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In vitro and *in vivo* inhibition of plant polyamine oxidase activity by polyamine analogues ☆

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ABSTRACT

Polyamine oxidase from *Avena sativa* L. cv. Cristal seedlings was purified to homogeneity using a simple four-step purification protocol including an infiltration washing technique. The enzyme had a high affinity for spermidine and spermine ($K_m \sim 5.5$ and 1.2 μM, respectively), and also oxidized norspermidine ($K_m \sim 64.0$ μM). Natural and synthetic diamines, cyclohexylamine, the putrescine analogue 1-aminooxy-3-aminopropane, and several polyamine analogues had inhibitory effects on polyamine oxidase activity and none were substrates. No inhibitory effect was observed on spermidine oxidation when the reaction product 1,3-diaminopropane was added. By contrast, 1-aminooxy-3-aminopropane showed mixed inhibition kinetics and a K_i value of 0.113 mM. In addition, *in vitro* enzymatic activity assays showed that the oligoamine [3,8,13,18,23,28,33,38,43,48-deca-aza-(*trans*-25)-pentacontene], the tetramine 1,14-bis-[ethylamino]-5,10-diazatetradecane, and the pentamine 1,19-bis-[ethylamino]-5,10,15-triazanonadecane, displayed potent competitive inhibitory activities against polyamine oxidase with K_i values of 5.8, 110.0 and 7.6 nM, respectively, where cyclohexylamine was a weak competitive inhibitor with a K_i value of 0.5 mM. These analogues did not inhibit mycelial growth of the fungus *Sclerotinia sclerotiorum* (Lib.) De Bary and the bacterium *Pseudomonas viridiflava* (Burkholder) Dowson *in vitro*. On the contrary, with concentrations similar to those used for polyamine analogues, guazatine (a well-known fungicide and at the same time, a polyamine oxidase inhibitor) inhibited (~85%) *S. sclerotiorum* mycelial growth on Czapek-Dox medium.

Finally, the analogue 1,19-bis-ethylamino-5,10,15-triazanonadecane inhibited polyamine oxidase activity observed in segments of maize leaves *in vivo*. The results obtained provide insights into research on the influence of polyamine oxidase activity on plant biotic and abiotic stresses.

1. Introduction

Polyamines have frequently been associated with processes of cell growth, division and differentiation. Their levels are finely controlled through a network regulatory system that includes pathways for polyamine biosynthesis and degradation (Tiburcio et al., 1997). The enzymes involved with the catabolism of these substances are widely distributed in living organisms and have been extensively studied in plants (Smith, 1985; Cohen, 1998).

There are two main types of plant amine oxidases: the copper-containing amine oxidases (Cu-AOs), commonly known as diamine oxidases (DAOs; EC 1.4.3.6), and the flavin-containing amine oxidases (FAD-AOs), generically known as polyamine oxidases (PAOs; EC 1.5.3.11). Plant DAOs catalyze oxidative deamination of physiological diamines putrescine (Put) and cadaverine (Cad), producing the corresponding aminoaldehydes, ammonia, and hydrogen peroxide (H_2O_2), as well as oxidizing some synthetic diamines. In contrast, plant PAOs preferentially cleave the aminopropyl moiety of spermidine (Spd) and spermine (Spm) chains on secondary amino groups, generating 1,3-diaminopropane (DAP), H_2O_2, and 1-pyrroline or 1-(3-aminopropyl)pyrroline, respectively (Cohen, 1998; Šebela et al., 2001). PAO activity involved in the back conversion of polyamines has been recently reported in *Arabidopsis thaliana* (Tavladoraki et al., 2006). Historically, plant PAOs have been described as mostly associated with cell walls of monocotyledonous species (Kaur-Sawhney et al., 1981), especially in cereals belonging to the Gramineae family (Federico and Angelini, 1991; Cona et al., 2006).

This paper introduces a novel and simple method to purify polyamine oxidases in monocotyledonous species. In addition, using a partially purified preparation, PAO inhibition by the natural diamines (DAP, Put, Cad), the synthetic amines **9** and **10**, and the polyamine analogues **11**; **13**; 14 and the oligoamine **15** was assayed (Fig. 1). The results obtained support the hypothesis that

☆ In memoriam of Professors Rosalia and Benjamin Frydman.

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these synthetic drugs are strong inhibitors of spermidine and spermine oxidation by plant PAOs.

