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FEMS Microbiology Letters 230 (2004) 115–121

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Ornithine and arginine decarboxylase activities and effect of some polyamine biosynthesis inhibitors on *Gigaspora rosea* germinating spores

Analía I. Sannazzaro [a], Cora L. Álvarez [a], Ana B. Menéndez [a,b], Fernando L. Pieckenstain [a], Edgardo O. Albertó [a], Oscar A. Ruiz [a,*]

[a] *Instituto de Investigaciones Biotecnológicas, Instituto Tecnológico de Chascomús (IIB-INTECh), Universidad Nacional de General San Martín, Consejo Nacional de Investigaciones Científicas y Técnicas (UNSAM-CONICET), Casilla de Correo 164, 7130 Chascomús, Buenos Aires, Argentina*

[b] *Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

Received 28 July 2003; received in revised form 14 November 2003; accepted 17 November 2003

First published online 4 December 2003

Abstract

The pathways for putrescine biosynthesis and the effects of polyamine biosynthesis inhibitors on the germination and hyphal development of *Gigaspora rosea* spores were investigated. Incubation of spores with different radioactive substrates demonstrated that both arginine and ornithine decarboxylase pathways participate in putrescine biosynthesis in *G. rosea*. Spermidine and spermine were the most abundant polyamines in this fungus. The putrescine biosynthesis inhibitors α-difluoromethylarginine and α-difluoromethylornithine, as well as the spermidine synthase inhibitor cyclohexylamine, slightly decreased polyamine levels. However, only the latter interfered with spore germination. The consequences of the use of putrescine biosynthesis inhibitors for the control of plant pathogenic fungi on the viability of *G. rosea* spores in soil are discussed.

Keywords: Arbuscular mycorrhizal fungus; Arginine decarboxylase; Cyclohexylamine; α-Difluoromethylarginine; α-Difluoromethylornithine; Ornithine decarboxylase

1. Introduction

Polyamines are small aliphatic polycationic compounds ubiquitous in all living organisms [1]. They bind to polyanionic macromolecules such as DNA, RNA and phospholipids [2] and thus are essential for growth and differentiation, though their precise role in these processes is mostly unknown. The diamine putrescine can be synthesized directly from ornithine by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) or indirectly, via a series of intermediates following decarboxylation of arginine by arginine decarboxylase (ADC, EC 4.1.1.19) (Fig. 1). In turn, spermidine and spermine are synthesized from putrescine by successive additions of aminopropyl groups provided by decarboxylated *S*-adenosylmethionine, a metabolite synthesized by the enzyme *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) (Fig. 1). The aminopropyl additions to putrescine are catalyzed by the aminopropyltransferases spermidine (EC 2.5.1.16) and spermine synthases (EC 2.5.1.22) (Fig. 1).

Both ADC and ODC pathways are present in higher plants and bacteria, but in mammalian cells and many fungi, ODC is the sole pathway for putrescine production. Therefore, it has been proposed that specific inhibitors of ODC activity could be used for the control of plant pathogenic fungi without affecting their hosts, in which putrescine would be synthesized by the ADC pathway [3–7]. Several studies have shown that in vitro exposure to α-difluoromethylornithine (DFMO), a suicide inhibitor of ODC activity (Fig. 1), reduces growth and differentiation of a number of fungi [8–10]. α-Difluoromethylarginine

* Corresponding author. Tel.: +54 (2241) 424049; Fax: +54 (2241) 424048.
E-mail address: ruiz@intech.gov.ar (O.A. Ruiz).

