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Methyl jasmonate protects Norway spruce [*Picea abies* (L.) Karst.] seedlings against *Pythium ultimum* Trow.

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The effect of methyl jasmonate (MeJA) was tested on the resistance of *Picea abies* seedlings against *Pythium ultimum*. Treatments with volatile MeJA during 3 days (25 μl 100 l^{-1} air) protected seedlings up to 75%. This effect was unlikely to result from a direct fungitoxic effect of MeJA, as under the same conditions of treatment, growth *in vitro* of *Pythium ultimum* was not affected. Observations of possible changes on histological barriers such as lignin deposits showed no differences between control and treated plants. MeJA induced the accumulation of free salicylic acid (SA) in all parts of the seedlings, whereas bound SA only increased in hypocotyls and cotyledons. An increase in chitinase activity was detected in cotyledons already 2 days after exposure to MeJA. The results suggest that MeJA acts by stimulating defence responses of the host plant. No increase in endogenous jasmonic acid was found in spruce seedlings inoculated with *Pythium* nor MeJA.

Keywords: damping off; defence responses; chitinase; jasmonate; methyl jasmonate; salicylic acid; soil-borne fungi.

INTRODUCTION

Jasmonates, first detected in essential oils of *Jasminum grandiflorum* L. [*8*] are widespread natural regulators involved in many processes during plant development [*6, 23*]. Recently, jasmonates have also been implied in the signalling pathway mediating induced defence responses in pathogen- or insect-attacked plants [*6, 11, 23*]. Treatment with jasmonates was shown to induce various responses including the accumulation of a ribosome-inactivating protein [*3*], serine proteinase inhibitors [*10*], leucine aminopeptidase and threonine deaminase [*14*], phenylalanine ammonia-lyase, thionin [*1*], chalcone synthase, vegetative storage protein and a proline-rich cell wall protein [*6*]. A number of secondary metabolites were also shown to accumulate in cultured cells of various plant species upon treatment with JA [*13*]. Many studies on the mode of action of jasmonates in plant-pathogen interactions are based on model systems, such as detached leaves or suspension-cultured cells treated with elicitors [*2, 9, 18*]. The application of jasmonate to tomato and potato induces local and systemic protection against *Phytophthora infestans* [*5*]. However, jasmonates had no effect on the local resistance of barley against powdery mildew [*21*]. Strong support for the role of jasmonates in pathogen defence was provided with *Arabidopsis* mutants that cannot accumulate jasmonic acid [*26*]. Such *fad* mutants were extremely susceptible to *Pythium mastophorum* and exogenous application of methyl jasmonate (MeJA) protected mutants to a level close to that of wild type controls [*26*]. Here we describe results indicating that MeJA can stimulate protection of *Picea abies* seedlings against *Pythium ultimum* causing damping-off disease. We have observed no direct effect of MeJA on the growth and development of *Pythium in vitro* and we propose that the protection is due to the induction of plant resistance mechanisms. This notion is supported by results showing induction of chitinase activity in cotyledons of MeJA-treated spruce seedlings.

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Abbreviations used in text: MeJA, methyl jasmonate; SA, salicylic acid.

MATERIALS AND METHODS

Biological material

Seeds of Norway spruce [*Picea abies* (L.) Karst.] were obtained from the Swiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf, Switzerland. They were collected from the experimental spruce stand in Tägerwilen, Switzerland. *Pythium* sp. isolates were obtained from culture collections of Novartis, Basle, Switzerland.

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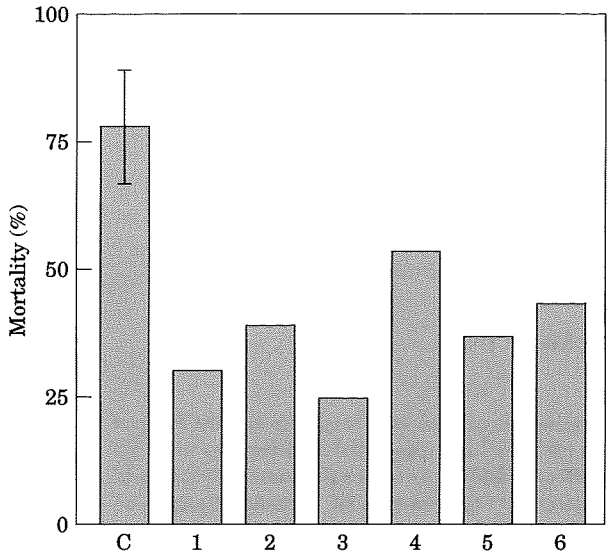