2. Results and discussion

2.1. Infiltration washing solution and PAO purification

PAO of 15-day-old oat seedlings was purified from intercellular fluids obtained from primary leaves by vacuum infiltration, using a high-salinity buffer solution to efficiently release the enzyme from the cell wall. This procedure was demonstrated to reduce PAO contamination with cytoplasmic proteins and also to prevent the dilution of PAO activity (Li, 1993). The precipitation caused by the addition of cold Me_2CO, ion exchange, and gel filtration chromatographic steps completed the purification protocol used, yielding a 22.7 fold purification and a specific activity of 2181 nkat/mg (Table 1). SDS-PAGE analysis indicated a single band with a *M*r of ca. 66 kDa both in the DEAE-Sephacel and the gel filtration fractions. These results are similar to those reported by Federico et al. (1989) and Li (1993). This protocol for purification of oat PAO was initially applied to low amounts of protein (Table 1), and further adjusted for the higher protein amounts used in the experiments described in this work. In addition to the high level of purification achieved, no DAO activity was detected in the DEAE Sephacel fraction. Therefore, this fraction was used for further kinetic studies.

2.2. Substrate specificity and kinetic characterization of purified oat PAO

The apparent K_m values, obtained from the fluorometric assay and calculated from Lineweaver-Burk plots, were 5.5 and 1.2 μM for Spd and Spm, respectively. These values were similar to those reported by Smith (1977) and Federico et al. (1989), obtained from colorimetric and oxygen electrode determinations.

When assaying norspermidine as substrate of oat PAO, a K_m of 64 μM was obtained, with activation energy equal to 96 kJ/mol. Interestingly enough, when spermidine was used, the activation energy was 64 kJ/mol. The enzymatic activity using spermidine as substrate was linear when the temperature ranged between 23 °C and 35 °C, showing the highest activity at 41 °C, whereas norspermidine displayed a similar value at 41 °C and 35 °C. The varia-

