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Research article

Evidence of parietal amine oxidase activity in *Solanum torvum* Sw. stem calli after *Ralstonia solanacearum* inoculation

Marcel Aribaud [a,b], Michel Noirot [b], Anne Gauvin [c], Christine Da Silva-Robert [d], Isabelle Fock [a], Hippolyte Kodja [a,*]

[a] Université de La Réunion, Faculté des Sciences et Technologies, UMR "Peuplements végétaux et bioagresseurs en milieu tropical" Université de La Réunion-CIRAD, 15 avenue René Cassin, BP 7151, 97715 Saint Denis messag cedex 9, La Réunion, France
[b] CIRAD, UMR "Peuplements végétaux et bioagresseurs en milieu tropical" Université de La Réunion-CIRAD, Pôle de Protection des Plantes, Ligne Paradis, 97410 Saint Pierre, La Réunion, France
[c] Université de La Réunion, Faculté des Sciences et Technologies, Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments, 15, Avenue René Cassin, BP 7151, 97715 Saint-Denis messag cedex 9, La Réunion, France
[d] Université de La Réunion, Faculté des Sciences et Technologies, Laboratoire de Biochimie et de Génétique moléculaire, 15, Avenue René Cassin, BP 7151, 97715 Saint-Denis messag cedex 9, La Réunion, France

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ABSTRACT

Calli induced from *Solanum torvum* stem explants were inoculated with *Ralstonia solanacearum* under partial vacuum. All calli showed a hypersensitive response after infiltration. Furthermore, amine oxidase activity with aldehyde and H_2O_2 production was detected in semi-purified cell walls of calli infiltrated by the bacteria. Due to its preferential affinity for monoamines, this enzyme is supposed to have monoamine oxidase-like (MAO-like) activity. Moreover, the presence of hydroxyl radicals in the aromatic cycle alters the oxidative deamination kinetics of potential substrates. Indeed, the oxidation of dopamine (+2, OH) was shown to be faster than that of tyramine (+1, OH), which in turn was faster than that of phenylethylamine (0, OH). The MAO-like catalytic activity was significantly inhibited by some reducing agents such as sodium bisulphite and cysteine, and also by tryptamine under anaerobiosis. This latter result suggested that the prosthetic group of the MAO-like enzyme could be a tyrosine-derived 6-hydroxytopaquinone structure. Finally, the sigmoid kinetics of the MAO-like enzyme in semi-purified cell walls did not correspond to that expected for a purified MAO, suggesting that the kinetics were affected by some factors present in cell walls.

1. Introduction

Bacterial wilt, caused by *Ralstonia solanacearum*, is one of the most dramatic plant diseases, affecting over 250 different species, including some economically important Solanaceae crops [1–3]. Bacterial wilt may cause up to 100% yield loss in *Solanum melongena* (eggplant) or *Solanum lycopersicum* (tomato). Nevertheless, tolerance to *R. solanacearum* exists in *Solanum torvum* [4,5], a wild species native to India and closely related to eggplant [6]. This species, widely distributed in tropical and subtropical areas such as in the Réunion Island (France), constitutes a diploid model for studying disease-resistance genes in the *Solanum* genus.

Plant resistance to pathogens is often associated with hypersensitive responses (HRs). Production of reactive oxygen intermediates, such as hydrogen peroxide (H_2O_2), is one of the earliest central events during HR. It restricts pathogens by directly attacking them [7], by strengthening the cell wall proteins through peroxidative cross-linking by inducing the deposition of related wall-bound phenolics [8,9], and by acting as a diffusible signal in cells adjacent to HR lesions [10]. H_2O_2 is directly produced by amine oxidases (AOs) [11–13], which catalyse the oxidation of biogenic amines.

Amine oxidase activity was first described in the liver [14], where the oxidative deamination of tyramine led to the production of oxygen and ammonia. Since then, there has been an emphasis on the production of aldehyde concomitant with oxygen (as H_2O_2) and ammonia [15–17]:

$$R{-}CH_2{-}NH_3^+ + O_2 \rightarrow R{-}CHO + H_2O_2 + NH_4^+$$

* Corresponding author. Tel.: +33 262 93 81 71; fax: +33 262 93 81 19.
E-mail address: hippolyte.kodja@univ-reunion.fr (H. Kodja).

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The presence of several types of AO, depending on the substrate, has also been shown [14,15,17–19]: the monoamine oxidases (MAOs), which oxidize tyramine, dopamine, and phenylethylamine, the diamine oxidases (DAOs) using substrates such as diaminopropane and putrescine, and the polyamine oxidases (PAOs) requiring for example spermine and spermidine as substrates. AOs can also be classified according to their cofactor: the copper-containing amine oxidases (Cu-AOs, EC 1.4.3.6) [20] and the flavin-containing amine oxidases (FAD-AOs, EC 1.4.3.4; FAD-PAOs, EC 1.5.3.11) [21].

Cu-AOs are found in more than 25 plant species, such as in *Avena sativa*, *Helianthus tuberosus*, *Nicotiana tabacum*, and *Pisum sativum* [22]. In addition to the presence of the cupric ion, Cu-AOs contain a cofactor, the 2,4,5-trihydroxy-phenylalanyl quinone or topaquinone (TPQ) [20,23]. Their substrates are primary amines, including mono-, di-, and polyamines [24], and secondary amines, such as spermine and spermidine [24,25]. In plants, the enzyme is known to be located in the membranes and cell walls of the epicotyle as soluble proteins [26,27] and in the xylem of the root [22]. Cu-AO activity is inhibited by tryptamine under anaerobic conditions [28], *para*-hydroxyphenylacetaldehyde (*p*HPA) [29], pyridine derivatives, oximes, copper chelators, and 2-bromoethylamine [24].

FAD-PAOs with noncovalent binding form the second group of amine oxidases known in plants. To date, they have been reported to be present only in monocots such as *A. sativa* [30], *Hordeum vulgare* [31], *Oryza sativa* [32], and *Zea mays* [33]. They are specific to the secondary amine functional group present in polyamines and are found both in the cytoplasm and in intercellular spaces [24,34]. However, the oxidation of the secondary amine function does not release ammonia [35]. Some inhibitors of FAD-PAOs have no effect on Cu-AOs, and *vice versa* [24,28,29,36,37]. This property facilitates the use of tryptamine under anaerobic conditions in order to differentiate Cu-AOs from FAD-PAOs.

In plants, AOs are mainly associated with the primary and secondary cell walls of tissues undergoing lignification, suberization, and wall stiffening (such as xylem parenchyma, endodermis, and epidermis). Their association with cortical parenchyma cell walls during specific developmental stages has also been reported [38–40]. AO activities are enhanced during incompatible interactions between plants and pathogens [26,27,41]. For example, PAO activity increases up to 3-fold above the basal level during the HR against tobacco mosaic virus infection in *N. tabacum* [42].