FIG. 1. Effect of MeJA on the mortality of *P. abies* seedlings inoculated with *P. ultimum*. 1–6: independent experiments each consisting of 250 seedlings treated with MeJA prior to inoculation with *P. ultimum*. C: control. Data represent means of three independent experiments each consisting of 250 untreated seedlings (±SD).

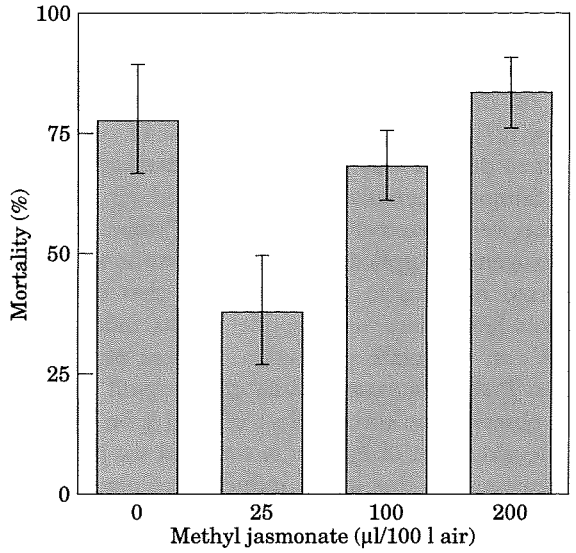

FIG. 2. Dose-dependence of MeJA on mortality of *P. abies* seedlings inoculated with *P. ultimum*. Data represent means of three independent experiments, each consisting of 250 seedlings (±SD). Control plants were inoculated with *P. ultimum* but not pretreated with MeJA.

Culture conditions

Seeds were surface-sterilized by dipping for 10 min in 30% (v/v) hydrogen peroxide followed by washing in four changes of sterile distilled water. Seeds were germinated on wet filter papers in the dark at room temperature for 8–10 days, they were then transferred into pots (10 cm × 25 cm, 1000 ml) containing a sand-vermiculite mixture (1:1, v/v). Plants were grown in a growth chamber (22 °C day/18 °C night temperature, 80% rel. humidity, 14 h light).

Pythium ultimum was maintained in Petri dishes on potato carrot agar (PCA) [2%, w/v, using a 1:1 potato and carrot homogenate and 1·8%, w/v, bacteriological agar (Unipath Ltd., Hampshire, U.K.)], in darkness at room temperature.

Inoculation with Pythium ultimum

For routine infection tests, seedlings were used 10 days after emergence; at a later time they were seen to develop age-related resistance. Infections with *Pythium ultimum* were carried out by pouring a suspension of sporangia around the base of each plant. The concentration of sporangia was 25000 spores ml^{-1} of water.

Methyl jasmonate treatment

Spruce seedlings were used 7 days after emergence and placed in plastic chambers (volume: 100 l) and treated for 3 days with methyl jasmonate (MeJA) (Serva, Feinbiochemica GmbH & Co., Heidelberg, Germany). Routinely, 25 µl of MeJA was added on each of the 3 days of treatment using cotton wool to allow evaporation. After 3 days, treated seedlings were removed from the chambers and inoculated one day later with *Pythium ultimum*. For chitinase and salicylic acid (SA) analysis, roots, hypocotyls and cotyledons were collected 2, 4 and 6 days after the end of the MeJA treatment.

For mycelial weight estimation *Pythium ultimum* was grown on PCA medium placed in plastic chambers with the same concentration of MeJA as for seedlings treatment. After 3 days of treatment, they were scraped off the Petri dishes and the wet weight of mycelia was estimated as well as sporangia production and sporangia germination.