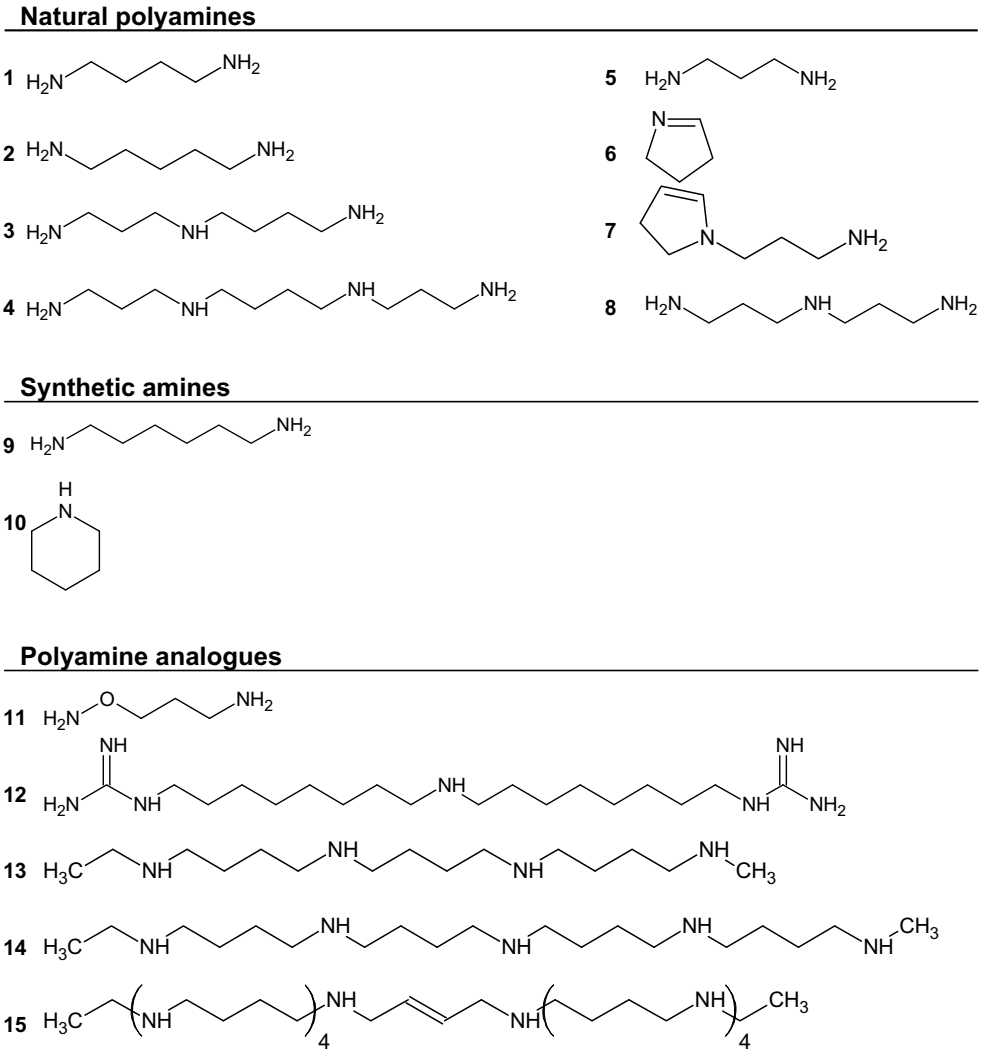

Fig. 1. Structural representation of natural and synthetic amines and polyamine analogues used in this study. (**1**) 1,4-diaminobutane (putrescine); (**2**) 1,5-diaminopentane (cadaverine); (**3**) 1,8-diamino-4-azaoctane (spermidine); (**4**) 1,12-diamino-4,9-diazadodecane (spermine); (**5**) 1,3-diaminopropane (DAP); (**6**) 1-pyrroline; (**7**) 1-(3-aminopropyl)-pyrroline; (**8**) 1,7-diamino-4-azaheptane (norspermidine); (**9**) 9; (**10**) cyclohexilamine (CHA); (**11**) 1-aminooxy-3-aminopropane (APA); (**12**) 2[8[8 (diaminomethylideneamino)octylamine]octyl]guanidine (guazatine); (**13**) tetramine 1,14-bis-[ethylamino]-5,10-diazatetradecane (SL-11156); (**14**) pentamine 1,19-bis-[ethylamino]-5,10,15-triazanonadecane (SL-11061); (**15**) oligoamine [3,8,13,18,23,28,33,38,43,48-deca-aza-(*trans*-25)-pentacontene] (SL-11144).

Table 1
PAO purification from oat seedlings

Purification step	Volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg)	Purification (fold)	Yield (%)
IWS[a]	18.00	0.5526	53.16	96.20	1.00	100.0
Me CO precipitation	3.00	0.2493	43.32	173.76	1.80	81.5
DEAE Sephacel	3.00	0.0658	32.95	500.22	5.20	62.0
Gel filtration	0.26	0.0069	15.21	2181.00	22.72	28.6

[a] IWS = infiltration washing solution.

tion of Spd/norspermidine activity decreased as temperature was shifted from 23 °C to 35 °C (Fig. 2). This result was consistent with the higher activation energy associated with the oxidative catalysis of norspermidine, the Q_{10} for Spd and norspermidine being 2.347 and 3.550, respectively. These observations reinforce the previous idea related to the high specificity for polyamine oxidation by PAO, since the backbones of these two polyamines differ only in one methylene group.

2.3. Diamines, polyamine analogues and derivatives as inhibitors of PAO activity

In agreement with Federico et al. (1989), N^1 acetylspermine (2 μM) was enough to inhibit PAO activity *in vitro*, when evaluated in pure preparations of the enzyme. PAO activity was recovered after dialysis of the purified enzyme fraction incubated with N^1 acetylspermine. In addition, PAO activity was found not to be inhibited by N^1acetylspermine when crude extracts were used as a source of enzyme (data not shown). As a whole, these results suggest that inhibition was actually caused by the aldehyde generated as a consequence of *N*-acetyl polyamine oxidation.

No activity was detected when Put, Cad, and **9** were used as substrates (data not shown), but all of them inhibited Spd oxidation (Table 2). PAO inhibition by Put has been previously reported by Radová et al. (2001).

The elucidation of the structure of the catalytic tunnel of maize PAO (Binda et al., 1999) provided the theoretical basis for a model that correlates the degree of PAO inhibition caused by the above mentioned diamines with the amount of carbon atoms in their molecules, by assuming that hydrophobic interactions between the diamine carbon backbone and the aromatic residues located in the enzyme inner funnel take place. Results obtained in the present regarding oat PAO inhibition by diamines with different carbon chain lengths are in good agreement with those obtained for maize PAO by Cona et al. (2004). Therefore, it is tempting to speculate that the catalytic tunnel of oat and maize PAOs share similar structural features.

