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LACCASE ACTIVITY COULD CONTRIBUTE TO CELL-WALL RECONSTITUTION IN REGENERATING PROTOPLASTS

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; laccase activity; cell-wall regeneration; protoplasts; wounding.

Abstract—Laccase (EC 1.10.3.1) activity was measured in regenerating and non-regenerating protoplasts isolated from tobacco (*Nicotiana tabacum*, L.) leaves. Laccase activity diminished soon after isolation; thereafter, it steadily increased in regenerating protoplasts during a 6-day culture period, whereas it was undetectable in non-regenerating protoplasts. A different pattern of isoforms was expressed in protoplasts by comparison with the donor tissue. Polyphenol oxidizing activity was detected also in the spent medium but it was not possible to definitely determine if it was due to laccase or to peroxidase (POX, EC 1.11.1.7) activity. New isoPOXs were quickly expressed soon after protoplast isolation as well, but POX activity remained negligible during the first day in culture. Leaf wounding induced an immediate stimulation of laccase activity whereas POX activity increased very slowly and peaked only after 4 days. Therefore, laccase could be the only effective polymerizing enzyme during the first day of protoplast culture and could contribute in the first steps of healing in wounded leaves, substituting for POX activity in cell wall reconstitution when hydrogen peroxide is not yet available. © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Laccase activity has been demonstrated to contribute to lignin deposition in higher plants [1]. Laccases oxidize phenolics, with preference for the derivatives of the hydroxycinnamic acid, and the resulting radicals couple non-enzymatically in a random fashion [2]. Dehydrogenation is performed in the absence of hydrogen peroxide and therefore monolignol polymerization catalysed by laccases is supposed to function during cell wall lignification, whenever the low H_2O_2 concentration could prevent peroxidase activity [3]. Some laccase isoforms have been recovered from the spent medium of sycamore cells [3–5]; they were able to oxidize many phenolics *in vitro*, even though such substrates were probably not the natural ones in cell suspension culture.

Protoplast regeneration depends upon cell wall reconstitution [6, 7] and seems to rely on POX activity [8]. Recently, the importance of POX activity for protoplast regeneration has been confirmed by the observation that protoplasts failed to divide if H_2O_2 was removed from the medium or if POX activity was directly inhibited [9]. We have also shown that non-regenerating protoplasts have a lower POX activity when compared with regenerating ones [10, 11]. However, there is no information on the possible implication of any other enzyme(s) in the polymerization process during cell wall reconstitution of protoplasts. In this work we have shown that laccase activity is detectable during protoplast culture and we propose its possible role in cell wall regeneration. Furthermore, since the process of protoplast isolation can be considered an extreme case of wounding, laccase activity has been determined in wounded leaves and compared with the course of POX activity stimulation.

RESULTS AND DISCUSSION

The process of protoplast isolation from tobacco leaf mesophyll inhibited laccase activity; in regenerating protoplasts such activity was around 15% of that measured in the donor tissue, but it slowly increased during the first 6 days of culture (Fig. 1). No laccase activity was detected in the spent medium the first day after isolation but it significantly increased starting from day 3 of protoplast culture (Fig. 2). Three isoforms were detectable when a native gel loaded with leaf extract was stained for laccase activity; only the isoform with the highest mobility was identified on a native gel loaded with protoplast extract but its activity was apparently higher than in

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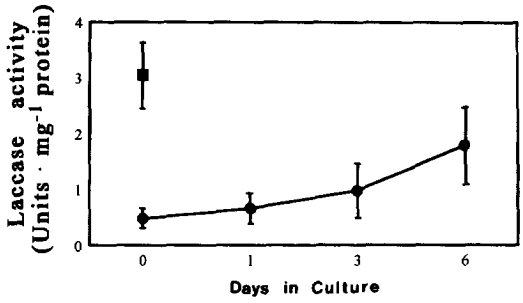

Fig. 1. Laccase activity in crude extract from tobacco leaves (■) and in regenerating protoplasts during the first 6 days in culture (●). Data are the means from three independent experiments.