Chitinase activity

Picea abies seedlings were homogenized in liquid nitrogen, resuspended in phosphate buffer (0·5 g per 2 ml of buffer, 50 mM) at pH 7·0, and centrifuged for 10 min at 5000 ***g***. The supernatant was used for chitinase assay [*16*]. One hundred microliters of supernatant, 50 µl of 50 mM phosphate buffer at pH 7·0 and 100 µl of ^{3}H-chitin were incubated for 30 min at 37 °C. The reaction was stopped with 250 µl of 1M trichloroacetic acid. The mixture was then centrifuged at 5000 ***g*** for 10 min and the radioactivity was measured in a 250 µl aliquot of the supernatant using a scintillation counter. Proteins were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Germany).

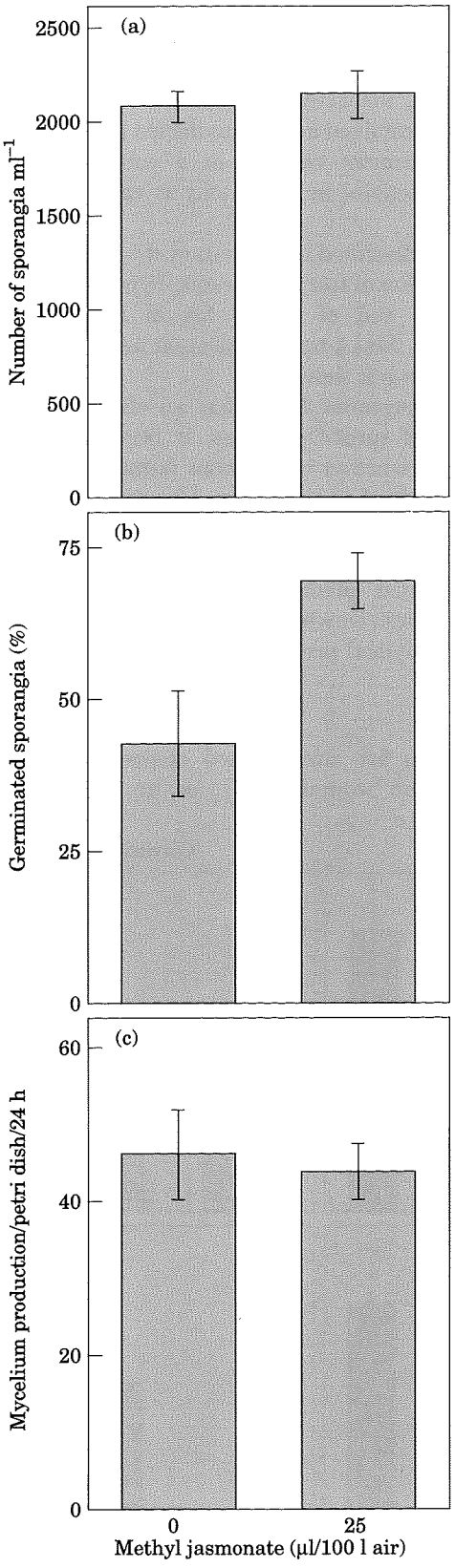

FIG. 3. Effect of MeJA on *P. ultimum*: (**a**) sporangia formation, (**b**) germination of sporangia, (**c**) mycelium formation (wet weight). Data represent means of three independent experiments, each consisting of six Petri dishes (±SD).