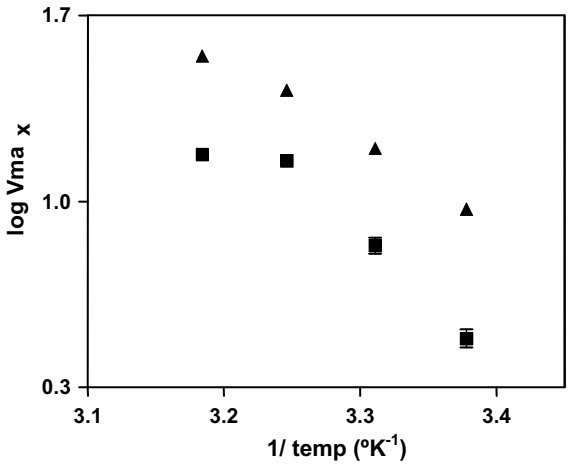

Fig. 2. Arrhenius plot for purified PAO activity. Enzymatic assays were set up in triplicate using the fluorometric assay. Spermidine (triangles) and norspermidine (squares) were used as substrates, both at 1 mM. Each experiment was conducted 3 times and results are reported as means ± s.d.

Table 2
Relative *in vitro* PAO activity as affected by diamines or APA

Treatment	Spermidine	Spermine
Diaminopropane	101.0 ± 8.0	98.0 ± 5.0
Putrescine	58.6 ± 4.0	88.3 ± 0.3
Cadaverine	39.5 ± 1.0	68.9 ± 2.9
1,6-Diaminohexane	0.0 ± 0.0	0.0 ± 0.0
APA	20.0 ± 3.0	32.0 ± 3.0

Enzymatic assays were carried out in triplicate using the fluorometric assay. Spermidine and spermine (1 mM) were used as substrates and diamine and APA concentrations were 1 mM. PAO activity is expressed as a percentage of the activity detected for a given substrate in the absence of inhibitors. Each experiment was conducted 3 times, and results are reported as means ± s.d.

Similarly to the previously mentioned diamines, the Put analogue **11** inhibited PAO activity, inhibition being higher than that caused by Put. It is important to consider that the oxygen confers structural rigidity to the **11** molecule, thus reducing the probability of being displaced by substrate molecules once in the catalytic site. This diamine analogue has been reported as a potent competitive inhibitor of mammalian (Khomutov et al., 1985) and bacterial ornithine decarboxylase (EC 4.1.1.17). This drug has also been described as a potent competitive inhibitor of mammalian spermidine synthase (EC 2.5.1.16) and liver adenosylmethionine decarboxylase (EC 4.1.1.50), but does not affect spermine synthase activity (EC 2.5.1.22) (Khomutov et al., 1985). Aminooxy compounds such as **11** are known to be carbonyl reagents harboring reactivity against enzymes that utilize pyridoxal phosphate as a cofactor (Hyvönen et al., 1988). However, no evaluation of the effect of **11** on flavin-containing enzymes was done prior to the present. In this work, this Put analogue was found to inhibit (ca. 80%) of *in vitro* oat PAO activity at 1 mM (Table 2). Interestingly, with the same concentration, DAP had no inhibitory effects on oat PAO. By contrast, the polyamine analogues **15**, 13 and **14** at 0.1 mM were enough to inhibit (ca. 100%) *in vitro* PAO activity. The K_i values obtained for polyamine analogues and the amine synthetic **10** are shown in Table 3. All the amines assayed were competitive inhibitors excepting for **11**, which was a mixed inhibitor with a dissociation constant (α) of 9.469.

The absence of primary amine groups in the structures of polyamine analogues **15**, 13 and **14** prevents them from being substrates of PAO, in accordance with the observations reported by Binda et al. (1999) after the evaluation of structurally related compounds. In addition, the results obtained in the present work suggest a direct relationship between the number of amine groups, molecular size, and inhibitory capacity, which is consistent with the hypothesis that the positively charged amine groups may compete for the negative charges of the surface of entrance of the catalytic tunnel (Binda et al., 1999). It is also possible that the major backbone of the analogues establishes a strong interaction with the hydrophobic amino acids that cover the first part of the cata-

Table 3
Ki values for oat PAO inhibition by APA, polyamine analogues and CHA

Inhibitor	K_i value (M)
APA	1.2×10^{-4}
SL-11144	5.8×10^{-9}
SL-11156	1.1×10^{-7}
SL-11061	7.6×10^{-9}
CHA	5.0×10^{-4}

Enzymatic assays were carried out in triplicate using Spd (1 mM) as a substrate. Results are expressed as means ± s.d.

lytic tunnel, which would affect the interaction of PAO with its natural substrates Spd and Spm. Similar considerations may explain the weak competitive inhibition ($K_i \sim 0.5$ mM) observed for **10**.