Abbreviations: ADC, arginine decarboxylase; AM, arbuscular mycorrhizal; CHA, cyclohexylamine; DFMA, α-difluoromethylarginine; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase

0378-1097/03/$22.00
doi:10.1016/S0378-1097(03)00880-2

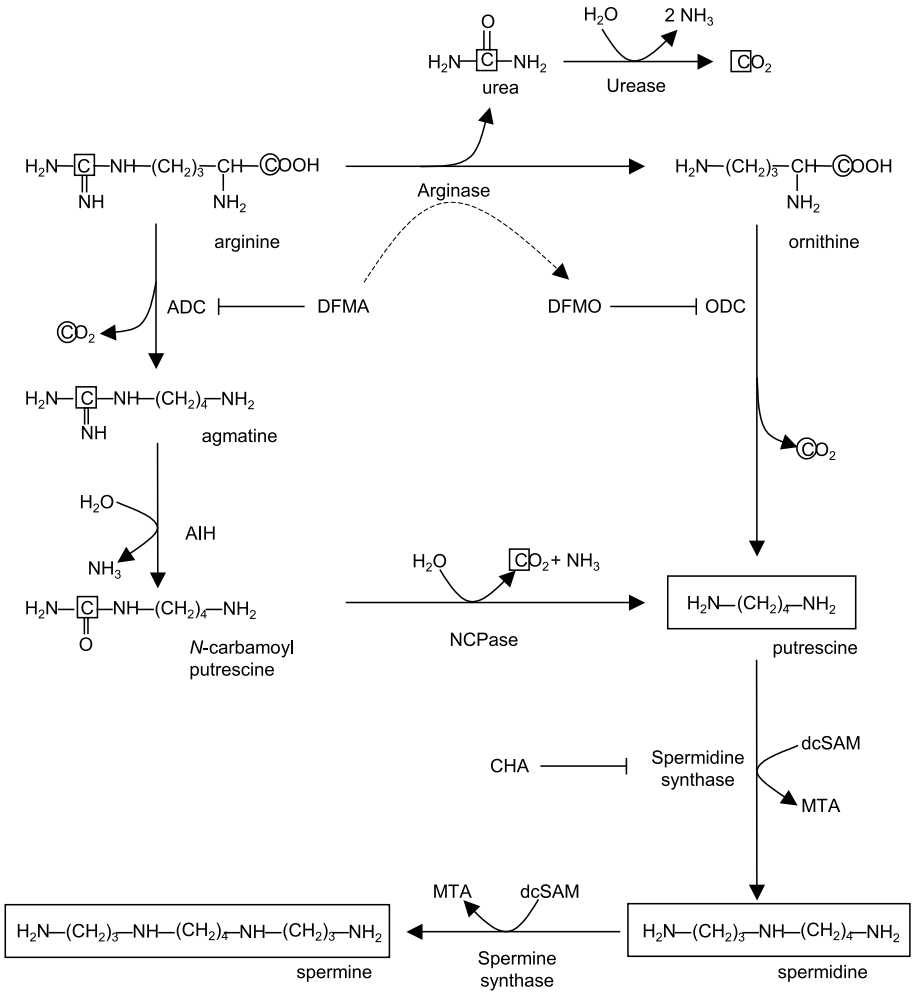

Fig. 1. Pathways of biosynthesis of the major plant polyamines and target enzymes for the action of polyamine biosynthesis inhibitors. Different carbon atoms that are released as CO_2 are enclosed in circles and squares. Putative arginase-mediated conversion of DFMA into DFMO is shown with a dashed line. AIH, agmatine iminohydrolase; dcSAM, decarboxylated *S*-adenosylmethionine; MTA, 5-methylthioadenosine; NCPase, *N*-carbamoyl-putrescine amidohydrolase.

(DFMA), a suicide inhibitor of ADC (Fig. 1), also inhibits growth of some fungi. However, it has been proposed that this effect is the result of the conversion of DFMA into DFMO via arginase (EC 3.5.3.1, Fig. 1), an enzyme known to occur in fungi and higher plants [11–13]. Nevertheless there is some evidence for the existence of ADC activity in some fungi [14,15] and therefore, inhibition of this enzyme by DFMA could reduce fungal growth in such cases. Cyclohexylamine (CHA) is a powerful inhibitor of spermidine synthases (Fig. 1) from mammalian cells, trypanosomes, plants, certain bacteria [16] and fungi [17], and its inhibitory effect on mycelial growth of plant pathogenic fungi has also been reported [10,17,18].

