First report of the inhibition of arbuscular mycorrhizal infection of *Pisum sativum* by specific and irreversible inhibition of polyamine biosynthesis or by gibberellic acid treatment

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Received 20 November 1995; revised version received 13 March 1996

Abstract DFMO (α-DL-difluoromethylornithine), a specific irreversible inhibitor of ornithine decarboxylase (ODC), a polyamine biosynthetic pathway enzyme, strongly inhibits root growth and arbuscular mycorrhizal infection of *Pisum sativum* (P56 myc$^+$, isogenic mutant of cv. Frisson). This inhibition is reversed when exogenous polyamine (putrescine) is included in the DFMO treatment, showing that the effect of DFMO on arbuscular mycorrhizal infection is indeed due to putrescine limitation and suggesting that ODC may have a role in root growth and mycorrhizal infection. However, treatment with gibberellic acid (GA3) which increased root titers of polyamines strongly inhibited arbuscular mycorrhizal development. The possible role of polyamines in the regulation of the development of arbuscular mycorrhizal infection is discussed.

Key words: Arbuscular mycorrhizal infection; Putrescine; Polyamine biosynthesis inhibitor; Gibberellic acid; *Pisum sativum*; *Glomus mosseae*

1. Introduction

Polyamines are simple aliphatic compounds present in all living cells and required for optimal growth and development in many biological systems [1-4]. The diamine putrescine is synthesized from arginine and ornithine via the rate-limiting enzymes arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), respectively. Higher plants and bacteria use both the ADC and ODC pathways for synthesising polyamines, while fungi possess only ODC for polyamine biosynthesis [3,4]. It should be possible, therefore, to control fungal polyamine biosynthesis by specifically inhibiting ODC without affecting plant growth and development [5-8].

Recently, we showed that ODC also regulates putrescine biosynthesis during hyphal growth in the arbuscular mycorrhizal fungus *Glomus mosseae* [9]. Low endogenous concentrations of the polyamines putrescine and spermidine can be growth limiting, and appear essential for hyphal growth. Furthermore, a marked inhibition of germination and hyphal growth was observed after application of exogenous polyamines at a concentration above 5 mM.

We previously reported that polyamine treatment at concentrations below 1 mM significantly increased the frequency of arbuscular mycorrhizal infection in myc$^+$ pea genotypes (cv. Frisson and P56, an isogenic mutant of cv. Frisson) [10]. Are polyamines essential for arbuscular mycorrhizal infection, or are they simply secondary by-products of the process? One way to approach this question is by studying the effect of specific irreversible inhibitors of putrescine synthesis and exogenous polyamines on arbuscular mycorrhizal development.

Plant hormones clearly play important roles in the control of plant growth and development [11]. Changes in polyamine levels and their biosynthetic enzymes accompany many hormonal responses, and in a few cases polyamines appear to mimic the effects of plant hormones [12]. This has led some researchers to suggest that polyamines mediate the action of plant hormones or are part of their signal response pathways [13]. In animal systems polyamine biosynthesis increases in response to hormones, and the polyamines produced are necessary for hormone action [14,15]. Since the early work of Slankis [16], phytohormones produced by the plant partner have been suspected to be involved in some of the morphological and/or anatomical modifications characteristic of the ectomycorrhizal association. Furthermore, in a variety of systems (especially in *Pisum sativum*) gibberellin applications are followed by an increase in polyamine levels and the activities of their biosynthetic enzymes. Smith et al. [17] report that polyamines may be required for the full expression of gibberellin-induced internode elongation in peas. Based on their experiments, Kaur-Sawhney et al. [18] suggested that polyamines may mediate the gibberellin-induced growth response in the germination of pea seedlings. We have used this approach to study the effect of the application of exogenous gibberellic acid (GA3) on arbuscular mycorrhizal infection of *P. sativum* (P56, myc$^+$) and polyamine metabolism.

In order to assess the importance of polyamines on arbuscular mycorrhizal infection via specific irreversible inhibition of putrescine biosynthesis (via the ODC pathway) and after gibberellic acid (GA3) treatment, we measured the effects of these inhibitors and GA3 on polyamine titers, mycorrhizal infection and root development in *P. sativum*.