2.4. In vivo PAO activity determination and effect of polyamine analogue **14**

Assuming similar catalytic properties and molecular structures for maize and oat PAOs, it would be interesting to comparatively analyse the effect caused by polyamine analogues on both activities. Bearing this in mind, maize PAO was purified from whole leaves using a protocol identical to that described for oat. The inhibition order for the analogues (10 μM) was identical to the one previously observed for oat PAO, their values being ~88.5%, 29.79% and 88.53% for **15**, 13 and **14** respectively, as compared with the control.

The polyamine analogue **14** was selected for subsequent experiments due to its great inhibition capacity and relatively small size, which allows using it for the study of *in vivo* PAO activity in whole segments of maize leaves.

To that end, four 10-mm long successive segments cut from the blade base towards the apex of maize leaves were used to evaluate PAO activity, by determining the fluorescence generated in the presence and absence of Spd at 1 mM and homovanillic acid (HVA) (Fig. 3). PAO activity proved to be very low in the absence of Spd, but significantly increased when this polyamine was added

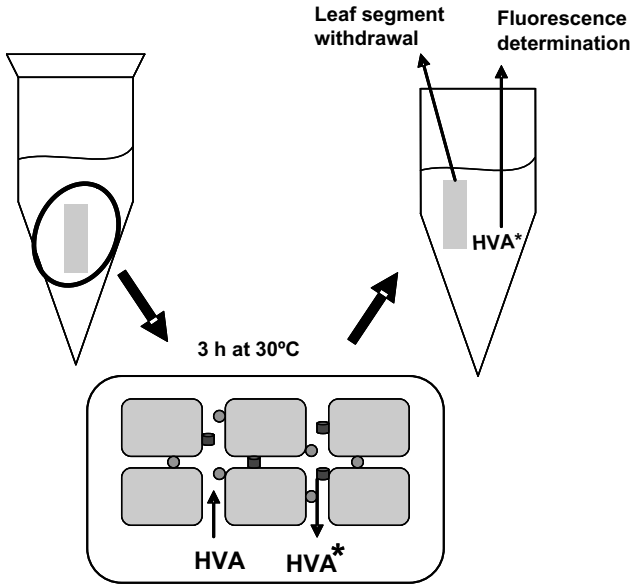

Fig. 3. Schematic representation of *in vivo* PAO activity determination. Segments of the third leaf of actively growing maize plants were used. In this measurement, exogenous peroxidase was not added. Black squares represent PAO enzyme, which is located in intercellular spaces. Gray circles represent endogenous peroxidase, HVA is homovainillic acid, HVA* represents the dimeric fluorescent form of HVA.

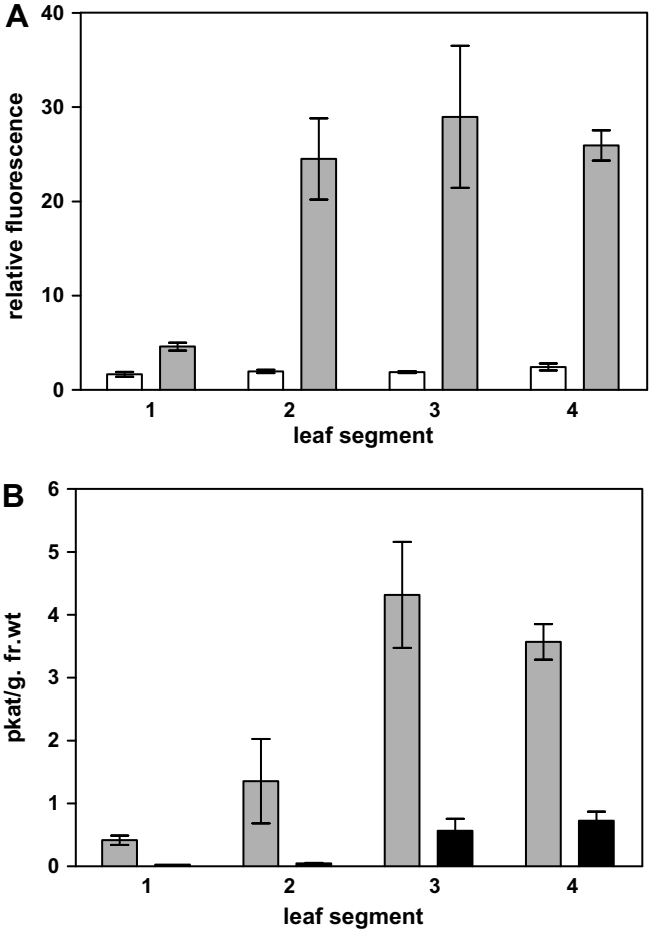

Fig. 4. *In vivo* PAO activity (A) and inhibition by the polyamine analogue SL-11061 (B). A – PAO activity was determined in 10 mm-long segments from maize leaves and is expressed as relative fluorescence. White bars represent basal activity and gray bars represent the activity after Spd addition (1 mM). Results are means ± s.d. ($n = 3$). B – PAO activity is expressed in pkat/grams fresh weight. 1,2,3,4 are 10-mm-long segments sequentially obtained from the blade base. Gray bars represent the activity of control segments with 1 mM Spd and black bars represent the activity of segments treated with 1 mM spermidine plus 10 μM SL-11061. Results are means ± s.d. ($n = 3$).