Callus culture is a good model for studying bacteria–plant cell interactions because most bacteria are localized in the intercellular space of inoculated tissues and do not penetrate the host cells [43]. This is true for *R. solanacearum*, which invades intercellular spaces and binds to cell walls. The first aim of this study is to study an AO-like activity in the walls of calli after *R. solanacearum* inoculation, calli being derived from *S. torvum* stems. This *in situ* characterization contrasts with the classical biochemical *in vitro* approach [22,23,26,29,36], leading to the elucidation of some basic characteristics of the enzyme's activity in real biological systems. Furthermore, an AO-like activity with a preferential affinity for aromatic monoamines was observed, and the inhibition of this activity using reducing agents or an irreversible TPQ inhibitor was attempted.

2. Results

In all the results of the current experiments, the instantaneous production of hydrogen peroxide (IPHP) and aldehyde (IPA) is expressed as nanomoles per gram of semi-purified cell wall (S-PCW) per hour (nmol g^{-1} h^{-1}).

2.1. Preliminary experiments

2.1.1. Absence of soluble proteins in S-PCWs after washing

AO activity can arise from either S-PCWs or soluble proteins present on S-PCWs. It was therefore necessary to verify the absence of soluble proteins on S-PCWs after 10 washings.

Before the first washing, i.e. in the supernatant, there were 4.13 mg mL^{-1} of soluble proteins. This content started to decrease strongly as soon as the first washing (0.48 mg mL^{-1}) was undertaken to reach 0.0013 mg mL^{-1} at the fourth washing. No soluble proteins were detected from the fifth to the tenth washing. Nevertheless, absence of detection did not necessarily mean absence of soluble proteins. Consequently, the soluble protein content (y) was expressed as a function of the washing number (x): $y = 3.48\ e^{(-1.9469x)}$ ($R^2 = 0.99999$). This allowed us to estiamate the soluble protein content after 10 washings: $1.22.10^{-8}$ mg mL^{-1}. Obviously, such expected soluble protein content can be considered as negligible.

2.1.2. Is there some AO-like activity in soluble proteins?

Even if soluble proteins present on S-PCWs can be considered as negligible after 10 washings, it is necessary to verify the presence or not of an AO-like activity in soluble proteins. This was tested first in the presence of tyramine: there was no IPHP from 0 to 96 h, at either pH 5.8 or pH 8. In contrast, an IPHP was observed when using diamines and polyamines as substrates, but only at pH 8. In that case, there was no difference between aliphatic amines and the IPHP was residual (6.96 nmol g^{-1} h^{-1}). Note that in untreated calli, IPHP in soluble proteins in the presence of di- and polyamines was quasi-null (0.05 nmol g^{-1} h^{-1}).

Consequently, if an AO-like activity had been subsequently observed in S-PCWs in the presence of tyramine, its parietal origin would have been taken into account.

2.2. Evidence of parietal amine oxidase-like activity in calli infected by R. solanacearum

In all the experiments, the AO-like activity was estimated through the IPHP and the IPA.

2.2.1. Is there some AO-like activity in the S-PCWs of untreated 6-week-old calli?

The aim of the experiment was to record the basic AO-like activity in S-PCWs of untreated 6-week-old calli in the presence of tyramine. In fact, IPA was found to be zero, whereas IPHP was residual (23 nmol g^{-1} h^{-1}). Two hypotheses can be put forward in order to understand such a result: (1) tyramine was not the best substrate for revealing the AO-like activity; and/or (2) the AO-activity was not enhanced in the absence of bacteria inoculation.

2.2.2. Is there some AO-like activity in the S-PCWs of calli stressed by the application of the inoculation protocol without bacteria?

When AO-like activity is observed on inoculated calli, its origin could be the stress due to the inoculation protocol. It was therefore important to record AO-like activity in S-PCWs for which the inoculation protocol was carried out using TRIS buffer, but without bacteria. Such an experiment was called pseudo-inoculation.

IPA varied significantly from $T = 0$ to 96 h ($F_{6,14} = 13.6$; $p = 0.00004$) (Fig. 1). Showing about 12 nmol g^{-1} h^{-1} just after the pseudo-inoculation, IPA reached a peak at about 24–28 h (62 nmol g^{-1} h^{-1}), before decreasing by up to 8 nmol g^{-1} h^{-1} at $T = 96$ h.

IPHP also showed significant variations from $T = 0$ to 96 h ($F_{6,14} = 31.7$; $p < 0.000001$) (Fig. 2). The IPHP was about 130 nmol g^{-1} h^{-1} just after the pseudo-inoculation and then increased,

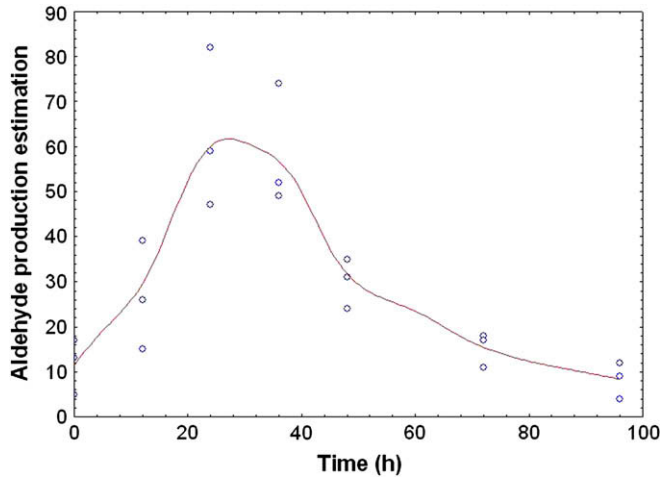

Fig. 1. Effect of a pseudo-inoculation on the instantaneous production of aldehyde (IPA) in S-PCWs. The inoculation protocol was carried out on calli, but without any bacteria in the TRIS buffer. S-PCWs were prepared from calli sampled between $T = 0$ and 96 h after the pseudo-inoculation. IPA was recorded at $t = 120$ min and was expressed in nmol g^{-1} h^{-1} of formaldehyde equivalent. Tyramine (15 mM) was used as the substrate.

reaching 349 nmol g^{-1} h^{-1} after 12 h. Later, the IPHP decreased quickly from 12 to 48 h and then more slowly during the subsequent period (>48 h), to reach 38 nmol g^{-1} h^{-1} at 96 h.

For instance, both IPHP and IPA increased just after the application of the inoculation protocol and this could result from a stress. Nevertheless, IPHP and IPA differed in two ways: (1) the IPA was 4–6 times lower than the IPHP, and (2) its peak was delayed (28 vs. 12 h). The difference could be due to aldehyde trapping by bovine serum albumin (BSA) and other proteins.