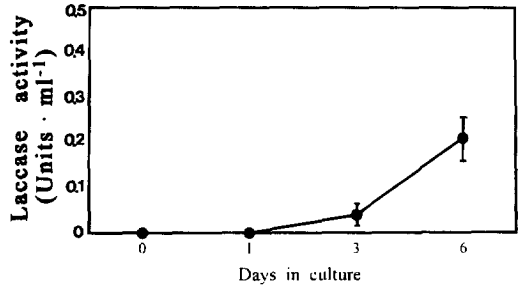

Fig. 2. Laccase activity in spent medium recovered from regenerating protoplasts during the first 6 days in culture. Data are the means from three independent experiments.

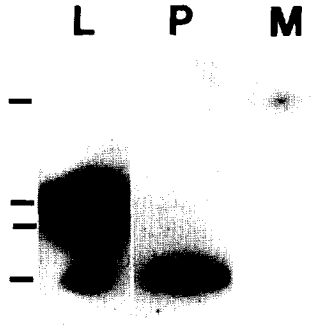

Fig. 3. Laccase isoforms separated by native PAGE using crude extract from tobacco leaves (L), regenerating protoplasts (P) and their corresponding spent medium (M). 50 μg of leaf and protoplast protein and 100 μl of spent medium were loaded.

the leaf extract (Fig. 3). A new band of activity, of lower mobility, was detected in gels loaded with spent medium (Fig. 3). In non-regenerating protoplasts isolated from both tobacco and *Vitis*, laccase activity was very low soon after protoplast isolation and became undetectable one day later, whereas no laccase activity was found in the corresponding spent medium throughout the culture period (data not shown).

Mayer and Harel [12] suggested that the laccase-like-activity measured in the first studies dealing with plant laccase(s) could be partially due to POX activity in the presence of endogenously produced H_2O_2. However, protoplast laccase and POX activities showed a

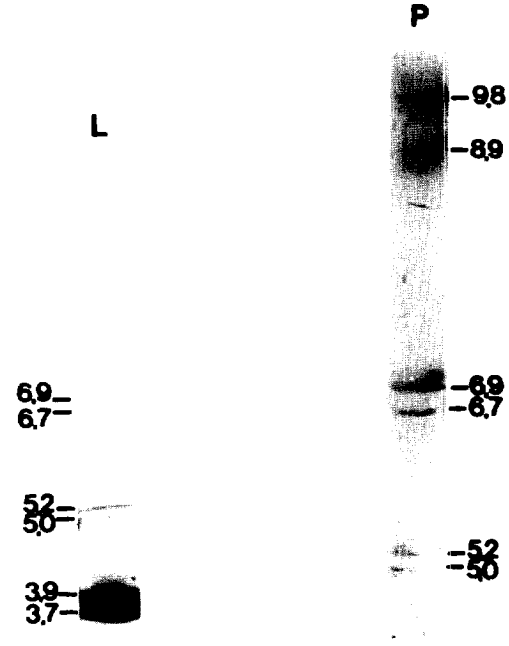

Fig. 4. POX isoforms separated by IEF using tobacco leaves (L) and in cultured protoplasts collected 2 hr after isolation (P). 50 μg of leaf crude extract and 40 μl of partially purified fraction enriched in POX activity by Concanavalin-A chromatography were loaded.

different behaviour in response to isolation and culture, since POX activity was almost undetectable soon after isolation and increased 10-fold during protoplast culture [11], whereas laccase activity maintained an appreciable level of acitivty after isolation and increased only 3- to 4-fold during the culture period (Fig. 1). Furthermore, substrate oxidizing activity was not inhibited by the addition of 60 units ml^{-1} catalase to the reaction medium, which would prevent any H_2O_2 accumulation and consequently impair POX activity [9]. In our experiments laccase isoforms separated on native gels were detected using catechol, that is substrate for catechol/polyphenol oxidase (PPO) activity as well. However, tropolone (100 μM), a specific inhibitor of PPO [13], did not inhibit substrate oxidation at pH 5.0 and no SDS-dependent stimulation of oxidizing activity was measured at pH 6.5, as could be expected if PPO was involved in the reaction [14]. These results seem to rule out the possibility that what has been measured in regenerating protoplasts as laccase activity was actually due to POX or PPO. No substrate oxidation was measured when boiled enzyme was used.