(Fig. 4A). These results demonstrate that apoplastic polyamine concentration is a limiting factor for PAO activity, being consistent with the observations made by Rea et al. (2004) with tobacco leaf discs.

In these experiments, no interference by DAO activity was evident, since it was very low and only detected when using radiolabelled putrescine (data not shown).

The inhibitory effect of analogue **14** on PAO *in vivo* was also evaluated and the results obtained are shown in Fig. 4B. Inhibition was observed in all segments, with fluorescence not being detected in the first ones due to their low basal PAO activity. As a whole, the results obtained strongly suggest that this method allows *in vivo* determination of PAO activity and can contribute to the study of polyamine oxidation and its capacity to generate H_2O_2.

2.5. Effects of polyamine analogues on growth of Sclerotinia sclerotiorum and Pseudomonas viridiflava in vitro

Gárriz et al. (2003) evaluated the effect of **11** on growth and differentiation of the phytopathogenic fungus *Sclerotinia sclerotiorum*. The specific inhibition of polyamine biosynthesis caused by this diamine analogue on polyamine biosynthetic enzymes and poly-

amine levels reduced fungal growth significantly. Similar findings were reported for other polyamine biosynthesis inhibitors (Gárriz et al., 2004). These observations are consistent with those reported by other groups, suggesting that polyamine analogues can act as powerful fungicides (Rajam and Galston, 1985; Walters and Mackintosh, 1997). However, 10 and 100 μM of the polyamine analogues **13**, 14 and **15** did not exert any negative effects on *S. sclerotiorum* mycelial growth, in a similar way to the tetramine Spm (Fig. 5A). Although *in vitro* inhibition of mycelial growth is not necessarily the best indicator to predict *in planta* fungicidal activity, the results of the present work strongly suggest that these analogues could not be used as fungicides. As opposed to the above mentioned polyamine analogues, the addition of **12**, a powerful inhibitor of PAO activity with a $K_i \sim 10^{-8}$M (Šebela et al., 2001) and a well-known fungicide, significantly reduced mycelial growth of *S. sclerotiorum* ($P < 0.05$). This effect was not reverted by the addition of a similar concentration of Spm ($P < 0.05$, Fig. 5B).

Compound 12 (Fig. 1) presents a mode of action based on inhibition of lipid biosynthesis and oxygen uptake (Yagura et al., 1984). However, there are no reports on the influence of this fungicide on fungal polyamine metabolism. The fact that Spm was found not to be able to revert the inhibition of mycelial growth caused by **12** (Fig. 5B) demonstrates that this effect of **12** is not due to PAO inhibition.

As occurred when evaluated on growth of *S. sclerotiorum*, **14** (50 μM) did not affect *in vitro* growth of *Pseudomonas viridiflava*, whereas identical concentrations of **12** showed an inhibitory effect of ca. 80% (data not shown). In this way, the polyamine analogue **14** is not active against the two organisms above mentioned and therefore not of potential use as inhibitor of plant pathogens. Thus, the lack of inhibitory effects of this polyamine analogue on growth of plant pathogens, as well as its high selectivity and specificity, renders it as an interesting tool for the study of the role of plant PAOs in defense responses. On the contrary, the mostly used **12**-based product (Pestanal®) is not reliable for certain research applications, since it contains, besides **12** (Fig. 1), a mixture mainly represented by fully guanidated triamine, diamine and monoguanidated diamine (Dreassi et al., 2007). Similar considerations are also valid for both the commercial and standard (analytical grade) guazatine compositions available. Taken together, these results reinforce the idea that **12** would be inadequate for experiments aimed to study the role of polyamine catabolism in plant interactions with fungi and bacteria.