2. Materials and methods

Seeds of *P. sativum* L. P56 (nod$^-$myc$^+$), an isogenic mutant of cv. Frisson [19], were surface sterilized in 3.5% calcium hypochlorite solution for 20 min, rinsed with sterile water and germinated on a solid culture medium [20] under controlled conditions [21]. After 4 days, seedlings were transplanted into pots containing 200 g of a soil-based inoculum of *G. mosseae*, prepared as described by El Ghachtouli et al., [10], and terrageen (Oil Dry) mix (1:2, v/v). Plants were grown for 2 weeks in a constant environmental room (16 h, 20°C, 80% R.H., 320 μE m^{-2} s^{-2}). The protocol was 1 plant/pot, 5 replicates and daily drenching of soil with water (control) or with solutions of 2 mM DFMO, 2 mM DFMO + 5×10^{-4} M putrescine or at 10^{-7}, 10^{-6} M and 10^{-5} M gibberellic acid (GA3).

Plant growth was assessed by determining fresh weight. Polyamines were analysed in roots using published methods [22,23]. The develop-

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PII S0014-5793(96)00379-1

Table 1. Effects of 2 mM DFMO, 2 mM DFMO + 5×10^{-4}M putrescine and 10^{-7}, 10^{-6} or 10^{-5} M GA3 on root fresh weight (FW) of the *Pisum sativum* mutant P56 (myc$^+$), uninoculated (NI) or inoculated (I) by *Glomus mosseae*, after 2 weeks of culture

Treatments	Root FW (g)	
	NI	I
Control	1.45[a]	1.45[a]
2 mM DFMO	0.95[b]	1.01[b]
2 mM DFMO + 5×10^{-4}mM putrescine	1.25[ab]	1.23[ab]
10^{-7} M GA3	1.44[a]	1.46[a]
10^{-6} M GA3	1.40[a]	1.39[a]
10^{-5} M GA3	1.37[a]	1.38[a]

Values with different letters are significantly different ($P<0.05$).

ment of mycorrhizal infection in roots was estimated after staining with trypan blue [24] by the method of Trouvelot et al. [25], and expressed as *F*%, frequency of root infection, *M*%, colonization intensity of the whole root system and *A*%, frequency of endocellular arbuscule formation in the root system.

All data were analysed statistically using the Newman-Keuls test ($P = 0.05$) [26].

3. Results

3.1. *Effect of polyamine biosynthesis inhibition by DFMO and GA3 treatment on P. sativum root growth and development of arbuscular mycorrhizal infection*

DFMO caused a reduction of about 45% in root system fresh weight of uninoculated or *G. mosseae*-inoculated plants, after 2 weeks of culture (Table 1). This effect was partially reversed by adding putrescine at 5×10^{-4} M to DFMO treatments.