We have previously shown that most of tobacco leaf POX activity was due to two very acidic (Ip of 3.7 and 3.9, respectively) isoforms and no POX activity was visualized in protoplasts soon after isolation [11]; minor leaf isoforms with weakly acidic and neutral Ip became detectable only by using at least 40 μg protein from crude extract (Fig.4, L). Using affinity chromatography we have purified and concentrated the protein fraction from both regenerating and non-

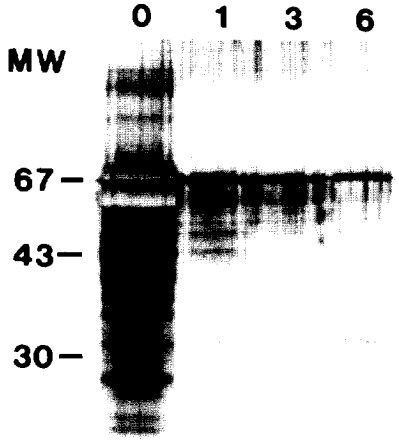

Fig. 5. Course of protein accumulation in spent medium during protoplast culture. 50 μl of spent medium recovered soon after protoplast isolation (0), and after 1, 3, and 6 days of protoplast culture were loaded on a SDS-denaturating gel and proteins were detected by silver staining.

regenerating protoplasts cultured for 2 hr. Three groups of isoPOXs became detectable, using protein from both samples (Fig. 4, P); the neutral and weakly acidic isoforms were similar to those found in the donor tissue (Fig. 4, L), whereas in a previous experiment the two basic isoPOXs were visualized by exploiting crude extract only after some days of protoplast culture [11]. The zymogram in Fig. 4 indicates that their expression is promptly stimulated by isolation, even though their activity remained almost undetectable during the first days of culture [11].

Both proteins with structural and enzymic functions are accumulated in protoplast culture medium during the first days after isolation and seem to play a crucial role in protoplast regeneration [8]. The rate of their accumulation diminished progressively during the culture period (Fig. 5), probably as a consequence of the increasing polymerization of the cell wall network, that progressively offers a more efficient structure to which excreted material can be tightly fixed [15]. In contrast with most of the other proteins, both laccase (Fig. 2) and POX (Fig. 6) activities recovered from the spent medium increased during protoplast culture. In our experiments laccase activity was measured in

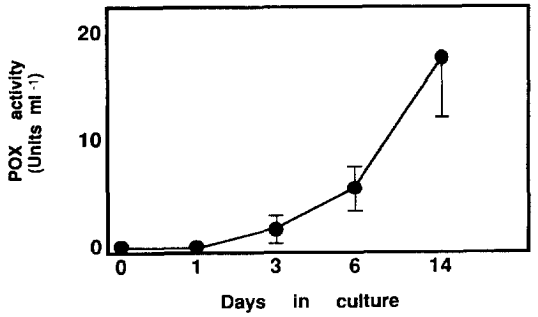

Fig. 6. POX acitivty in spent medium recovered from regenerating protoplasts during the first 6 days in culture. Data are the means from three independent experiments.

the spent medium also in the presence of catalase, which prevented POX activity, and tropolone, that would inhibit PPO activity [13], in the reaction mixture. Nevertheless, laccase and POX activities are both undetectable in the spent medium the first day after protoplast isolation, when no H_2O_2 is available [11], and thereafter increased at the same rate. Therefore it cannot be ruled out that the external polyphenol oxidizing activity was due to polyfunctional polymerizing enzymes possessing both POX and laccase activities [16, 17].

