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Jasmonates, together with zeatin, induce hypaphorine accumulation by the ectomycorrhizal fungus *Pisolithus microcarpus*

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Abstract

Jasmonates have been reported to stimulate accumulation of indole alkaloids in plant cells. We demonstrated that jasmonic acid and methyl jasmonate applications result in increased hypaphorine concentration in *Pisolithus microcarpus* hyphae. Looking for synergetic effects we found that jasmonic acid and zeatin may contribute additively to hypaphorine accumulation. Both plant hormones were found in root exudates and must contribute to induce hypaphorine over-accumulation in symbiotic hyphae. In return, fungal hypaphorine was shown earlier to regulate auxin activity in host plant tissues during early stages of ectomycorrhiza ontogenesis. Jasmonates and zeatin might therefore contribute to some steps of molecular dialogue, which modulates the development of functional ectomycorrhizae and maintain symbiotic hyphae in a characteristic juvenile stage.

Keywords: Jasmonic acid, cytokinin, hypaphorine, mycorrhiza, IAA

1. Introduction

The establishment of ectomycorrhizal associations implies substantial reorganisation of root tissues, and modification of the hyphal growth pattern. This must require mutual recognition and dialogue between both symbiotic partners. Signalling molecules are exchanged at very early stages of the interaction, prior to any physical contact between symbionts. There is much evidence of spore germination and hyphal growth stimulation by host plant root exudates (Melin and Rama Das, 1954; Fries et al., 1987; Fries, 1988), and a case of chemotropism has been reported (Horan and Chilvers, 1990). In forest soil, such activity on hyphal growth by root diffusible molecules could promote root fungus encounter and consequently mycorrhizal formation. Some active compounds are excreted by roots such as rutin (Lagrange et al., 2001) while others, such as cathechin (Koide et al., 1998), could be released by litter from the host tree. Active concentrations of these signalling compounds can be as low as nanomolar or even picomolar for rutin (Lagrange et al., 2001). We recently reported that zeatin, a cytokinin, present in *E. globulus* root exudates, mimicked root exudates activity on hyphal branching as well as on hyphal hypaphorine accumulation (Lagrange et al., 2005).

Hypaphorine is an indole alkaloid, accumulated by *Pisolithus microcarpus* hyphae in response to root contact (Béguiristain and Lapeyrie, 1997) that subsequently regulates root hair elongation and auxin activity in host plant tissues (Ditengou and Lapeyrie, 2000; Ditengou et al., 2000; Kawano et al., 2002; Ditengou et al., 2003; Jambois et al., 2004). Hypaphorine is the first-identified fungal molecule regulating expression of symbiosis-related genes in host plant roots (Nehls et al., 1998; Tagu et al., 2003).

Jasmonates (JAs), including jasmonic acid (JA) and methyl jasmonate (MeJA) act as plant hormones by regulating developmental processes and responses to environmental cues. These include root growth, pollen development, abscission, senescence, or responses to wounding and UV irradiation (Creelman and Mullet, 1995, 1997; Farmer, 1994; Reymond and Farmer, 1998). Since jasmonates induce plant defence responses against pathogens (Reymond and Farmer, 1998) or herbivorous insects (Creelman et al., 1992), and since methyl jasmonate is a strong candidate molecule for airborne signals that mediate interplant communication in response to aggressions (Farmer and Ryan, 1990), jasmonates might contribute as well to the molecular dialogue between host plant and fungus during ectomycorrhiza ontogenesis.

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Here we aimed at investigating further molecular dialogue between symbionts. Considering that, (i) fungal hypaphorine accumulation is under zeatin control, (ii) together with zeatin, jasmonic acid was reported to enhance indole alkaloid accumulation by periwinkle (*Catharanthus roseus*) hairy root culture or suspension-cultured cells (Rijhwani and Shanks, 1998, Menke et al., 1999), here we tested the activity of jasmonates on hypaphorine accumulation by fungal hyphae looking for synergy between jasmonates and zeatin.