Exogenous application of DFMO (2 mM) to plants affected

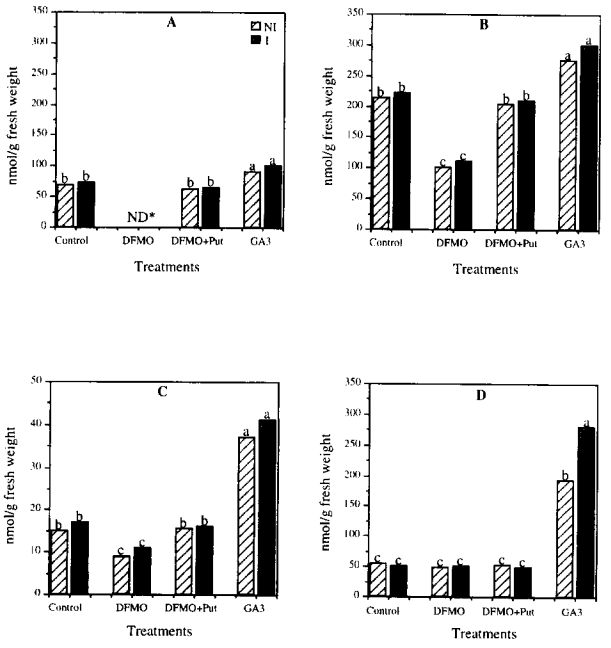

Fig. 1. Root polyamine content (A, putrescine; B, spermidine; C, spermine; D, cadaverine) in the *Pisum sativum* mutant, P56 not inoculated (NI, light bars), or inoculated (I, dark bars) with *Glomus mosseae* and treated or not (control), with 2 mM DFMO, 2 mM DFMO + 5×10^{-4} M putrescine (PUt) and 10^{-6} M GA3, at day 14 of culture. For the same polyamine, values with different letters are significantly different ($P=0.05$). ND*, not detected.

Table 2. Effects of 2 mM DFMO, 2 mM DFMO + 5×10^{-4} M putrescine and 10^{-7}, 10^{-6} or 10^{-5} M GA3 on arbuscular mycorrhizal infection (*F*%, infection frequency; *M*%, colonization intensity of the whole root system; *A*%, arbuscule frequency) of the *Pisum sativum* mutant P56 (myc$^+$), inoculated with *Glomus mosseae*, after 2 weeks of culture

Treatments	Arbuscular mycorrhizal infection		
	F%	*M*%	*A*%
Control	44.5[a]	8.13[a]	4.6[a]
2 mM DFMO	20.7[b]	3.93[b]	2.3[b]
2 mM DFMO + 5×10^{-4} mM putrescine	38.6[a]	8.15[a]	4.6[a]
10^{-7} M GA3	46.0[a]	8.00[a]	0.0
10^{-6} M GA3	33.2[b]	4.97[b]	0.0
10^{-5} M GA3	0.00	0.00	0.0

For the same infection parameter, values with different letters are significantly different ($P<0.05$).

the arbuscular mycorrhizal infection measured 2 weeks after inoculation, by reducing the frequency of infection (*F*%), colonization intensity (*M*%) and arbuscule formation (*A*%) by about 50% (Table 2). These inhibitory effects either on root and mycorrhizal development were reversed when 5×10^{-4} M putrescine was added in the DFMO treatment.

Treatment with GA3 did not modify root growth (Table 1). When applied to plants at 10^{-7} M, GA_3 selectively inhibited arbuscule formation, mimicking the phenotype of myc^{-2} mutants [27] (Table 2). Application of 10^{-6} M GA3 to plants had an inhibitory effect on arbuscular mycorrhiza frequency (*F*%) (reduction by about 25%), on colonization intensity (*M*%) (reduction by about 40%) and suppressed arbuscule formation (*A*%) (Table 2), whilst at 10^{-5} M, GA3 completely inhibited the development of mycorrhizal infection (Table 2).

3.2. *Effect of polyamine biosynthesis inhibition by DFMO and GA3 treatment on root P. sativum polyamine titers*

In order to determine the nature and the extent of DFMO action, we examined the polyamine content of all plants. Putrescine was absent from uninoculated or inoculated roots after addition of DFMO (Fig. 1A). This inhibitor decreased spermidine content of uninoculated and inoculated plants by 50% (Fig. 1B) and spermine was reduced by about 35% (Fig. 1C). Cadaverine, synthesized via lysine decarboxylase, remained unchanged (Fig. 1D). The polyamine pool (putrescine, spermidine and spermine) was reduced by about 60%. Application of exogenous putrescine prevented the decreases in spermidine and spermine caused by DFMO, and led to restoration of 90% of putrescine titers (Fig. 1).

Addition of 10^{-6} M GA3, the concentration which decreased all mycorrhizal infection parameters, increased the putrescine level by 40% (Fig. 1A), the spermidine level by 36% (Fig. 1B) and the spermine level by 140% (Fig. 1C) in uninoculated or inoculated roots. The titer of cadaverine was increased approx. 6-fold in inoculated roots and 4-fold in uninoculated ones (Fig. 1D). The total overall polyamine pool was increased 2-fold.

