Rojas et al., 1997). The phenol activator effects here reported were inversely related to the efficacy of oxidation of these compounds by oat wall peroxidases in the presence of H_2O_2. In the latter reaction, oat ionic wall peroxidases oxidized CA very efficiently with a V_{max}/K_m value of 12.4×10^{-2} s^{-1} per g fw, while *E*-FA and *p*-CA were utilized with V_{max}/K_m values of 2.2×10^{-2} and 0.3×10^{-2} s^{-1} per g fw, respectively. Covalently bound oat wall peroxidases showed a similar specificity for phenol oxidation with H_2O_2 (unpublished). These results are in agreement to our previous results with HRP (Rojas et al., 1997). In contrast, *E*-FA and *p*-CA, that are good substrates for lupine peroxidase in the presence of H_2O_2, have been found to be efficient activators of NADH oxidation (Pedreño, Sabater, Muñoz, & García-Carmona, 1987). The results described for tobacco wall peroxidases and CA (Mäder, Ungemach, & Schloß, 1980) also differ from our findings since CA at a concentration of 10^{-4} M effectively enhanced H_2O_2 formation by tobacco peroxidases while we found no activating effect for this phenol in the range of 10^{-6}–10^{-3} M (Table 1). The inverse correlation found by us between the efficacy of phenol peroxidation and the effectiveness in activating NADH oxidation, can be interpreted in terms of inhibition of NADH oxidation by phenolic compounds that have high affinity for the intermediates of the peroxidative cycle and thus could decrease the level of CoIII.

Oat ionically and covalently bound cell wall fractions utilized DIMBOA as a substrate of the peroxidative cycle with hyperbolic kinetics, giving a product that absorbed light at 345 and 430 nm. In the presence of 0.5 mM H_2O_2 maximum rate values for the oxidation of this hydroxamic acid by the ionic and covalent fractions were 7.9×10^{-11} and 0.2×10^{-11} mol·s^{-1} per g fw, respectively. Semisaturating DIMBOA concentrations were 5.7 and 0.5 mM for the respective wall fractions. Thus, the rate of NADH oxidation in the presence of saturating DIMBOA was considerably higher than the maximum rate of DIMBOA peroxidation (38 and 1047 times for the ionic and covalent fractions, respectively). On the other hand, the oxidase activating effect was obtained at concentrations of about 100 μM DIMBOA while this hydroxamic acid was oxidized by H_2O_2 at concentrations above 500 μM. Thus, DIMBOA resulted to be a poor substrate for oat wall peroxidases in the presence of H_2O_2 and an efficient activator of NADH oxidation, supporting the correlation described above for the phenolic

substrates. The above results indicate that the main effect of DIMBOA at the cell wall level would be to generate H_2O_2 from O_2 which then would be used for the oxidative coupling of cell wall phenols as *E*-FA residues or CA to give DFA bridges and lignin respectively. The efficient utilization of CA and *E*-FA by oat wall peroxidases in the presence of H_2O_2 support the latter hypothesis.

2.3. Compound III decomposition by DIMBOA

The mechanism of NADH oxidase activation by DIMBOA could be similar to that found for *p*-CA or for other phenols with HRP (Gross et al., 1977; Pedreño et al., 1987; Hasegawa et al., 1992). These electron donors enhance the rate of decomposition of CoIII into active ferric peroxidase (Yokota & Yamazaki, 1965; Ricard & Nari, 1967; Halliwell, 1978; Smith, Morrison, & Milham, 1982; Metodiewa, Pires de Melo, Escobar, Cilento, & Dunford, 1992), reaction which has been demonstrated to be the limiting step when this enzyme acts as an oxidase to generate H_2O_2 (Tamura & Yamazaki, 1972; Nakajima, Hoshino, & Yamazaki, 1991). Since CoIII could not be monitored with our partially purified oat cell wall fractions, we studied the effect of DIMBOA on CoIII prepared from horseradish peroxidase. HRP was incubated with NADH at pH 5.6 to obtain CoIII that was monitored at 418 nm. Under these conditions all of the enzyme was transformed into this intermediate that was stable at least for 30 min. Addition of DIMBOA in the range of 1–130 μM to the reaction mixture effectively increased the rate of decomposition of HRP CoIII with saturating kinetics (Fig. 3A). Data in Fig. 3 suggest the formation of a CoIII–DIMBOA complex that breaks down later to ferric peroxidase

$$\text{CoIII} + \text{DIMBOA} \leftrightarrows \text{CoIII} - \text{DIMBOA} \rightarrow \text{ferric peroxidase} + \text{DIMBOA}.$$