2. Materials and Methods

Biological material and growth conditions

The strain 441 of the ectomycorrhizal fungus *Pisolithus microcarpus*, was isolated from a sporocarp collected under *Eucalyptus citriodora* in Brazil (at Sao Paulo, by M. Ivory). Glass flasks were filled with 10 ml of low-sugar modified Pachlewski liquid medium (KH_2PO_4, 7.3 mM; ammonium tartrate, 2.7 mM; $MgSO_4.7H_2O$, 7.3 mM; glucose, 100 mM; bacteriological malt extract Difco, 3 g l^{-1}; thiamine-HCl, 9 mM; trace element stock solution (Kanieltra Co.), 0.1 ml l^{-1}) supplemented or not with jasmonic acid or methyl jasmonate (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M, final concentration), or zeatin (10-6 M, final concentration) solutions sterilised by filtration, and inoculated with *Pisolithus microcarpus* agar plugs (one plug per flask). After 2 weeks, colony ergosterol content and hypaphorine concentration were assessed. Seven replicate colonies were analysed per treatment.

Hypaphorine and ergosterol quantification in fungal colonies

Each colony was collected, quickly dried on absorben paper and homogenised in methanol (400 µl) in an Eppendorf tube. Following sonication (10 min) and centrifugation (10 min, 10,000 rpm), supernatants were analysed by HPLC. To quantify simultaneously hypaphorine and ergosterol, a protocol was developed combining previously reported methods for ergostero (Martin et al., 1990) or hypaphorine (Béguiristain et al. 1995) purification. HPLC analyses were performed with a Beckman's System Gold including a binary pump and a Thermo Quest automatic injector (20 µl). The instrument was equipped with a UV detector, a data processing station, and fitted with a 250×4 mm C18 end-capped ODS AQ YMC 5 µm column (Interchim). Hypaphorine (retention time: 11 min) and ergosterol (retention time: 25 min) were eluted (flow rate: 1 ml min^{-1}) by a gradient of two solvents, A (H_2O 100%) and B (methanol 100%). The initial conditions were 85% A + 15% B, the next step (30% A + 70% B) was reached in 11 min, the last step (100% B) was reached in 4 min and maintained for 13 min. Compounds were identified and quantified by comparison with standards. Ergosterol was purchased from Sigma and hypaphorine chemically synthesized according to Romburgh and Barger (1911).

3. Results

Fungal hypaphorine accumulation in response to jasmonic acid and methyl jasmonate

Jasmonic acid (10^{-7}–10^{-4} M) did not significantly affect *Pisolithus microcarpus* hyphal growth (assessed by ergosterol accumulation) on malt enriched nutrient medium (Fig. 1a). However, jasmonic acid 10^{-4} M induced a strong increase (2 fold) of hypaphorine concentration in hyphae (mol of hypaphorine per mol of ergosterol) (Fig. 1b). Comparison of jasmonic acid and methyl jasmonate activities showed that they were identical at the same optimal concentration tested, 10^{-4} M (Fig. 2).

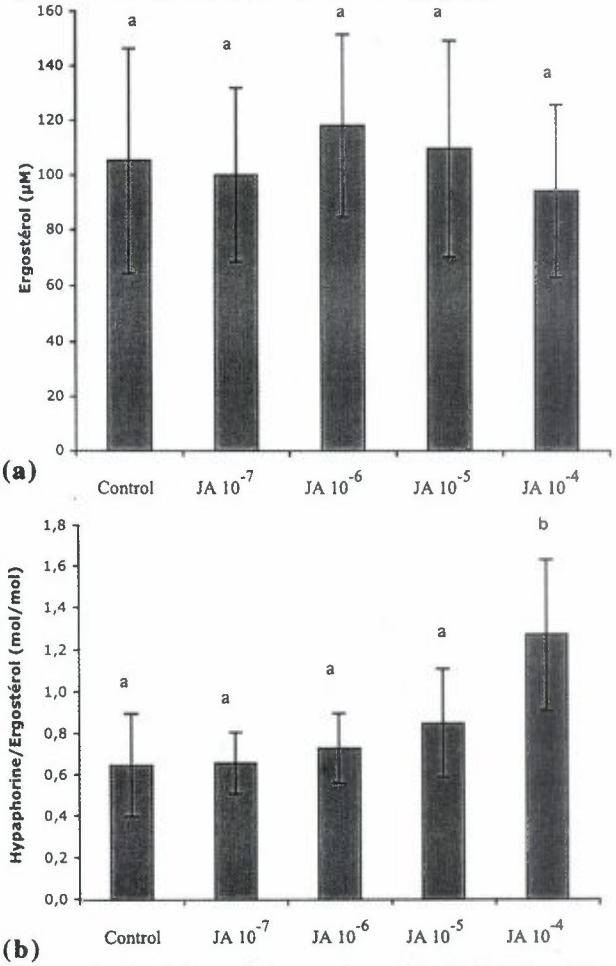
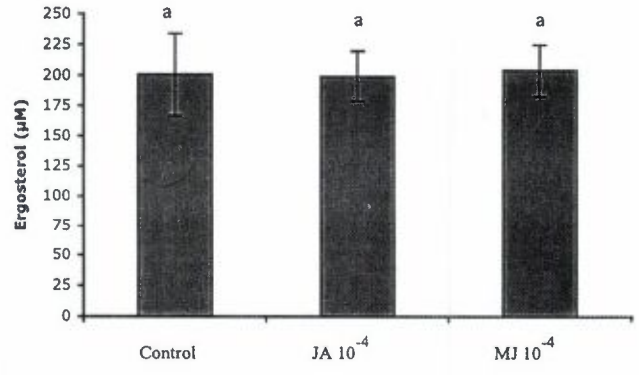