2.2.3. Is there an increase in AO-like activity in the S-PCWs of inoculated calli?

Evidence of a parietal AO-like activity in the S-PCWs of inoculated calli was obtained through IPA and IPHP when using tyramine as the substrate.

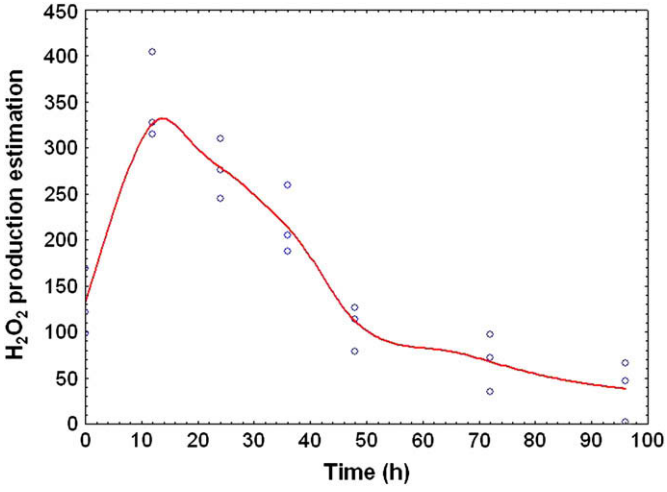

Fig. 2. Effect of a pseudo-inoculation on the instantaneous production of H_2O_2 (IPHP) in S-PCWs. The inoculation protocol was carried out on calli, but without any bacteria in the TRIS buffer. S-PCWs were prepared from calli sampled between $T = 0$ and 96 h after the pseudo-inoculation. IPHP was recorded at $t = 120$ min and was expressed in nmol g^{-1} h^{-1} of formaldehyde equivalent. Tyramine (15 mM) was used as the substrate.

In the S-PCWs of inoculated calli, IPA increased from $t = 20$ to 120 min ($F_{5,18} = 49.5$; $p < 1.10^{-6}$). The sigmoid function $y = M/[1 + e^{a(t-b)}]$ was used to fit data recorded on each sample (Fig. 3). This allowed us to emphasize kinetic differences between replicates (Fig. 3). Parameter M ranged from 604 to 734 nmol g^{-1} h^{-1} (mean 686 nmol g^{-1} h^{-1}). Means obtained for the parameters a and b were −0.036 and 36.8, respectively. It is important to know that parameters M, a and b can be interpreted, M and b representing the asymptotic production and the abscissa at the inflexion point, respectively, whereas parameter a was related to the slope at the inflexion point.

According to the fit, IPA would range from 120 to 180 nmol g^{-1} h^{-1} at time $t = 0$ (Fig. 3). In particular, IPA at $t = 120$ min would be quasi-defined by its value at time $t = 0$, and this value was characteristic of the callus from which the extract was derived. These points are further elaborated in Section 3.

IPHP also increased over the time course studied ($F_{5,18} = 24.0$; $p < 1.10^{-6}$). As for IPA, the results were fitted to a sigmoid function (Fig. 4). In this case, parameter M ranged from 1214 to 1495 nmol g^{-1} h^{-1} depending on the callus (mean 1302 nmol g^{-1} h^{-1}). Means obtained for the parameters a and b were −0.0485 and 48.9 nmol g^{-1} h^{-1}, respectively. The initial value would range from 100 to 150 nmol g^{-1} h^{-1} depending on the callus.

Differences were noted between the kinetics of IPA and IPHP (Figs. 3 and 4). In the case of IPA, the different curves were almost parallel (Fig. 3), whereas in the case of IPHP, the between-callus variance at $t = 0$ was amplified when reaching the asymptote ($t = 120$). The second kinetic difference concerned the abscissa of the inflexion point, which was 36 min for the IPA and 49 min for the IPHP. Finally, the third difference concerned the parameter M which was 1.9-fold higher in the case of IPHP.

Based on the experiment on the S-PCWs of untreated calli, two hypotheses were put forward: (1) the tyramine was not the best substrate for revealing the AO-like activity; and/or (2) the AO-activity was not enhanced in the absence of bacteria inoculation. Results of the current experiment allow the rejection of the second hypothesis: the AO-like activity would be enhanced by the bacteria. Concerning the first hypothesis, the response is incomplete: the tyramine would be a substrate of the AO-like enzyme, but is it the best?

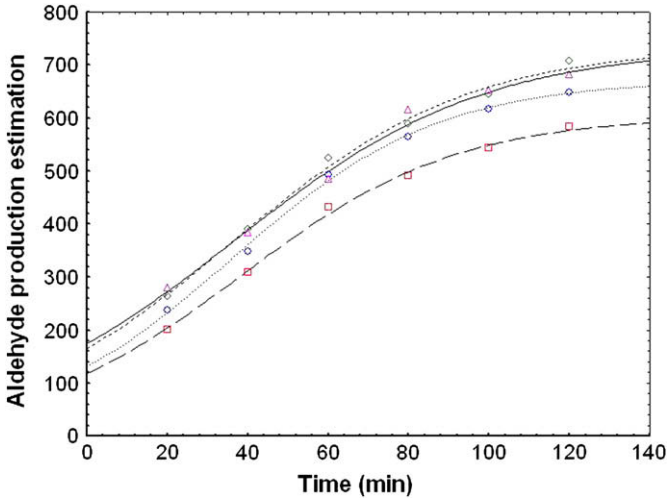

Fig. 3. Kinetics of the instantaneous production of aldehyde (IPA) of S-PCWs from inoculated calli. Tyramine (15 mM) was used as the substrate. The estimates are expressed in nmol g^{-1} h^{-1} of formaldehyde equivalent. The data were fitted using a sigmoid function. Each curve corresponds to an extract.

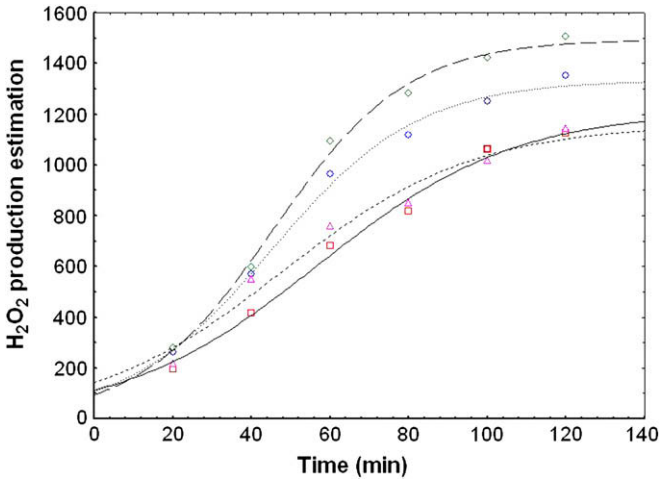

Fig. 4. Kinetics of the instantaneous production of H_2O_2 (in nmol g^{-1} h^{-1}) of S-PCWs from inoculated calli. Tyramine (15 mM) was used as the substrate. Data were fitted using the sigmoid function. Each curve corresponds to an extract.