In the case that polyamine inhibitors were applied to plants for the control of pathogenic fungi, their effects on mycorrhizal fungi should be considered. Most plant taxa establish symbiotic associations with these fungi and it has been extensively demonstrated that any reduction in the mycorrhizal status of the plant affects its growth. In vitro experiments carried out with diverse ectomycorrhizal species have shown different responses to polyamine biosynthesis inhibitors [19,20]. Regarding arbuscular mycorrhizal (AM) fungi, El Ghachtouli et al. [21] reported the presence of putrescine and spermidine in *Glomus mosseae* and studied the effect of DFMA and DFMO on spore germination and hyphal growth. However, the biochemical pathways leading to putrescine synthesis in AM fungi, as well as the effect of polyamine biosynthesis inhibitors on growth and development of AM species other than *G. mosseae*, have not hitherto been studied. Thus, the aim of the present study was to contribute to a better understanding of polyamine metabolism in AM fungi and to determine whether polyamine biosynthesis inhibitors may cause negative effects on spore germination and hyphal growth of *Gigaspora rosea*, an AM species not studied so far.

2. Materials and methods

2.1. AM fungal strain

The isolate BEG 9 of *G. rosea* Nicholson and Schenck was used for all the experiments. Spores were obtained from cultures of *Sorghum halepensis* and *Trifolium repens* by wet-sieving of soil and then purified by centrifugation in a sucrose gradient [22]. Spores were rinsed in sterile, distilled water containing 0.05% of Tween 80 and immediately surface disinfected for 8 min in a solution of 2% chloramine T, 0.02% streptomycin and 0.01% gentamicin [23] and then thoroughly rinsed in sterile, distilled water in a Petri dish and used within 24 h. After 1 week of incubation, spores that showed any sign of external contamination were discarded in all experiments.

2.2. Chemicals

DFMA and DFMO were kindly supplied by Merrell Dow Pharmaceuticals. CHA and all the other chemicals used in this study were obtained from Sigma. Inhibitors were dissolved in distilled water, filter-sterilized and stored at −20°C. L-[U-^{14}C]Ornithine (50 nCi ml^{-1}, specific activity: 52 mCi mmol^{-1}) was purchased from NEN Life Sciences. L-[1-^{14}C]Arginine (100 nCi ml^{-1}, specific activity: 55 mCi mmol^{-1}) and L-[guanido-^{14}C]arginine (100 nCi ml^{-1}, specific activity: 50 mCi mmol^{-1}) were purchased from American Radiolabeled Chemicals. [^{14}C]Urea (500 nCi ml^{-1}, specific activity: 52 mCi mmol^{-1}) was obtained from Amersham Pharmacia Biotech.

2.3. In vivo decarboxylation of radioactive substrates

Surface-disinfected spores were disposed into sterile 96-well microtiter plates (6 mm diameter each well) containing 100 μl of semisolid medium consisting of 0.4% gellam gum (Gel-Gro™, ICN) in 10 mM 2-[*N*-morpholino]-ethanesulfonic acid buffer, pH 7 (eight spores per well). After 1 week of incubation at 25°C, germinated spores (700–800) were transferred with a micropipette to sterile 15-ml glass tubes containing 200 μl of the semisolid medium described above. Tubes were supplemented with 1 μl of L-[U-^{14}C]ornithine, L-[1-^{14}C]arginine, L-[guanido-^{14}C]arginine or [^{14}C]urea. When necessary, non-radioactive ornithine or urea was added to the tubes at 10 mM final concentration. A Whatman 3 MM paper disc soaked in 2 N KOH was fitted on the top of each glass tube and sealed with Parafilm®. After 18 h of incubation, discs were removed and $^{14}CO_2$ determined using 200 μl of scintillation fluid in a Beckman LS 5000 TD scintillation counter. Reaction tubes containing 200 μl of semisolid medium (without spores) were used as controls. Three replicate tubes were used per treatment and the experiment was performed twice.