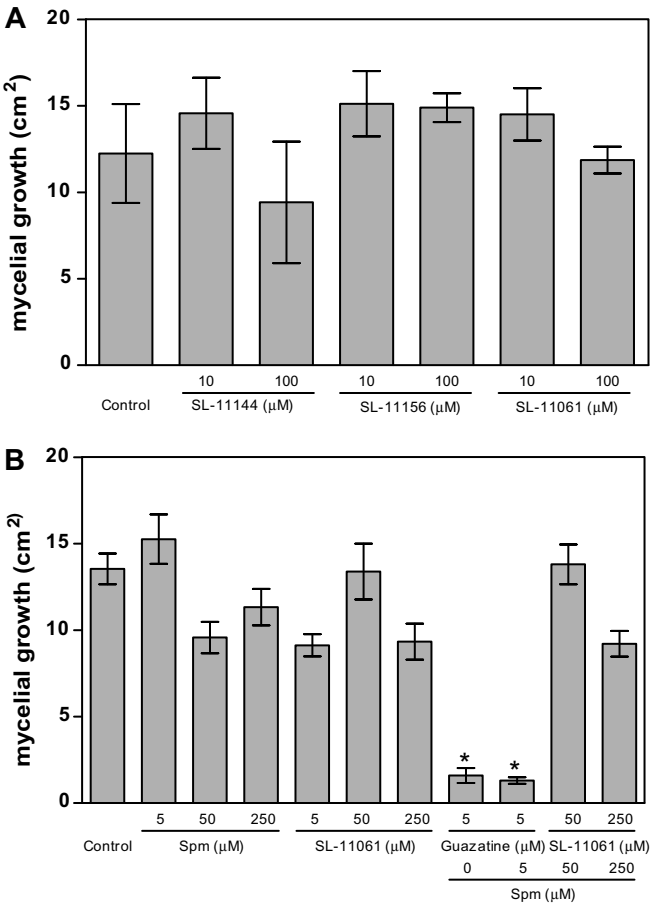

Fig. 5. Effect of polyamine analogues SL-11144, SL-11156, and SL-11061 (A) and effects of SL-11061, spermine and guazatine (B) on *Sclerotinia sclerotiorum* mycelial growth.-The concentrations and combinations of compounds used are indicated. Growth was expressed as the area of fungal colonies (cm^2). The values reported are means ± s.d. ($n = 4$) and were analyzed by one-way ANOVA and Tukey post test using GraphPad Prism software. Statistically significant differences between treatments and the control ($P < 0.05$) are indicated as *.

3. Concluding remarks

The PAO inhibitor **12** was used by Yoda et al. (2003) to study the role of the H_2O_2 derived from PAO activity in the hypersensitive cell death of tobacco plants reacting to tobacco mosaic virus infection. In the present work, it was demonstrated that **12** would be inappropriate for studies on either plant–bacteria or plant–fungi interactions, since it sharply reduced growth of *S. sclerotiorum* and *P. viridiflava*. By contrast, **14** did not affect growth of the above mentioned microorganisms.

Taking into account the high efficiency and specificity of the polyamine analogue **14** regarding its inhibitory effect on PAO activity *in vitro* and *in vivo*, as well as its innocuousness on *S. sclerotiorum* and *P. viridiflava* growth, it can be suggested that this compound could be an useful tool to evaluate *in vivo* the relevance of polyamine oxidation (and the H_2O_2 generated in this reaction) during plant responses to infection by phytopathogenic fungi and bacteria. Therefore, **14** could be used as an alternative to **12** for further research on PAO inhibition in bacterial or fungal-plant interactions. Moreover, the ability of this polyamine analogue to inhibit PAO activity *in vivo* suggests that it could be used to study the participation of PAO-derived H_2O_2 in developmental processes, as well as in plant responses to abiotic stresses. These findings are clearly significant and could have an impact on the study of plant PAOs and other flavin-containing amine oxidases.

4. Experimental

4.1. General experimental procedures

Putrescine, spermidine, spermine, N^1-acetylspermine, and other chemicals were obtained from Sigma Chemical Co. Guazatine (Pestanal®) was purchased from Riedel-de-Haën (Germany). The synthetic diamine **11** was kindly supplied by Dr. Alex Khomutov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow). The polyamine analogues **15**; 13 and **14** were synthesized by SLIL Biomedical Corporation (Madison, WI) as previously described (Reddy et al., 2001; Valasinas et al., 2001; Mitchell et al., 2002) and were kindly gifted by Dr Benjamin Frydman. Enzymatic assays were set up in triplicate and each experiment was conducted three times with similar results. Results are reported as means of triplicates corresponding to a representative experiment.