2.3. Comparison between substrates

As previous experiments showed that IPHP was a better criterion by which to measure the AO-like activity than IPA, it was retained for all the experiments that followed.

2.3.1. Comparison between aromatic monoamines differentiated by their hydroxylation level

Dopamine (I), tyramine (II), and phenylethylamine (III) (Fig. 5) were tested as substrates for the enzyme extract. They were selected on the basis of the hydroxylation level of their aromatic cycles: 0, 1, and 2 for phenylethylamine, tyramine, and dopamine, respectively.

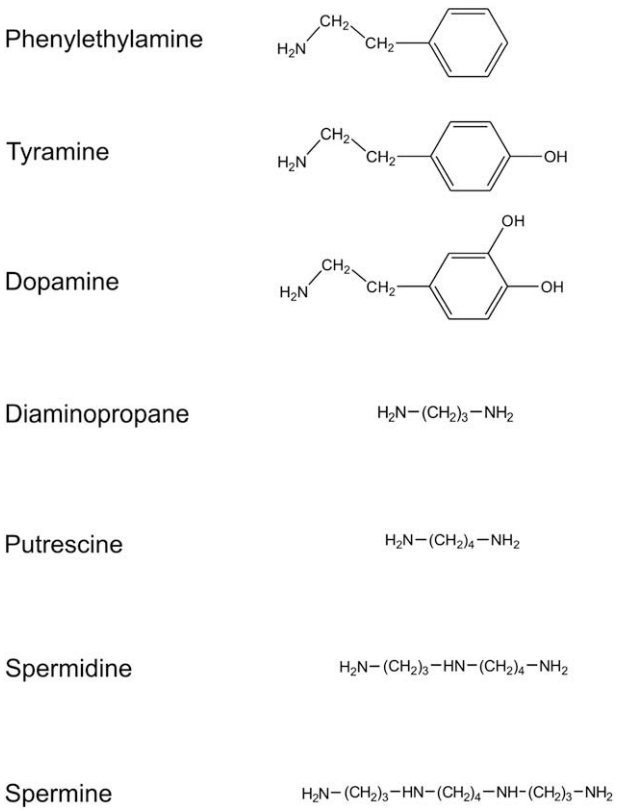

Fig. 5. Chemical formula of amine oxidase substrates used in the experiments.

Table 1
Instantaneous H_2O_2 production (in nmol g^{-1} h^{-1}) of S-PCWs from calli, just after the inoculation (T_0) and 96 h later (T_{96}). The substrates included three monoamines (phenylethylamine, tyramine, and dopamine), two aliphatic diamines (diaminopropane and putrescine) and two aliphatic polyamines (spermidine and spermine) (in nmol g^{-1} h^{-1}). Substrate concentration was 40 mM.

		T_0	T_{96}
Aromatic monoamines	Phenylethylamine	10.1	476.4
	Tyramine	58.2	2668
	Dopamine	54.6	3478
Aliphatic diamines	Diaminopropane	0.0	2.6
	Putrescine	0.0	3.5
Aliphatic polyamines	Spermidine	0.0	7.4
	Spermine	0.0	2.0

IPHP increased over the duration of study for the three substrates ($F_{1,24} = 455$; $p < 1.10^{-6}$), but with some differences between the levels obtained from these substrates ($F_{2,24} = 40.5$; $p < 1.10^{-6}$) (Table 1). Moreover, IPHP after 96 h of reaction linearly depended on the hydroxylation level of the aromatic cycle (Fig. 6).

2.3.2. Other potential substrates

Aliphatic amines [diaminopropane (IV), putrescine (V), spermidine (VI) and spermine (VII)] (Fig. 5) were compared with the aromatic monoamines to test the effects of the aromatic cycle and the aliphatic chain length on IPHP.

An initial statistical analysis confirmed the production of H_2O_2 for the seven substrates ($F_{6,28} = 560$; $p < 1.10^{-6}$). At $T = 0$, there was no production using aliphatic substrates, whereas a low production level was observed when using aromatic substrates (Table 1). At $T = 96$ h, IPHP from aliphatic amine substrates was residual relative to that observed with aromatic amines, thus highlighting the marked effect of the aromatic cycle on IPHP in cell walls.

A second statistical analysis (nested ANOVA) with respect to only the aliphatic substrates showed no difference between the di- and polyamines ($F_{1,2} = 0.11$; $p = 0.77$). In contrast, some variations were observed within each type of substrate ($F_{2,8} = 5.99$; $p = 0.025$); when using diamines, an increase in the chain length had a positive effect on IPHP, whereas in the case of polyamines, the addition of an aminopropyl radical on spermidine reduced IPHP 3-fold.

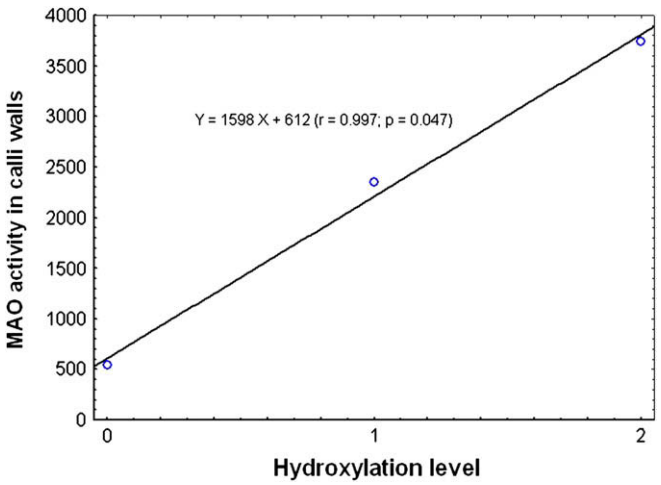

Fig. 6. Relationship between the instantaneous production of H_2O_2 (in nmol g^{-1} h^{-1}) of S-PCWs from inoculated calli and the hydroxylation level of the aromatic cycle. Substrate concentration was 40 mM.

2.4. Inhibition of amine oxidase-like activity

2.4.1. Using tryptamine under anaerobic conditions

In Cu-AOs with TPQ, tryptamine gives the indolacetaldehyde which reacts with the prosthetic group to form a covalent link under anaerobic conditions, inhibiting the enzyme activity. Inversely, tryptamine under anaerobic conditions did not inhibit the enzyme activity of FAD-PAOs. In the present experiment, tryptamine pretreatment under anaerobiosis induced a decrease in IPHP with tyramine as the substrate (Fig. 7). Fittings yielded a value of $M = 3705$ nmol g^{-1} h^{-1}, $a = 0.138$, and $b = 17.0$ (at I_{50}). There was no relationship between the initial IPHP and the I_{50} (concentration of substrate at which inhibition was 50%). Moreover, IPHP was nullified at 60 mM of tryptamine. Consequently, the parietal AO-like activity seems to be a Cu-AO-like activity with TPQ as cofactor.