From the rate at saturating DIMBOA concentrations (Fig. 3A, 5.86×10^{-11} mol/ml/s = 5.86×10^{-2} μM/s) at which the concentration of complexed CoIII was 1.2 μM (total enzyme concentration), a kinetic constant of 4.88×10^{-2} s^{-1} was obtained for the decomposition of the CoIII–DIMBOA complex. We also tested the effect of increasing concentrations of *p*-CA on HRP CoIII decomposition Fig. 3 (A and B) and a rate constant of 0.34×10^{-2} s^{-1} was obtained for the breakdown of the CoIII complex with this hydroxycinnamic acid. *p*-CA is known to be an effective activator of NADH oxidation catalized by peroxidases (Halliwell, 1978; Pedreño et al., 1987; Rojas et al., 1997). Thus, our results show that DIMBOA is 14 times more effective than *p*-CA in promoting CoIII decomposition and suggest that this mechanism operates in DIMBOA stimulation of NADH oxidation by oat wall peroxidases. From data in Fig. 3A, a semisaturating DIMBOA concentration of 14.9 μM

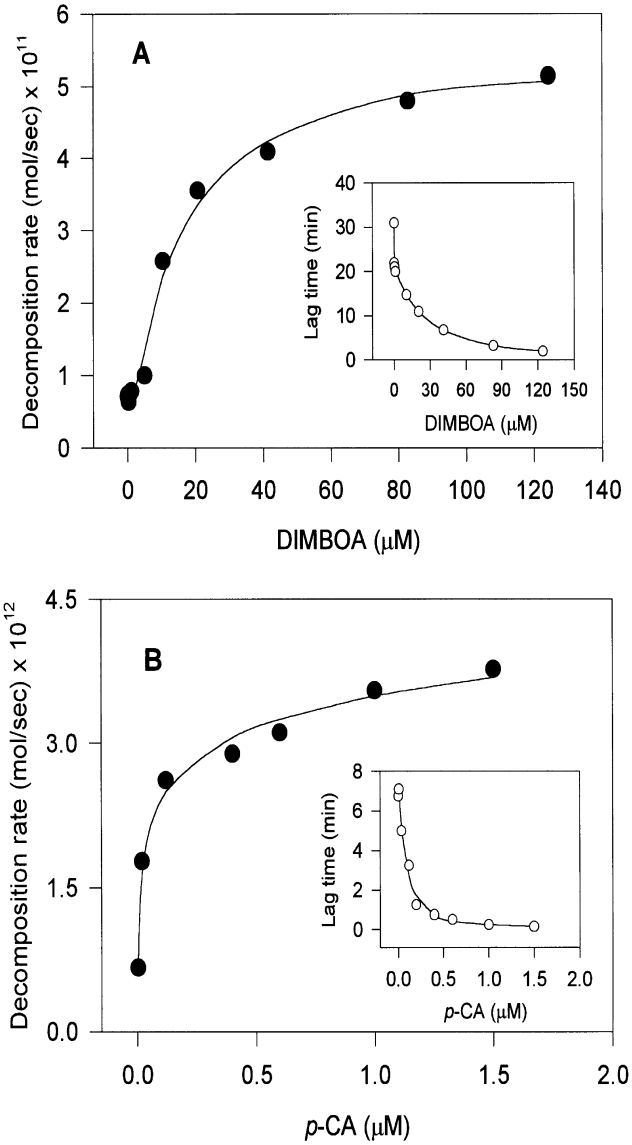

Fig. 3. Effect of DIMBOA (A) and of *p*-coumaric acid (*p*-CA) (B) on compound III decomposition. Reaction conditions as described in Section 3. Inserts: lag time on compound III decomposition dependent on the DIMBOA and *p*-CA concentrations.

was calculated, while a value of 0.16 μM was obtained for *p*-CA evidencing differences in the affinity of both activators for HRP CoIII. It is interesting that progress curves for CoIII breakdown showed lag times that depended inversely on DIMBOA or *p*-CA concentrations (Fig. 3, insert), which can be interpreted as accumulation of radical species generated from both CoIII oxidants.