4.3. Biological material

Avena sativa L. cv. Cristal seeds were treated with 1% NaOCl solution and sown directly on pots filled with a mixture of sand and

perlite (1:1) with a light/dark cycle of 16 h/8 h at 23 °C. Seedlings were irrigated with 0.5× Hoagland's solution. Primary leaves from 14-day-old seedlings were harvested. *Zea mays* L. cv. DK 752 MG seeds were sown directly on pots filled with a mixture of perlite and vermiculite (1:1) and grown under conditions similar to those used for *A. sativa*. The third leaf was used for PAO purification, as well as for the determination of PAO activity *in vivo* (see below). An isolate of *Sclerotinia sclerotiorum* (Lib.) de Bary from the IIB-INTECh Fungal Culture Collection (IFCC 458/02) and *Pseudomonas viridiflava* (Burkholder) Dowson strain Pvalb8 (Alippi et al., 2003) were used for mycelial and bacterial growth inhibition assays.

4.4. Extraction and purification of Avena sativa polyamine oxidase

Extraction of apoplastic protein was performed as described by Li (1993) with slight modifications. The abaxial epidermal sheet of oat leaves was peeled and leaves were washed in distilled H_2O. The peeled leaves were then vacuum infiltrated with 200 mM NaCl in 5 mM K-Pi buffer at pH 6.5 for 15 min, followed by breaking and reestablishing the vacuum every 5 min; this process being conducted 3 times. The infiltration washing solution (IWS) was collected, cooled to 0 °C, and 1 volume of Me_2CO, pre-cooled to -20 °C, was added. The ppt. obtained by quick centrifugation was resuspended in 20 mM Bis-Tris-propane buffer pH 6.5 and applied to a DEAE-Sephacel column (1 × 2 cm) equilibrated in the same solution. Fractions containing the highest PAO activity after anion exchange chromatography were pooled, concentrated using Centricon® (14 kDa cut-off membrane) and injected into a FPLC-Superose 6® system. A flow rate of 0.5 mL/min of K-Pi buffer (50 mM; pH 6.5) containing 0.15 M NaCl was used and ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine albumin (66 kDa) ovoalbumin (54 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) were used as reference standards. Fractions (1 mL) showing PAO activity were pooled and concentrated as described above. A sample of this preparation was analysed by SDS-PAGE and stained with 0.1% Coomassie Brilliant Blue solution in 7% HOAc.

4.5. In vitro polyamine and diamine oxidase assay methods

The H_2O_2 produced by PAO activity was routinely determined by coupling the reaction with horseradish peroxidase and guaiacol (Smith, 1983). The absorbance increase was recorded at 470 nm. In inhibition assays, inhibitors were preincubated for 1 min. The calibration curve was obtained by using standard H_2O_2 solution as substrate. In addition, PAO activity was determined by a fluorometric assay using 0.4 mM HVA as substrate for the peroxidase-H_2O_2 coupled reaction. The reaction was interrupted by adding NaOH soln. (50 μL, 5 N) and the fluorescence recorded using 323 nm for excitation and 426 nm for emission in a Bio-Tek Kontron SFM 25 spectrofluorometer. The calibration curve was performed using known H_2O_2 levels as a substrate.

DAO activity was determined as described by Maiale et al. (2004). Protein concentration was determined as described by Bradford (1976), using bovine serum albumin as standard.

4.6. In vivo polyamine oxidase assay methods

Ten-mm-length segments cut from the blade of the third leaf of *Z. mays* plants were used, as described by Rodríguez et al. (2002). HVA was used, but in this case for whole segments. The samples were introduced in a 1.5 mL microtube (with or without 1 mM of Spd. and **14**), without exogenous peroxidase. Subsequently, the segments were infiltrated for 1.5 min and incubated during 3 h at 30 °C, following which the segments were removed and the H_2O_2 levels in the infiltration solution was determined using the protocol previously described. An alternative protocol for the determination of PAO *in vivo*, based on the use of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenosulfonic acid yielded similar results, but the sensitivity was lower and therefore was not used (data not shown).

4.7. Effect of polyamine and polyamine oxidase inhibitors on Sclerotinia sclerotiorum and Pseudomonas viridiflava growth

The effects of Spm, 12 and polyamine analogues on *in vitro* growth of *S. sclerotiorum* and *P. viridiflava* were recorded. To evaluate mycelial growth, the protocol described by Pieckenstain et al. (2001) was used. The area of fungal colonies grown in solid Czapek-Dox medium was evaluated using the *Image program Pro Extra 5.1* (Average Inc. Cybernetics). Growth of *P. viridiflava* in glass tubes containing liquid King B medium was evaluated by the determination of the absorbance at 600 nm.

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