Figure 1. Activity of jasmonic acid (0.1–100 µM) on *Pisolithus microcarpus* in pure culture. (a) Biomass of colonies in pure culture estimated by ergosterol concentration in extracts. (b) Hypaphorine concentration in hyphae related to ergosterol content (mol.mol^{-1} ergosterol). Means of 7 replicates ± SD, compared by t-test (P=0.05); different letters above columns indicate significantly different means.



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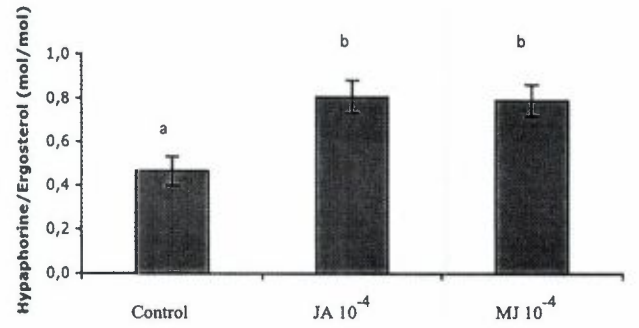
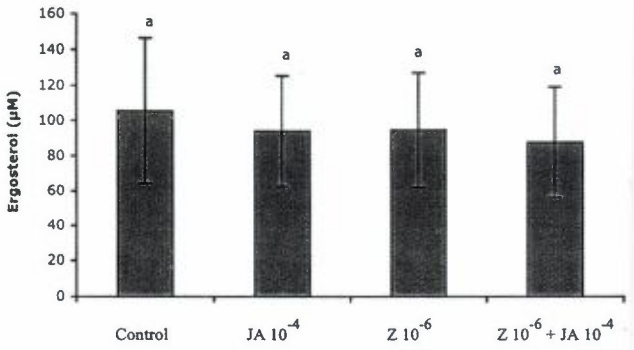
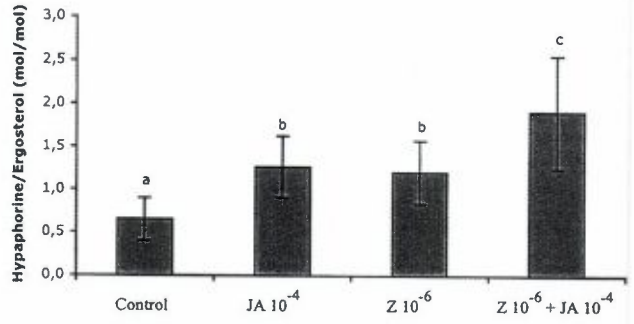


(b)

Figure 2. Activities of jasmonic acid and methyl jasmonate 100 µM on *Pisolithus microcarpus* in pure culture. (a) Biomass of colonies in pure culture evaluated by ergosterol concentration in extracts. (b) Hypaphorine concentration in hyphae related to ergosterol content (mol.mol^{-1} ergosterol). Means of 7 replicates ± SD, compared by the t-test (P=0.05); different letters above columns indicate significantly different means.



(a)



(b)

Figure 3. Activities of jasmonic acid (100 µM) and zeatin (1 µM) on Pisolithus microcarpus in pure culture. (a) Biomass of colonies in pure culture evaluated by ergosterol concentration in extracts. (b) Hypaphorine concentration in hyphae related to ergosterol content (mol.mol^{-1} ergosterol). Means of 7 replicates ± SD, compared by the t-test (P=0.05); different letters above columns indicate significantly different means.