2.4.2. Using sodium bisulphite and cysteine

As Cu-AO-like activity depends on the presence of topaquinone (TPQ) at the active site, TPQ reduction by sodium bisulphite or cysteine should decrease the enzyme activity.

A preliminary experiment was carried out to check whether cysteine and sodium bisulphite could reduce IPHP in the presence of parietal peroxidase. As expected, cysteine and natrium bisulphite were not oxidized by H_2O_2 (cysteine: $F_{1,32} = 0.0003$, $p = 0.98$; sodium bisulphite: $F_{1,32} = 0.75$, $p = 0.39$). Consequently, the decline in IPHP in further experiments on AO-activity inhibition could not be explained by the activity reduction due to the inhibitor.

Indeed, sodium bisulphite decreased the level of IPHP (Fig. 8). For each extract, the observed data were fitted to a sigmoid function, leading to the deduced values of $M = 3593$ nmol g^{-1} h^{-1}, $a = 0.214$, and $b = 12.7$. The parameter b indicated the concentration at which the decrease was 50% (I_{50}). Three important points need to be emphasized here: (1) the initial IPHP ranged from 3153 to 4054 nmol g^{-1} h^{-1}, depending on the extract; (2) the initial IPHP increased as the I_{50} decreased ($r = -0.96$; $p = 0.01$); and (3) the IPHP was zero in the presence of 40 mM bisulphite.

A similar result was observed in the presence of increasing concentrations of cysteine (Fig. 9). In this case, the sigmoid fitting yielded values of 3508 nmol g^{-1} h^{-1}, 0.14, and 23.4 for M, a, and b, respectively. As discussed previously, the initial IPHP varied depending on the extract. By contrast, the initial IPHP increased as the I_{50} increased.

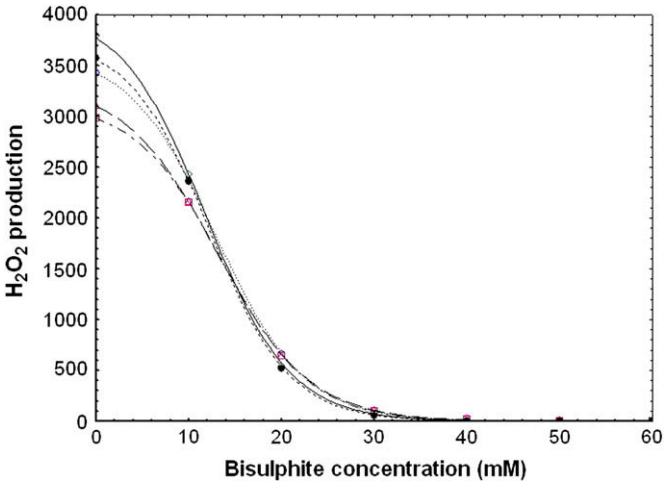

Fig. 8. Effect of the sodium bisulphite concentration on the instantaneous production of H_2O_2 (in nmol g^{-1} h^{-1}) of S-PCWs from inoculated calli (initial tyramine concentration was 40 mM).

A comparison between the two inhibitors for the values of M, a, and b was carried out. As expected, there were no differences with respect to the initial IPHP (M) ($F_{1,9} = 0.19$; $p = 0.67$). By contrast, a and b differed for the two inhibitors (a: $F_{1,9} = 160$, $p < 10^{-6}$; b: $F_{1,9} = 504$, $p < 10^{-6}$). Cysteine had a lower inhibiting activity: 65–70 mM of substance was required to halt IPHP.

3. Discussion

This is the first report of a parietal AO-like activity in plants, induced by *R. solanacearum*. Indeed, to date, only soluble amine oxidases from the cell walls of chickpeas, lentils, and etiolated seedlings of the soybean have been studied [26–28].

3.1. Evidence of AO-like activity in callus cell walls

Evidence for the presence of AO activity was obtained from the production of aldehyde and H_2O_2 over time in the callus cell walls of *S. torvum* inoculated with *R. solanacearum*. Nevertheless, as AO activity was detected in the nonpurified enzyme extract, the term "AO-like activity" seems more suitable. Moreover, it is unlikely that

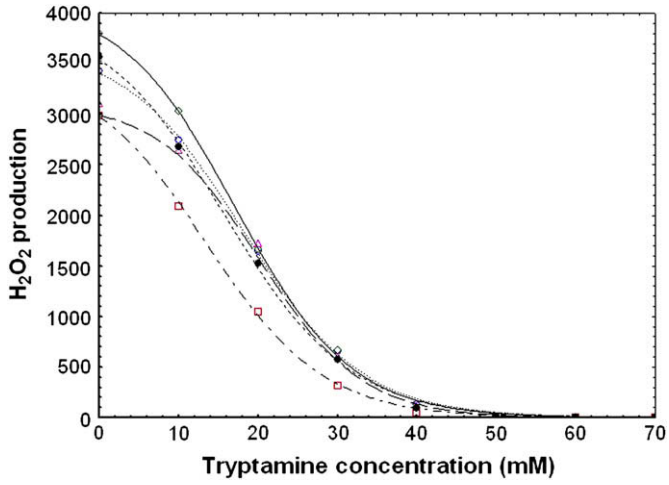

Fig. 7. Effect of the tryptamine concentration during the pretreatment under anaerobiosis on the instantaneous production of H_2O_2 (in nmol g^{-1} h^{-1}) of S-PCWs from inoculated calli and the (initial tyramine concentration was 40 mM).

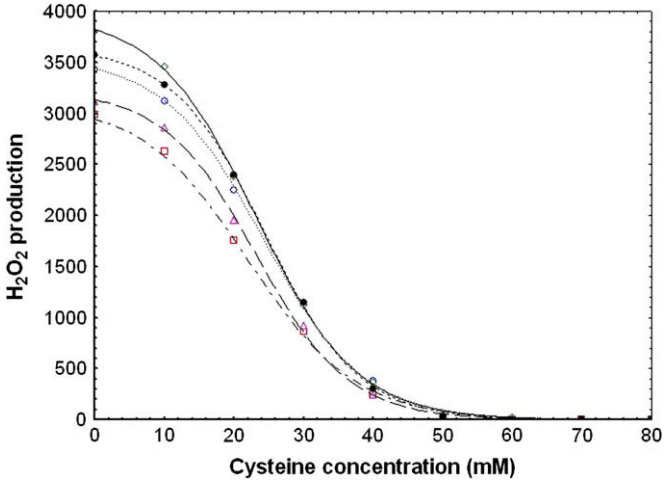

Fig. 9. Effect of the cysteine concentration on the instantaneous H_2O_2 production (in nmol g^{-1} h^{-1}) of S-PCWs from inoculated calli (initial tyramine concentration was 40 mM).

such activity could arise from residual soluble AOs. Cell walls were indeed washed 10 times and no soluble proteins were eluted from the last washing. In addition, only DAO- and PAO-like activities were recorded in soluble proteins, whereas the parietal AO-like activity was mainly specific to aromatic amines.