2.4. Effect of DIMBOA on diferulate and lignin levels

Incubation of oat coleoptile segments with 75 μM DIMBOA induced an increase in the levels of DFA and lignin in the cell walls (Fig. 4). After 5 h of treatment, DFA and lignin levels were 56 and 30% higher in coleop-

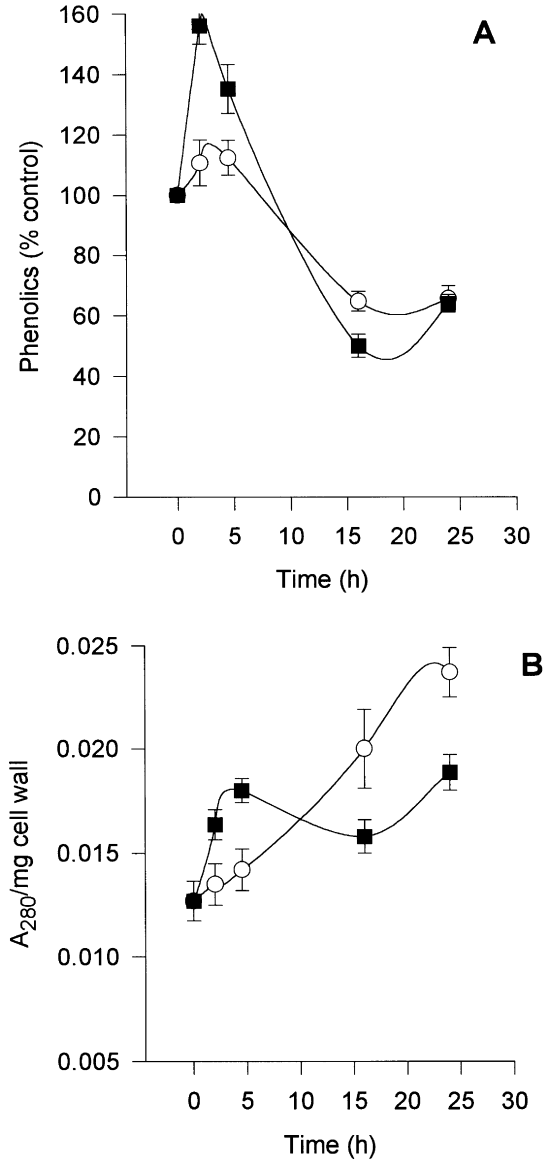

Fig. 4. (A) Diferulate (■) and ferulic acid (○) levels expressed as percent of the respective controls (incubations in the absence of DIMBOA). At $t=0$ DFA and *E*-FA levels in the cell walls were 0.095 and 1.78 μg per mg of cell walls, respectively. (B) Lignin levels in coleoptile segments incubated with 75 μM DIMBOA (■) or in the absence of DIMBOA (○).

tile segments treated with DIMBOA than in the respective controls (Fig. 4). The *E*-FA levels, in contrast, increased by only 11% after 5 h of incubation in the walls of DIMBOA-treated coleoptiles (Fig. 4A). These results correlate well with the above described effect of DIMBOA on oat cell wall peroxidases, that would generate through the oxidative cycle, the H_2O_2 required for DFA and lignin synthesis from the respective phenolic precursors. At reaction times exceeding 10 h, the lignin and DFA levels in the treated coleoptiles were lower than in the controls, possibly due to oxidative processes induced by H_2O_2 accumulation that could alter metabolic processes related to phenolics synthesis or incorporation into the cell walls. In fact, oat coleoptiles after 4 h of treatment with DIMBOA showed an oxidative damage as evidenced by the accumulation of both lipids and proteins oxidatively modified (unpublished).

In summary, our results suggest that cell wall peroxidases could be main targets for DIMBOA in oat coleoptiles by binding this hydroxamic acid to CoIII in the oxidative cycle, thus promoting its decomposition and H_2O_2 synthesis. This effect would contribute to the growth inhibitory effect of this hydroxamic acid by allowing cell wall phenol coupling and cell wall rigidity. At long treatment times, secondary effects induced by H_2O_2 accumulation could also contribute to growth inhibition.

3. Experimental

3.1. Reagents

All reagents used were analytical grade. Horseradish peroxidase (Type X, $R/Z=3.0$) was obtained from Sigma. DIMBOA was obtained from maize seedlings according to the procedure already described (Queirolo, Andreo, Niemeyer, & Corcuera, 1983).

3.2. Plant material

Six day old coleoptiles of *A. sativa* L. cv Nehuen grown at 22–25°C in the dark were used.

3.3. Preparation of peroxidase cell wall fractions

Oat coleoptile segments were ground in a mortar with 50 mM Tris–HCl pH 7.2 containing 0.1 mM phenylmethylsulphonylfluride (PMSF), 5% insoluble polyvinylpolypirrolidone, 5 mM $MgCl_2$ and sand. Cell walls were collected by centrifugation at $1000 \times g$ for 5 min and washed with Triton X-100 according to Ros Barceló, Muñoz and Sabater, (1987). Ionically bound (ionic) cell wall peroxidases were obtained by incubation with 1 M KCl while covalently bound (covalent) cell wall peroxidases were solubilized from the remaining pellet by treatment with pectinase plus cellulase in acetate buffer (0.1 M, pH 5.0) (Ros Barceló et al., 1987), including 1 mM PMSF, 2 mM benzamidine and 1 μM pepstatin A in the reaction mixture.