Wounding of tobacco leaves induced both laccase and POX activities, but laccase stimulation was very rapid and reached the maximum already after 6 hr, whereas the highest POX activity was measured only on day 4 after wounding (Table 1). Laccase is known to catalyse the oxidation of mono- and polyphenolics in the presence of O_2; the resulting phenoxyl radicals couple non-enzymically to form lignin oligomers [2]. The localization of laccase and laccase-like activities in lignifying tissues [1, 18] confirms its role in polymerization of secondary cell wall. However, laccases were recovered in cell suspension [3, 5] and in cultured protoplasts (Figs 1–3), where lignification is highly improbable, even though it was reported that lignin-like material accumulated in Norway spruce (*Picea abies*) cultured cells [19].

Even though laccase activity is usually measured using monolignols, laccases are capable of oxidizing a wide variety of substrates, including tyrosine [20]. Structural glycoproteins, like extensin, contain many tyrosine residues, which are used to form cross-links between proteins [21] and are supposed to contribute to cell-wall architecture [22] and to act as a barrier in case of wounding or pathogen infection [23]. These protein bridges have been claimed to constitute the initial network during cell wall reconstitution [7, 15] and the successive expression of two different extensins is induced after protoplast isolation and in response to wounding [24]. POXs have been shown to catalyse the tyrosine cross-links that lead to extensin polymerization [22] and protoplast isolation triggers the expression of new isoPOXs (Fig. 4), but they depend on H_2O_2 for oxidizing the phenolic groups. On the first day following isolation, there was no H_2O_2-accumulation and POX activity decreased in regenerating protoplasts to only 3% of the corresponding activity in the donor tissue [11]. In contrast, even though the total contribution of laccase to the polymerization of structural elements in the cell wall seems to be minor compared with POX activity [11], laccase activity was still efficient the first day after protoplast isolation (Fig. 1), namely when POX activity was negligible [11]. Therefore, laccases could function during the first stages of polymerization of cell wall elements, when H_2O_2 concentration is too low to ensure POX activity [3, 11]. Furthermore, POX activity alone could be not sufficient to ensure cell wall reconstitution. In fact, recalcitrant protoplasts isolated from tobacco and *Vitis* lacked in laccase

Table 1. Laccase and POX specific activities in crude extract from wounded tobacco leaves recovered at successive times. Data are expressed in units × mg^{-1} protein and are the means from five independent experiments

	Time after wounding					
Control	3 hr	6 hr	1 day	2 days	4 days	6 days
Laccase						
3.1±0.7 (100%)	5.3±1.3 (171%)	5.4±1.2 (174%)	4.7±1.2 (152%)	4.7±1.3 (152%)	4.5±1.1 (145%)	4.1±1.1 (132%)
POX						
9.2±0.9 (100%)	9.6±1.2 (104%)	11.9±1.5 (128%)	23.5±2.3 (255%)	50.3±6.0 (547%)	64.6±7.6 (702%)	52.9±5.8 (575%)

activity and were not able to accomplish cell wall reconstitution and to divide, even though during the first two days in culture they showed the same level of POX activity measured in regenerating protoplasts [11].

We propose that laccase activity detected in cultured protoplasts and soon after wounding could contribute to the polymerization of structural tyrosine-rich proteins instead of monolignol oxidation and its physiological role could be to ensure the cross-linking of cell wall structural proteins when H_2O_2 is not available. A possible two-step polymerization model, in which laccases and POXs control two successive levels of polymerizing reactions, could also explain the data showing that wounding induced a prompt activation of laccase activity whereas POX activity was stimulated only very lately (Table 1). However, laccase could also be involved in further steps of primary cell wall reconstitution and tightening, due to the uncertain H_2O_2 availability *in vivo* in all the cell wall compartments and the possible direct inhibition of POXs [16, 25–27].