The presence of a TPQ moiety at the active site was indirectly suggested in our experiments using tryptamine in anaerobiosis, which would be a specific inhibitor of Cu-AO activity [29,37], although identification of the TPQ structure using spectrophotometric methods was not carried out [44,45]. In anaerobiosis, this substrate is oxidized in indolacetaldehyde, which forms a covalent link with the carbonyl bearing the quinone function, thus avoiding regeneration of the TPQ structure [29]. The reversible inhibition of the copper-free enzyme contrasts to the irreversible inhibition of the native enzyme, showing the importance of copper in the irreversible covalent link formation [29].

The inhibition of AO-like activity was also tested using some reducing agents such as sodium bisulphite and cysteine. This reduction of enzyme activity was expected to be concentration dependent, and this was indeed the observed pattern. Moreover, inhibition was stronger with sodium bisulphite ($I_{50} = 12.7$ mM) than with cysteine ($I_{50} = 23.4$ mM), and this difference in their effect could be due to the higher reducing power of sodium bisulphite (the oxidoreduction coefficient for the bisulphite is 0.12 V as compared to 0.08 V for cysteine).

3.2. Variations in the affinity of AO-like activity to different substrates

The highest variations in affinity of the experimental AO-like activity were noted when comparing aromatic and aliphatic monoamines. Indeed, the presence of the aromatic cycle caused a 100- to 1000-fold increase in the activity. Nevertheless, affinity variations were also noted among the aromatic monoamines in relation to their hydroxylation level, and among aliphatic monoamines according to their chain length.

3.2.1. Affinity to aromatic monoamines increased with the hydroxylation level

The intensity of parietal AO-like activity depended on the aromatic monoamine that was used as the substrate. In addition, there was a linear and positive regression between the recorded parietal AO-like activity and the hydroxylation level of the aromatic cycle. A similar pattern was observed in previous reports when comparing tyramine ($K_M = 710$ nM) and phenylethylamine ($K_M = 520$ nM) in lentil seedlings [37]. Two hypotheses could be put forward on the basis of these observations:

(1) the presence of hydroxyl radicals would affect the enzyme–substrate complex formation. Low AO-like activity (phenylethylamine) and a low enzyme concentration imply a great relative importance of the enzyme–substrate complex. An increase in the number of activating substituent groups induces an increase in the electron density of the aromatic cycle, and could thus decrease the enzyme–substrate binding strength. A similar effect could be observed in lentils while using an aromatic amine with the indole cycle, such as for tryptamine, 5-hydroxytryptamine, and 5-methoxytryptamine [29]. In these cases, the presence of the hydroxyl and methoxy radicals at position 5 of the indole cycle also increases the electron density of the resultant structure.
(2) hydrophobic properties of the benzene cycle and the aliphatic chain would decrease when the number of hydroxyl radicals increases. Thus, the activating substituents would favour substrate penetration into the cellulopectic structure of plant walls and the insertion of the substrate within the active site of the AO-like enzyme. When considering aromatic amines, both hypotheses are not exclusive.

3.2.2. Affinity variations among aliphatic polyamines

Parietal AO-like activity increased in relation to the aliphatic chain length. This was noted for diaminopropane (3-carbon skeleton), putrescine (4-carbon skeleton) and spermidine (with a total of seven carbons); however, an increase in the chain length should increase the hydrophobic properties and, according to the second hypothesis, should decrease the activity. Consequently, the comparison of AO-like activities between the aliphatic polyamines facilitates the rejection of the second hypothesis concerning the possible effect of hydrophobic properties as an explanation for the affinity variations.

Nevertheless, spermine is a special case, with a higher carbon number (total of 10) and lower AO-like activity. In fact, this molecule has two secondary-amine functions that might be responsible for a spatial dimension incompatible with optimal adaptation to the active site.

3.3. AO-like activity kinetics

The fact that AO-like activity kinetics were fitted by a sigmoid function, which was not expected from a Michaelian enzyme, was an important finding. This discrepancy and its biological implications form the first point addressed in this discussion.

3.3.1. The choice of the sigmoid function for fitting activity kinetics

Theoretically, AO is a Michaelian enzyme [24,46,47]. Nevertheless, all observed kinetics fitted a sigmoid function. In particular, the statistical validations using residual analysis showed that only the sigmoid model could be applied. This finding is discussed herein in terms of the biological implications.

With the sigmoid model, an increase in IPHP over a course of time (t; $t + \Delta t$) depends not only on the substrate quantity, but also on the intensity of IPHP at time t. At the beginning of the reaction, IPHP is not greatly related to the quantity of the substrate present in the medium, it is mainly dependent on the intensity of IPHP at time t, and it increases exponentially. Conversely, at the end, deterioration of the substrate stock prevails, leading to asymptotic activity.

The sigmoid function also enables the extrapolation of the IPHP value when $t = 0$ and of M (asymptotic IPHP). All the fits that were obtained indicated that IPHP would not be nil at $t = 0$, i.e. AO-like activity would preexist at $t = 0$, which is a paradox for a Michaelian enzyme. Although the choice of the fitting function seemed suitable between $t = 20$ min and $t = 120$ min, caution is required concerning extrapolations made beyond the studied time range (before $t = 20$ min and after $t = 120$ min). The theoretical absence of IPHP at $t = 0$ would imply the presence of a second biological process between $t = 0$ and $t = 20$ min. This also implies that such a process had stopped between 20 and 120 min, i.e. when data were recorded. This assumption seems unlikely.

The other alternative is to consider that extrapolations at $t = 0$ were possible and consequently to look for some biological explanation for the gap between the theoretical model and the observed data. The marked difference noted between the experimental results and classical enzymatic kinetics was the sampling of the S-PCWs instead of a purified enzyme. The comparison between the IPA and IPHP kinetics could give an indication. In both estimations, there were kinetic differences between the S-PCW samples. In other words, the activity at $t = 0$ and the asymptotic activity depended on the origin of the S-PCWs. The observed difference between the expected isosteric behaviour and the observed

allosteric pattern could be due to the integration of AOs into the wall, possibly as a multienzyme complex. This does not refute the Michaelian hypothesis, which is still applicable to purified enzymes.