2.4. Effect of inhibitors on spore germination, mycelial growth and free polyamine levels

Surface-sterilized spores were transferred to 96-well microtiter plates (three spores per well) containing 100 μl of the semisolid medium described in Section 2.3 supplemented with 0, 0.05, 0.5 or 5 mM of polyamine biosynthesis inhibitors and incubated in the dark at 25°C for 1 week. Afterwards, germination percentage, hyphal length and the number of ramifications were determined on 144 spores. Hyphal length was measured with a camera connected to a binocular microscope and the images were analyzed with Image Pro-plus software [24]. Spores were then withdrawn from the microtiter plates and free polyamine levels were determined as described below on duplicate pools of 72 spores each.

2.5. Polyamine content of spores

Spores were ground with a pistil in a microfuge tube (1.5 ml) containing 200 μl of 5% perchloric acid and incubated overnight at 4°C. Following centrifugation at 10 000 × *g* for 15 min, 5 μl of 100 μM 1,7-heptane diamine were added to the supernatant as internal standard. Then, 200 μl of saturated Na_2CO_3 and 400 μl of dansyl chloride (20 mg ml^{-1} acetone) were added and the mixture was incubated in the dark at room temperature for 16 h. The reaction was stopped by adding 100 μl proline (100 mg ml^{-1}). After 30 min of incubation in the dark at room temperature, dansylated polyamines were extracted in 500 μl of toluene. The organic phase was separated and vacuum-evaporated. Polyamine standards were treated in the same way. Dansylated polyamines were dissolved in 100 μl acetonitrile and analyzed by high performance liquid chromatography [25].

2.6. Statistical analysis

Data were subjected to analysis of variance and post-hoc comparisons with Duncan's test. Experiments were conducted two to three times with similar results and those of representative experiments are shown.

3. Results

3.1. In vivo enzyme assays

Incubation of *G. rosea* spores in the presence of either L-[U-^{14}C]ornithine or L-[1-^{14}C]arginine resulted in the release of $^{14}CO_2$ (Table 1). The addition of 10 mM non-radioactive ornithine to the incubation medium did not reduce the release of $^{14}CO_2$ from L-[1-^{14}C]arginine (Table 1). Radioactive CO_2 was released by *G. rosea* spores when they were incubated with L-[-guanido-^{14}C]arginine and this reaction was not affected by the addition of 10 mM non-

Table 1
Production of $^{14}CO_2$ from radioactive substrates by *G. rosea* spores

Substrate	Amendment	$^{14}CO_2$ (nmol)
L-[U-^{14}C]Ornithine		40.7 ± 8.3
L-[1-^{14}C]Arginine		64.4 ± 10.7
L-[1-^{14}C]Arginine	10 mM ornithine	79.4 ± 13.2
L-[guanido-^{14}C]Arginine		12.2 ± 4.4
L-[guanido-^{14}C]Arginine	10 mM urea	13.6 ± 2.0
[^{14}C]Urea		911.1 ± 168.7

Spores (700–800) germinated under aseptic conditions were incubated for 16 h with radioactive substrates at 25°C and the release of $^{14}CO_2$ was determined as described in Section 2. Results are the mean of three replicates ± S.E.M.

radioactive urea (Table 1). High levels of $^{14}CO_2$ were produced after the incubation of spores with [^{14}C]urea (Table 1).

3.2. *Effect of polyamine biosynthesis inhibitors on spore germination and hyphal development of* G. rosea

After 1 week of incubation, almost 50% of spores had germinated when no inhibitors were added to the growth medium (Table 2). None of the concentrations of DFMA and DFMO tested, either alone or in combination, significantly affected spore germination (Table 2). On the other hand, spore germination was significantly reduced by all the concentrations of CHA used in this assay (Table 2). The addition of 0.05 mM DFMA to the growth medium increased germ tube length three-fold, but higher concentrations (0.5 and 5 mM) of this inhibitor had no effect on germ tube elongation (Table 2). None of the concentrations of DFMO tested significantly affected germ tube elongation (Table 2). However, hyphal length was two-fold increased when 0.5 mM DFMO was used in combination with the same concentration of DFMA (Table 2). None of the compounds evaluated significantly inhibited hyphal branching. Moreover, spores incubated with 5 mM of either DFMA or DFMO actually developed a higher number of branches than controls (Table 2).