Additivity of zeatin and jasmonic acid activities

Neither zeatin (10^{-6} M, the optimal concentration previously reported by Lagrange et al., 2005) nor a combination of zeatin (10^{-6} M) and jasmonic acid (10^{-4} M) significantly affected *Pisolithus microcarpus* hyphal growth (Fig. 3a). In agreement with earlier results (Lagrange et al., 2005), zeatin (10^{-6} M) stimulated hypaphorine accumulation by 2 fold as for jasmonic acid (10^{-4} M) (Fig 3b). When zeatin (10^{-6} M) and jasmonic acid (10^{-4} M) were supplied simultaneously, hypaphorine accumulation was stimulated by 3 fold (Fig. 3b).

4. Discussion

The role of jasmonates during plant responses to wounding and pathogen attacks is well established (Wasternack and Hause, 2002). While molecular signalling during mycorrhizal interactions is poorly understood, the involvement of jasmonic acid has been suggested. Jasmonic acid treatment accelerated the fastening of *Pisolithus tinctorius* or *Laccaria laccata* hyphae to *Picea abies* roots (Regvar and Gogala, 1996; Regvar et al., 1997). The accumulation of secondary metabolites can be induced in non-mycorrhizal barley roots by jasmonate treatments as well as during arbuscular mycorrhizal colonisation (Peipp et al., 1997). The present data provide further evidence. Jasmonates, just as *Eucalyptus globulus* root exudates or zeatin, a cytokinin present in root exudates of that host plant (Lagrange et al., 2005), stimulate hypaphorine accumulation by the ectomycorrhizal fungus *Pisolithus microcarpus*. Upstream, the biosynthesis of jasmonate in mycorrhizal barley roots has been shown to be induced during root colonisation by *Glomus intraradices* (Hause et al., 2002). The activities of jasmonic acid and zeatin on accumulation of indole alkaloids, ajmalicine and serpentine, in periwinkle hairy root or cell cultures were reported earlier (Decendit et al., 1992; Garnier et al., 1996; Rijhwani and Shanks, 1998; Menke et al., 1999), suggesting that common regulation pathways might be involved in some plants and fungi.

Since zeatin mimics the activity of *E. globulus* ssp *bicostata* roots (Béguiristain and Lapeyrie, 1997) and root exudates on *Pisolithus microcarpus* hyphae hypaphorine accumulation (Lagrange et al., 2005), it has been proposed

that zeatin from root exudates contributes to some steps of functional ectomycorrhiza development. However, zeatin from root exudates was not sufficient to induce the full response observed (Lagrange et al., 2005). This suggested then that root exudates may be regarded as a cocktail of active molecules. Considering the activity of jasmonates on hypaphorine accumulation and the additivity of zeatin and jasmonate activities, at the concentrations tested, we assume that jasmonates could be a component of that active molecule cocktail.

While jasmonic acid and zeatin transferred from roots to colonizing hyphae might contribute to induce hypaphorine accumulation in hyphae, in return, fungal hypaphorine should regulate auxin activity in plant tissues during early stages of ectomycorrhiza ontogenesis (Nehls et al., 1998; Ditengou and Lapeyrie, 2000; Ditengou et al., 2000; Kawano et al., 2002; Ditengou et al., 2003). As for previously described elicitor/chitinase interactions (Salzer et al., 1997), these jasmonate-zeatin/hypaphorine steps might be regarded as elements of the molecular dialogue between plant and ectomycorrhizal fungus. The fluxes of jasmonate and hypaphorine between symbionts still remain to be quantified.

Pisolithus microcarpus growth (estimated as ergosterol content) what not affected by jasmonic acid, methyl jasmonate or zeatin. Hypaphorine accumulation in hyphae or in some hyphae amongst a colony, could be regarded as a juvenility character since high hypaphorine concentrations have been detected in hyphal tips (the outer 2 mm of the colony) (Béguiristain and Lapeyrie, 1997), as well as in root colonizing hyphae whose juvenile stage (small vacuoles, organelle rich cytoplasm) has been repetitively attested by ultrastructural investigations (Dexheimer and Pargney, 1991; Kottke and Oberwinkler, 1986). Thus, root diffusible molecules, such as zeatin and jasmonates, might maintain the juvenility of symbiotic hyphae, characterized by high hypaphorine concentration.

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