To conclude, the problem under consideration is not overcoming a discrepancy between the observed data and a theoretical model, but rather coming up with a biological hypothesis to explain such a discrepancy. Hence, in this experiment, it was better to consider the kinetics of IPHP (or IPA) rather than the AO-like activity, even though this latter activity was responsible for IPHP.

3.3.2. Comparison between the kinetics of IPA and IPHP

Theoretically, after oxidization by AO, one tyramine molecule gives rise to one H_2O_2 molecule and one aldehyde molecule.

The relative efficiency of titration for IPA and IPHP is low in both cases. At $t = 0$, 400,000 nmol of tyramine per g of S-PCWs were estimated, whereas the asymptote M reached a mean of 686 and 1302 nmol g^{-1} h^{-1} for the IPA and IPHP, respectively. Although the efficiency was 2-fold higher in the case of IPHP, this was still very low (0.33%). Two hypotheses could explain this low efficiency. The first concerns an unknown product that is consumed by other parallel reactions. The second concerns the gradual loss of AO-like activity in cell walls with respect to IPA. In addition, both processes could be simultaneously involved in the low efficiency observed.

In practice, the asymptotic value M differed markedly when comparing IPA and IPHP. This was the main difference between the kinetics of the two products. The fact that the substrate concentrations were the same in both situations suggests that there was a higher loss of the aldehyde. The lower efficiency of the aldehyde titration could have resulted from its binding with proteins (Schiff reaction) [37].

Binding with proteins can also affect the enzyme, and consequently the AO-like activity. This impact was diminished by using BSA to trap aldehydes in place of the enzyme. This explains why IPHP was found to be 3-fold higher in the presence of BSA (data not shown). In all cases (with or without BSA), IPA would be underestimated and cannot give reliable results. This explains why AO activity in plants has only been estimated using the hydrogen peroxide assay, as for example in lentil seedlings [37] and *Lathyrus cicerea* [48]. However, even while using purified AO, the aldehyde production clearly points to gradual loss in enzymatic activity, in spite of the presence of BSA. K_M estimation would thus always be an underestimate, except at the first minute of the reaction when the IPA was found to be low relative to BSA concentration.

The second main difference between IPA and IPHP was the presence of a relationship between the values at $t = 0$ and the asymptote M in the case of IPA, while such a relationship was not clear for IPHP. This difference could also be related to the high between-extract diversity of the asymptote M, which was observed for IPHP.

4. Conclusion

S. torvum calli inoculated with the bacterium *R. solanacearum* expressed an HR in less than 96 h, resulting in an incompatible interaction involving a resistant host and a virulent bacterial strain. During the interaction, specific parietal AO-like activity was observed, which was the strongest when using aromatic monoamines as substrates, whereas it was still residual with aliphatic di- and polyamines. On the basis of this observation, it was concluded that the specific parietal AO-like activity was a central element in the HR cascade of *S. torvum* against *R. solanacearum*.

5. Material and methods

5.1. Plant material

5.1.1. Callus production and maintenance

In vitro plants of *S. torvum* (line STR-6) were grown on an agar basal medium (BM), including the macro- and micro-elements as per Murashige and Skoog [49], sucrose (30 g L^{-1}), and vitamins as per Morel and Wetmore [50] (7.5 g L^{-1}). Calli were induced from the stem explants of 3-week-old *in vitro* plants and grown on BM, supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 1 mg L^{-1} and benzylaminopurine (BAP) at 0.1 mg L^{-1}. One month later, the calli were transferred to a maintenance medium (BM with 2,4-D at 0.5 mg L^{-1}). For 6 months, the calli were subcultured monthly on the same type of fresh medium. The pH of all the media used was adjusted to 5.8 prior to autoclaving. All callus cultures were incubated at 27 ± 2 °C under darkness in a controlled culture room.

5.1.2. Notion of time

In all the experiments, there were two notions of time: (1) the lapse time with respect to the moment of the inoculation (even in controls without inoculation): calli were sampled for the preparation of S-PCWs before the determination of activity either at $T = 0$, 12, 24, 36, 48, 72, and 96 h or only at $T = 0$ and $T = 96$ h, depending on the experiment ($T = 96$ h corresponds to a full response of inoculated calli to the HR); and (2) the time lapse with respect to the beginning of the observations of enzyme activities: activities were recorded either at $t = 20$, 40, 60, 80, 100, and 120 min (kinetic), or only at the end ($t = 120$ min), depending on the experiment.

5.1.3. Inoculum preparation and callus inoculation

All inoculations were carried out under sterile conditions. The bacterial concentration of *R. solanacearum* (strain JT 519) was adjusted to 10^8 colony forming units (cfu) per mL of 1-mM TRIS buffer using a spectrophotometer (λ_{600}) (Genesys TM 10, Thermo Electron Corporation, Cambridge, UK).

One month before *R. solanacearum* inoculation, calli were transferred to BM medium without 2,4-D. For inoculation purposes, the selected calli were immersed in a bacterial suspension (0.1 g mL^{-1} of callus), under partial vacuum for 30 min. The infected calli were then harvested by filtration. The time point of the end of the inoculation protocol was considered as $T = 0$.

The samples ($T = 0$–96 h) were immediately stored at −80 °C to stop all enzymatic reactions.

5.2. Preparation of S-PCWs

Each callus batch (maintained at −80 °C) was submitted to S-PCW extraction according to the protocol of Chuan Chi Lin and Ching Huei Kao [51], but with slight modifications. After crushing the cell samples in liquid nitrogen, the powder was suspended in 0.1-M potassium phosphate buffer (pH 8), and subsequently centrifuged for 30 min at 400 g and 4 °C. The pellet obtained from this step contained the crude cell walls.

The next step consisted of eliminating proteins bound to the wall. The first two washings were carried out with 0.1-M potassium phosphate buffer (pH 8), in the presence of 1 M NaCl (20 mL of buffer per gram of calli). They were followed by four washings with 0.1-M sodium phosphate buffer (pH 5.8), in the presence of 1 M NaCl (20 mL of buffer per gram of calli). For the elimination of NaCl, four other washings were carried out using the same buffer, but a NaCl-free version.

After each washing, the pellet was resuspended in the buffer before centrifugation at 400 g for 30 min, then filtered and spin dried.

5.3. Kinetics observations: aldehyde and H_2O_2 measurement methods

For kinetics measurements, S-PCWs (0.1 g) were suspended in 2 mL of either 0.1-M sodium phosphate buffer at pH 5.8 (in the case of aromatic monoamines) or 0.1-M TRIS buffer at pH 8 (in the case of aliphatic amines). The medium was supplemented with substrate (15 or 40 mM depending on the experiment) and BSA (Albumin Bovine Factor V, Sigma) at 10 g L^{-1}. The enzymatic reaction was stopped just before measurements using 100 μL of 3 M acetic acid (Sigma–Aldrich).