3.3. *Effect of polyamine biosynthesis inhibitors on free polyamine content of* G. rosea *spores*

After 7 days of incubation in the absence of inhibitors, spermidine and spermine were detected in germinating control spores in concentrations of 0.039 and 0.012 pmol per spore, respectively (Fig. 2). Putrescine was not detected under the experimental conditions used. All the inhibitors evaluated diminished spermidine contents, but only the effects of 0.05 and 0.5 mM DFMA and 5 mM CHA were statistically significant (Fig. 2). Similarly, a slight decrease in spermine content was observed with all the inhibitors, although significant differences were evident only in the spores exposed to 0.05 and 0.5 mM DFMA (Fig. 2).

4. Discussion

The pathways involved in putrescine biosynthesis and the effect of polyamine biosynthesis inhibitors on the germination of *G. rosea* spores were investigated. As in many of the fungi studied so far, a pathway for putrescine biosynthesis through ornithine decarboxylase activity was found to operate in *G. rosea* spores, as demonstrated by the decarboxylation of L-[U-^{14}C]ornithine. To date, information regarding the pathways for polyamine biosynthesis in AM fungi was lacking. Thus, as an initial approach to gain insight into this subject, we intended to determine whether ODC is the sole pathway for putrescine biosynthesis in *G. rosea*. In this regard, spores were found to release $^{14}CO_2$ from L-[1-^{14}C]arginine. This result could have been due to the decarboxylation of L-[1-^{14}C]arginine by ADC or, alternatively, to the arginase-mediated conversion of L-[1-^{14}C]arginine to L-[1-^{14}C]ornithine and the

Table 2
Effect of polyamine biosynthesis inhibitors on germination and hyphal development of *G. rosea* spores

Inhibitor	Spore germination (%)	Hyphal length (cm)	Number of branches per germinated spore
None	47.9 ± 4.4	0.3 ± 0.1	0.3 ± 0.2
DFMA 0.05 mM	42.7 ± 1.0	1.0 ± 0.1*	2.3 ± 1.0
DFMA 0.5 mM	42.7 ± 22.5	0.5 ± 0.1	2.0 ± 0.0
DFMA 5 mM	35.6 ± 16.7	0.3 ± 0.1	5.2 ± 2.3*
DFMO 0.05 mM	38.3 ± 4.0	0.2 ± 0.1	2.0 ± 1.1
DFMO 0.5 mM	46.7 ± 5.0	0.2 ± 0.1	2.5 ± 1.2
DFMO 5 mM	51.7 ± 2.5	0.3 ± 0.2	2.0 ± 0.8*
DFMA+DFMO, 0.5 mM each	44.0 ± 3.9	0.6 ± 0.1*	0.8 ± 0.2
CHA 0.05 mM	9.4 ± 5.8*	N.D.	N.D.
CHA 0.5 mM	3.3 ± 0.8*	N.D.	N.D.
CHA 5 mM	10.0 ± 15*	N.D.	N.D.

Spores were incubated for 7 days in 96-well microtiter plates (three spores per well) containing growth medium amended with DFMA, DFMO or CHA. Afterwards, the percentage of germinated spores, hyphal length and the number of branches per germinated spore were determined under a binocular microscope. Results are the mean of 72 spores ± S.E.M. Statistical differences between control (no inhibitor) and treatments are shown as *$P \leq 0.05$. N.D., not determined.