4. Discussion

DFMO decreased root polyamine levels, and inhibited root growth and arbuscular mycorrhizal infection of *P. sativum*. These effects were reversed when exogenous putrescine was

included in the DFMO treatment, indicating that the effect of DFMO on arbuscular mycorrhizal infection was indeed due to putrescine limitation. These results suggest that ODC may play a role in root growth and in arbuscular mycorrhizal development. It is known that arbuscular mycorrhizal colonization is accompanied by chromatin decondensation of the plant nucleus which indicates an increased transcriptional activity [28]. Polyamines stabilize nucleic acids [29], and are essential to steps in transcription and translation [30]. Serafini-Fracassini et al. [31] suggested that newly synthesized polyamines become bound to RNA to make it active and protect RNA in vivo against stress-induced RNase activity. Inhibition of polyamine biosynthesis by DFMO may therefore affect the transcription process and consequently plant-mycorrhizal fungus interactions.

In order to investigate the possibility that root polyamine titers may regulate arbuscular mycorrhizal development, we assessed the effect of gibberellic acid, a phytohormone known to increase polyamine levels and the activities of their biosynthetic enzymes in a variety of systems [32]. Gibberellic acid treatment enhanced pea root polyamine levels, but contrary to expected results, this hormone led to suppression of the arbuscular mycorrhizal infection. This effect, however, depended on the concentration of GA3 used: at 10^{-5} M, GA3 completely inhibited arbuscular mycorrhizal development, while at 10^{-6} M, it reduced mycorrhizal infection frequency and intensity and suppressed formation of arbuscule, endocellular structures essential for symbiotic bidirectional nutrient exchange [27]. It is interesting to observe that arbuscule formation was selectively inhibited by the lowest concentration of GA3 (10^{-7} M). At this concentration, GA3 had no effect on infection frequency suggesting that GA3 does not directly affect the capacity of the fungus to infect the roots. Furthermore, we have shown that treatment of shoots with GA3 significantly inhibits arbuscule formation in roots (45% reduction with 10^{-6} M GA3) (unpublished data). These results indicate that GA3 should act via the plant and that it may affect the molecular dialogue between plant and fungal cells leading to arbuscule formation [27].

The opposite effects of DFMO and GA3 on root polyamine levels and the negative effect of both compounds on arbuscular mycorrhizal development, and also on nodule formation in *P. sativum* cv. Frisson (nod^+) (Martin-Tanguy, unpublished data), raise the question of the effective role of polyamines in the regulation of symbiotic interactions. Using different approaches to the determine whether gibberellins act by increasing polyamines levels, several authors have shown that polyamines mediate hormone-induced plant responses in many physiological processes including those of internode growth and dormancy break which are induced by gibberellic acid treatment [17,18,33]. It is possible that in the case of plant-arbuscular mycorrhizal fungus interactions, the very high GA3-induced polyamine levels may mediate inhibition of arbuscule formation by this hormone. We recently showed that although fungal endogenous concentrations of polyamines can be a growth limiting factor for *Glomus mosseae* [9], application of exogenous polyamines at high concentrations (above 5 mM) markedly inhibits of spore germination and hyphal growth [9]. In contrast, plant polyamine treatments at concentrations below 1 mM significantly increase arbuscular mycorrhizal infection in myc^+ pea genotypes (cv. Frisson and P56) [10]. In the present investigation, restoration of polyamine levels by exogenous putrescine application to the DFMO-inhibited system resulted in recovery of mycorrhizal development; a similar effect can be observed on nodule formation (Martin-Tanguy, unpublished data). These results suggest that optimal concentrations of polyamines are required for normal development of arbuscular mycorrhiza, and perhaps root symbioses in general. Further investigations are necessary in order to clarify the mechanism of GA3-inhibition of arbuscule formation and to appreciate the role of polyamines in this phenomenen.

Acknowledgements: We are grateful to the Merell Dow Research Institute (Ohio, USA) for a generous gift of DFMO and thank Dr. D. Morandi for stimulating discussions and Drs. D. Tepfer and V. Gianinazzi-Pearson for critically reading the manuscript.

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