5.3.1. Measurement using Schiff's reagent: aldehyde titration

Supernatant was collected by centrifugation at 20,000 *g* at room temperature. A 400-μL sample was added to 1600 μl of Schiff's reagent (Sigma–Aldrich.). Spectrophotometric (Hewlett–Packard UV/visible 453 model G1-103-A) measurements were carried out at $\lambda = 546$ nm. A calibration curve was obtained using formaldehyde (Sigma–Aldrich) in the presence of Schiff's reagent.

5.3.2. Measurement using guaiacol: H_2O_2 titration

As in Section 5.3.1, the supernatant was collected by centrifugation at 20,000 *g*. H_2O_2 was quantified through guaiacol (1% v/v) (Sigma–Aldrich) oxidation according to the modified protocol of Chuan Chi Lin and Ching Huei Kao [51]. The hydroxyguaiacol concentration was measured in the supernatant by spectrophotometry ($\lambda = 470$ nm) using the molar extinction coefficient $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$ [6].

5.4. Experimental designs

5.4.1. Amine oxidase substrate display

Seven substrates (Fig. 5) from Sigma–Aldrich were used in the following experiments: (1) the aromatic monoamines (phenylethylamine, tyramine, and dopamine, as monohydro-chloride); and (2) the aliphatic amines, including diamines (diaminopropane and putrescine, as dihydrochlorides) and polyamines (spermidine as trihydrochloride and spermine as tetrahydrochloride).

5.4.2. Preliminary experiments

The first experiment consisted of checking the quantity of soluble proteins after each washing during the preparation of S-PCWs. Soluble proteins were titrated using the Bradford method. Titration involved (1) the supernatant obtained after centrifugation; (2) the first two washings at pH 8 in the presence of NaCl; (3) the next four washings at pH 5.8, also in the presence of NaCl; (4) the last four washings at pH 5.8, but without NaCl (see Section 5.2 on the preparation of S-PCWs). Six replicates were made for each of the 11 steps.

The second preliminary experiment consisted of evaluating the AO-like activity in soluble proteins of calli cells. A crude extract was obtained in a buffer (pH 8) containing potassium phosphate (0.1 M), natrium ascorbate (1%), and PVP (0.5%). The first step was to eliminate all small soluble molecules (such as sugars, phenols, and amino-acids) by dialysis by using either a 0.1-M sodium phosphate buffer at pH 5.8 or a TRIS buffer at pH 8. In both cases, the dialysis buffer contained natrium ascorbate (0.01%). The "dialysis buffer:crude extract" ratio was 100:1 (v/v), and the dialysis duration was 12 h at 4 °C in darkness. Nevertheless, the dialysis buffer was renewed at 4 h and 8 h. Finally, the dialysed crude extract was centrifuged for 20 min at 20,000 *g* (4 °C). Soluble proteins were then titrated using the Bradford method.

AO-like activities were estimated on soluble proteins from calli sampled at $T = 0$, 12, 24, 36, 48, 72, and 96 h after inoculation. At each time *T*, three soluble protein preparations were obtained constituting three replicates. Only IPHP were estimated and this was done at $t = 120$ min in the presence of tyramine (15 mM). Other AO-like activities were also tested using putrescine, diaminopropane, spermidine, and spermine as substrates (15 mM).

5.4.3. Evidence of amine oxidase (AO)-like activity

The aim of the first experiment was to estimate the AO-like activity in S-PCWs extracted from untreated 6-week-old calli. This experiment was carried out without bacteria inoculation, but also without pseudo-inoculation, as in the third experiment. Avoiding stresses due to the inoculation protocol, the experiment constituted the absolute control. Twenty-one S-PCW preparations were obtained (replicates). AO-like activities were estimated by IPA and IPHP in S-PCWs after $t = 120$ min in the presence of tyramine (15 mM).

The second experiment consisted of estimating AO-like activity from S-PCWs for which the inoculation protocol was carried out, but without any bacteria in the TRIS buffer. Such a control allowed the testing of the effects of stress induced by both the TRIS buffer and the inoculation protocol. Activities were recorded on S-PCWs from calli sampled at $T = 0$, 12, 24, 36, 48, and 96 h after the pseudo-inoculation. At each time *T*, three S-PCW preparations were obtained constituting three replicates. AO-like activities were estimated by IPA and IPHP in S-PCWs after $t = 120$ min in the presence of tyramine (15 mM).

The aim of the next experiment was to observe an increased AO-like activity in S-PCWs from calli when they were inoculated with bacteria. This required four S-PCW preparations (replicates) of inoculated calli sampled at $T = 96$ h. For each preparation, two aliquots were obtained to estimate separately the IPA and the IPHP (total of eight aliquots). Kinetic observations were recorded every 20 min, from $t = 20$ to 120 min in the presence of tyramine (15 mM).

5.4.4. Substrate affinity: aliphatic polyamines and aromatic monoamines

Affinities of phenylethylamine, tyramine, dopamine, diaminopropane, putrescine, spermidine, and spermine were compared. For each substrate (40 mM), the experiment was carried out using two sampling times ($T = 0$ and $T = 96$ h). Three replicates were conducted for each of the 14 substrate-sampling time combinations.

5.4.5. Inhibition of AO-like activity

In this experiment, only the sample at $T = 96$ h was used and the initial tyramine concentration was 40 mM. The inhibitors tested (all from Sigma–Aldrich) were cysteine, sodium bisulphite, and tryptamine. The use of each inhibitor constituted an experiment.

For the tryptamine treatment, the buffer was supplemented with different concentrations of tryptamine (0–60 mM). The mixing was carried out under strict anaerobic conditions obtained using within-vial degassing by nitrogen. After sealing, these vials were incubated for 6 h at 28 °C in darkness. At the end of the incubation period, S-PCWs were recovered using centrifugation (20,000 *g*, 20 min, +4 °C).

For cysteine and natrium bisulphite, a preliminary experiment was carried out to check whether IPHP was reduced by the inhibitor. The buffer was supplemented with 20 mM of H_2O_2 and 20 mM of inhibitor. The results were compared with those obtained from the inhibitor-free control treatment.

In all the other experiments, inhibitor concentrations varied from 0 to 100 mM. Four replicates were conducted per concentration–inhibitor combination, i.e. $4 \times 3 \times 11 = 132$ aliquots. IPHP was recorded 1 h after the addition of the inhibitor.

5.5. Statistical analyses

Two main statistical methods were used for data analysis: an analysis of variance (ANOVA) and nonlinear least square regression.

5.5.1. Analysis of variance

All ANOVA models used were balanced with fixed effects. One-way ANOVAs and two-way ANOVAs were carried out.

5.5.2. Nonlinear least square regression

This was used for the fitting of kinetic observations to a sigmoid function $y = M/(1 + e^{a(t-b)})$. In each case, the validity of the model was confirmed by residual analysis relative to the observed values.

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