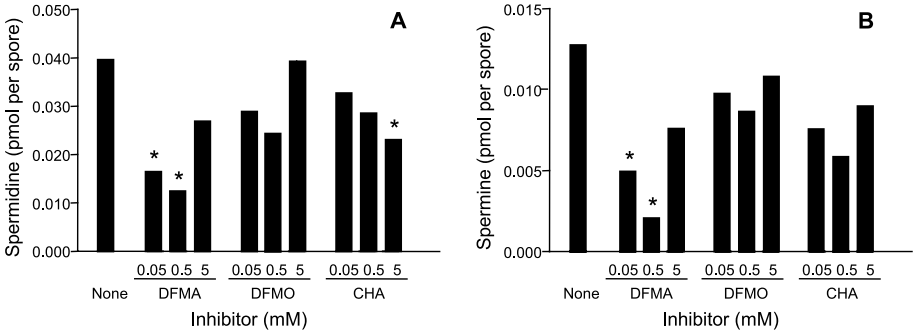

Fig. 2. Effect of polyamine biosynthesis inhibitors on spermidine (A) and spermine (B) concentrations in *G. rosea* spores. After incubation for 7 days in the presence of DFMA, DFMO or CHA, germinated spores were subjected to polyamine extraction and analysis as described in Section 2. Results are the means of two replicates and statistical differences between controls (no inhibitor) and treatments are shown as $*P \leq 0.05$.

subsequent release of $^{14}CO_2$ by ODC activity (Fig. 1). If the last hypothesis was correct, the addition of non-radioactive ornithine to the incubation medium should have diluted L-[1-^{14}C]ornithine formed by arginase activity, thus decreasing the release of $^{14}CO_2$ from L-[1-^{14}C]arginine (Fig. 1). However, this was not the case, in that non-radioactive ornithine had no effect on the levels of $^{14}CO_2$ produced from L-[1-^{14}C]arginine. In this way, our results suggest that ADC is active in *G. rosea* spores.

The incubation of spores with L-[guanido-^{14}C]arginine also resulted in the release of $^{14}CO_2$. This result could have been the consequence of two different processes. If a pathway for putrescine biosynthesis similar to that operating in plants exists in this fungus, L-[guanido-^{14}C]arginine would have been converted to [^{14}C]*N*-carbamoyl-putrescine by ADC and agmatine iminohydrolase (EC 3.5.3.12) (Fig. 1). Subsequently, [^{14}C]*N*-carbamoyl-putrescine would have been converted to non-radioactive putrescine and $^{14}CO_2$ by *N*-carbamoyl-putrescine amidohydrolase (EC 3.5.1.53). Alternatively, arginase would have converted L-[guanido-^{14}C]arginine to non-radioactive ornithine and [^{14}C]urea. Then, the latter would have been converted to $^{14}CO_2$ by urease (EC 3.5.1.5), an enzyme that is highly active in *G. rosea* spores, as demonstrated by the significant release of $^{14}CO_2$ from [^{14}C]urea. However, the addition of non-radioactive urea had no effect on the release of $^{14}CO_2$ from L-[guanido-^{14}C]arginine, thus suggesting that arginine is not efficiently converted to ornithine under the experimental conditions used. As a whole, the results obtained demonstrate the presence of ADC in *G. rosea* spores and suggest that enzymes similar to those operating downstream of ADC in plants could also be present in this fungus. In general, ODC is the sole pathway for putrescine biosynthesis in fungi [1,9]. However, there is some evidence for the existence of ADC in a few species, such as the plant pathogens *Colletotrichum truncatum* [4] and *Ophiostoma ulmi* [15] and the ectomycorrhizal fungus *Laccaria proxima* [20]. To our knowledge, this is the first report on the existence of ADC activity in AM fungi. Further work will be necessary to elucidate the complete set of reactions leading to putrescine biosynthesis from arginine in this organism. In this regard, it would be interesting to complement the information here obtained with the determination of enzyme activities in fungal extracts. However, the obligate symbiotic nature of AM fungi makes it difficult to conduct such an approach. Regarding the in vivo approach used in the present study, it should be taken into account that bacterial contamination of spores could be a source of interference, given that bacteria do have ADC activity. However, *G. rosea* spores were thoroughly disinfected and no signs of bacterial growth in the incubation medium were evident during the assay period. Additional interference could result from the presence of intracytoplasmic bacterium-like organisms [26], which have been shown to be present in some AM species. This possibility should also be discarded as an explanation for the presence of ADC in *G. rosea*, in view of the fact that confocal microscopy and molecular studies have shown bacterium-like organisms to be absent in this particular species [26,27].