3.4. Enzyme assays

NADH oxidation by oat cell wall fractions was recorded at 340 nm and 22° in a Shimadzu UV-120 spectrophotometer ($\varepsilon_{NADH,\ 340}=6.02\ mM^{-1}\ cm^{-1}$). The reaction medium contained in 0.1 M K–Pi buffer pH 7.0, 50 μM $MnCl_2$, 160 μM NADH, peroxidase wall fractions (0.5–1.5 enzyme units) and different concentrations of

the effectors. In the NADH oxidation assay in presence of DIMBOA as effector, there is no interference by the oxidation product of DIMBOA absorbing light to 345 and 430 nm, because both this oxidation product is accumulated after 87% of NADH is oxidized and the NADH oxidation rates were measured when less than 10% of NADH has been oxidized. One enzyme unit was defined as the amount of peroxidase that oxidizes 1 μmol of *o*-phenylendiamine (*o*-PDA) per min at pH 4.5 and 22°C ($\varepsilon_{\text{o-PDA},450} = 1.05\ \text{mM}^{-1}\ \text{cm}^{-1}$). NADH oxidation was also monitored by determining O_2 uptake with a Hansatech oxygraph model CB1. DIMBOA oxidation was monitored at 430 nm in 0.1 M K–Pi buffer pH 7.0 with 0.5 mM H_2O_2 and utilizing an extinction coefficient of 1260 $M^{-1}\ cm^{-1}$ (Rojas et al., 1997) for the oxidation product.

3.5. Kinetics of CoIII decomposition

CoIII was obtained by incubation of 1.2 μM HRP at 22°C with 1 mM NADH in 50 mM Na-malate buffer pH 5.6. Its decomposition was monitored at 418 nm after the addition of DIMBOA or *p*-CA to the reaction mixture. Rates were calculated from the slopes of progress curves after the lag phase, utilizing an extinction coefficient of 75 $mM^{-1}\ cm^{-1}$ for CoIII, determined from the A obtained when HRP was completely transformed into CoIII. V_{max} and semisaturating effector concentrations ($E_{0.5}$) were obtained by fitting the saturation curves with the program Table Curve (Jandel Scientific).

3.6. Preparation of cell walls for phenolics extraction

All procedures were carried out under fluorescent light. Oat coleoptiles were ground in a mortar with liquid nitrogen. The resulting fine powder was suspended and stirred vigorously for 15 min in 1.5 ml of 2% (w/v) Triton X-100, 50 mM azide and 1 M KCl in Eppendorf vials. After centrifugation (14,000 rpm, 5 min) the pellet was consecutively washed for 15 min with MeOH (twice), H_2O, 1% (w/v) SDS, H_2O (twice), EtOH, MeOH/$CHCl_3$ (1:1 v/v), EtOH and H_2O (twice). The remnant insoluble material was incubated with 5 ml α-amylase (1 mg/ml) in 50 mM Tris pH 7.0 for 6 h and then with 5 ml pronase E (0.5 mg/ml) in the same buffer overnight. Finally, cell walls were washed with H_2O, MeOH, MeOH/$CHCl_3$ (1:1 v/v) and dried at 40°C.

3.7. Determination of DFA, FA and lignin levels in oat cell walls

For diferulate and ferulate quantification, phenolics esterified to wall polysaccharides were liberated by alkaline hydrolysis. Dried cell walls (20–40 mg) were treated at room temp for 20 h with 2 M NaOH (5 ml, degassed) in vials sealed under N_2. *E*-3,4-OH-cinnamic acid was added to the vials as internal standard. After hydrolysis the mixture was centrifuged, acidified to pH 2.0 with HCl and then extracted with Et_2O. Ethereal extracts were vacuum-dried and stored in the dark. Samples were analyzed by HPLC on a Waters model 600E system controller fitted with a Waters Ultra Wisp model 715 sample processor and a photodiode array detector (Waters, model 990^+). Monitoring was at 310 nm using a reversed phase Waters Nova-Pak C_{18} (150×3.9 mm) column (flow rate 0.9 ml/min) and the elution was performed with a convex gradient of MeCN (solvent A) against 3% HOAc: 0–5 min, 5% A; 5–20 min, 5–10% A; 20–45 min, 10–20% A; 45–55 min, 20% A; 55–65 min, 20–50% A; 65–67 min, 50% A; 67–80 min, 5% A. The amounts of *E*-FA and DFA were calculated from the respective calibration curves and according to the yield of internal standard. Lignin was determined in the saponified cell walls by the method of Iiyama (Iiyama & Wallis, 1990) and expressed as the A at 280 nm per mg of cell walls.

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