EXPERIMENTAL

Plant material and isolation of protoplasts. Tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants were grown in a glasshouse at 25°, and 4–5 fully developed leaves were collected prior to blooming. Grapevine plants (*Vitis vinifera* L. cv Sultanina) were grown *in vitro* as previously described [28] and developed leaves were used for isolating protoplasts [10]. Protoplasts were isolated from tobacco and *Vitis* leaf mesophyll and cultured as described [9, 10, 30]; 4 hr maceration of tobacco leaf mesophyll tissue in hydrolytic soln (1% cellulase, 0.5% macerozyme) was used to recover regenerating protoplasts and 18 hr maceration in the same hydrolytic soln resulted in recalcitrant protoplasts [30]. In order to mimic wounding response, very narrow cuts were performed on the upper surface of the leaves, which were subsequently stored in H_2O in the dark.

Protein extraction. Crude extract from tobacco leaves was obtained by grinding the tissue in a mortar and pestle in the presence of 4 vol. of extraction buffer [50 mM K-Pi, pH 7.9, 20% sorbitol, 1 mM EDTA, 2 mM DTT, 10 μM leupeptin, 0.3% Triton X-100]. The homogenate was filtered through 8 layers of cheesecloth, centrifuged for 20 min at 18 000 *g*, and the supernatant was used for enzyme assays. Lysis of protoplasts collected at successive times during culture was accomplished by adding 3 vol. of extraction buffer and storing the suspension on ice for 30 min with periodic stirring. The soluble fr. was recovered by centrifugation (15 min at 18 000 *g*). The spent medium was sepd by pelleting the protoplasts for 10 min at 150 *g*, the remaining debris were recovered by centrifugation (20 min at 18 000 *g*) and the final supernatant was dialysed overnight at 4° against 50 mM K-Pi buffer, pH 7, 1 mM EDTA, before use.

Enzymic activities. Protein concn was quantified according to ref. [31]. Laccase activity was measured spectrophotometrically at pH 5.2 by following the oxidation of 2,2-azinobis-(3-ethyl-benzthiazoline-6-sulphonate) at 420 nm, according to ref. [5], using an extinction coefficient of 3.6×10^4 M^{-1} cm^{-1}. Tropolone (100 μM) was used as specific inhibitor of PPO [13]. Catalase (Sigma) was added to the reaction mixt. 60 units ml^{-1} to inhibit POX activity and SDS (1.5 mM) was used in a K-Pi buffer soln, at pH 6.5, to test PPO activity stimulation [14]. POX activity was measured according to ref. [32].

Gel electrophoresis. Both SDS-denaturating and native PAGEs were run by using a Bio-Rad minigel system, according to ref. [33]. Following non-denaturing electrophoresis, the slabs were stained for laccase activity by incubating them in 50 ml of 100 mM Na-Pi buffer, pH 6.8, 15 mg catechol, and 50 mg sulphanilic acid at 30° for 30–60 min. Total proteins were visualized in SDS-denatured gels by Ag staining. POX isoforms were sepd by IEF and specifically stained as previously described [11].

Peroxidase purification and concn. Crude extract from protoplasts cultured 6 days was used to recover the protein fr. by a double-step $(NH_4)_2SO_4$ pptn (40 and 75% satn, respectively); protein was resuspended in Concanavalin-A Sepharose buffer (50 mM Tris–HCl, pH 7.0, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$) and thoroughly dialysed against the same buffer before loading it on a Concanavalin-A

Sepharose column (Pharmacia). The column was washed with 15 vol. of Concanavalin-A Sepharose buffer and bound proteins were eluted using 5 vol. of a linear gradient of 0–200 mM α-methyl mannopyranoside. Frs showing POX activity were pooled, dialysed against 50 mM Tris–HCl, pH 7, 1 mM EDTA and concd using Centricon vials (Amicon).

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