As stated above, many fungi depend on ODC activity for putrescine biosynthesis, whereas in plants, two alternative pathways for the synthesis of this diamine are active, namely ODC and ADC. As a consequence, ODC inhibitors have been proposed as fungicides for the control of plant diseases. However, this approach could negatively affect the growth of beneficial fungi such as AM species. In addition, information about the effect of polyamine biosynthesis inhibitors on growth and development of AM fungi was scant. Therefore, an additional aim of the present study was to evaluate the effect of polyamine biosynthesis inhibitors on the germination of *G. rosea* spores. None of the two inhibitors of putrescine biosynthesis diminished spore germination, hyphal growth or branching. Curiously, both hyphal growth and branching were stimulated by certain concentrations of DFMA and DFMO. The mechanism underlying this stimulatory effect remains unclear, but it should be noticed that DFMA exerted a similar effect on other mycorrhizal fungi such as *G. mosseae* [21] and *L. proxima* [20] and on the oomycetous *Phytophthora infestans* [13]. It should be kept in mind that, although not always reaching statistical significance, spermidine and spermine levels were slightly decreased by DFMA or DFMO. This result demonstrated that these

compounds actually were incorporated by germinating spores and there reached target enzymes. Therefore, germination of *G. rosea* spores does not depend on putrescine biosynthesis, probably because this diamine is not important for the germination process. Failure to detect putrescine in *G. rosea* spores provides further support for this hypothesis. A different picture was described by El Ghachtouli et al. [21], who found putrescine to be the most abundant polyamine in *G. mosseae* spores. However, differences in free polyamine contents between fungal species have been reported [28]. In the above mentioned work by El Ghachtouli et al. [21], *G. mosseae* spores were exposed to low temperatures to induce germination. Similar cold treatments are known to increase polyamine levels in plants [29–31]. In the present work, *G. rosea* spores were harvested and immediately used without this kind of stratification. Hence, it should not be discarded that the differences in polyamine levels between the two species are due to the stratification treatment.

Taking into account that inhibitors of putrescine biosynthesis had no effect on spore germination, we decided to evaluate the sensitivity of this process to CHA, an inhibitor of spermidine biosynthesis. The highest concentration of CHA tested (5 mM) caused a decrease of spermidine and spermine levels similar to that caused by 0.05 or 0.5 mM DFMA. However, as opposed to DFMA, a strong inhibition of spore germination was observed in the presence of all the concentrations of CHA evaluated. In other fungi, inhibition of spermidine synthase by CHA [18] and synthetic putrescine analogues [32,33] leads to the accumulation of putrescine. Given that putrescine accumulation is known to have toxic effects in plants [34], the possibility that a CHA-induced increase in putrescine levels had inhibited the germination of *G. rosea* spores should not be discarded. However, this hypothesis cannot be confirmed because putrescine co-elutes with CHA under the chromatographic conditions used for polyamine analysis, thus preventing the determination of putrescine levels in CHA-treated spores. Further work will be necessary to validate this hypothesis. In addition, the possibility that CHA exerted non-specific effects on metabolic pathways other than polyamine biosynthesis should also be considered, taking into account the lack of information regarding the specificity of this compound [9].

In conclusion, putrescine biosynthesis seems to occur by two alternative pathways involving ODC and ADC activities in *G. rosea* and spore germination is not prevented by ODC inhibitors in this AM species. Therefore, the use of this kind of compound for the control of fungal plant diseases will probably have no deleterious effects on the viability of *G. rosea* spores.

Acknowledgements

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), the Agencia Nacional de Promoción Científica y Tecnológica (Pict 01-04510 ANPCYT, Argentina), the Third World Academy of Sciences (TWAS) and the Agencia Española de Cooperación Internacional (AECI). A.I.S., C.L.A. and F.L.P. are fellows of the CONICET, UNSAM and COREPRO, respectively. A.B.M. is a researcher of the Universidad de Buenos Aires (UBA). O.A.R. and E.O.A. are members of the research committee of CONICET.

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