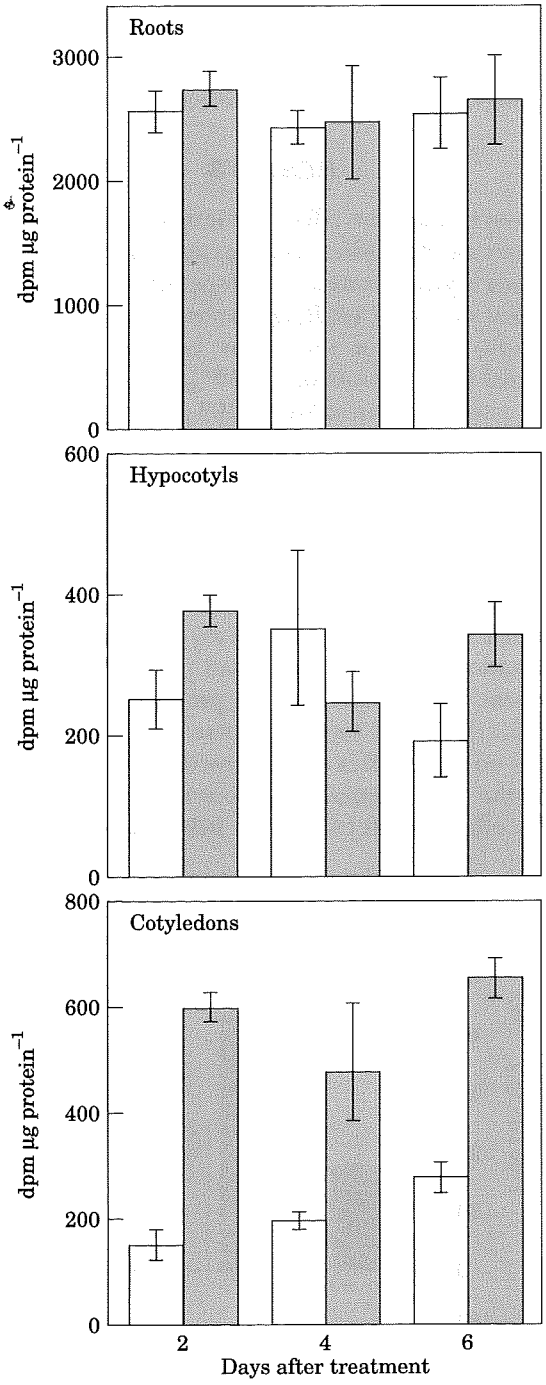

FIG. 4. Chitinase activity in different parts of spruce seedlings after treatment with MeJA (25 µl/100 l^{-1} of air). Data represent means of three independent experiments, each consisting of 250 seedlings (±SD). ▨, control; ■, MeJA.

Salicylic acid analysis

Extractions and analysis using HPLC (System Gold, Beckman, Nyon, Switzerland) were performed according to Meuwly *et al.* [*17*].

Analysis of jasmonic acid

The extraction and quantitative analysis of jasmonic acid were carried out as described earlier [19].

RESULTS

We have studied the effect of methyl jasmonate on the resistance of *Picea abies* seedlings against the soil-borne pathogen *Pythium ultimum*. Compared to controls, the mortality of young seedlings exposed to gaseous MeJA (26·8 ppb, based on the molar volume) over 3 days decreased from approximately 80% to 40% (Fig. 1). At higher concentrations of MeJA (108 ppb), spruce seedlings become more susceptible to *Pythium* infection (Fig. 2).

To test a direct effect of MeJA on the growth and development of *Pythium ultimum*, Petri dishes were inoculated with spores and placed under the same plastic chamber as MeJA-treated seedlings. After 3 days of exposure to 25 μl MeJA, the production of sporangia remained unaffected [Fig. 3(a)] whereas sporangia germination was slightly increased [Fig. 3(b)]. The same treatment had no effect on the production of mycelium [Fig. 3(c)]. Taken together, these results indicate that there is no direct effect on the growth of *Pythium* by MeJA under the experimental conditions used here.

The effect of MeJA on the host response in spruce seedlings was also investigated. Figure 4 shows that MeJA treatment has no effect on chitinase activity in roots that exhibit a high constitutive level of chitinase activity. In hypocotyls, the activity of chitinase was lower than in roots and remained mostly unaffected by MeJA. However, MeJA treatment resulted in a consistent increase in chitinase activity in cotyledons as early as 2 days after treatment.

Results described in Fig. 5 show that MeJA induced the accumulation of free SA in roots, hypocotyls as well as in cotyledons and of bound SA in hypocotyls and in cotyledons. MeJA had no effect on lignification in spruce tissue (data not shown).

Since exogenous MeJA has an effect on the defence response of spruce seedlings, it becomes important to determine whether endogenous jasmonic acid may be a signal involved in resistance. Changes in jasmonic acid levels after inoculation were monitored to test this hypothesis. Seedlings of control, *Pythium*-infected or MeJA-treated plants contain jasmonate levels below 10 ng g^{-1} fresh wt which is the quantitative detection limit of our analytical procedure (data not shown).

DISCUSSION

Jasmonates are stress-related compounds produced in plants upon wounding [7], or in elicitor-treated cell

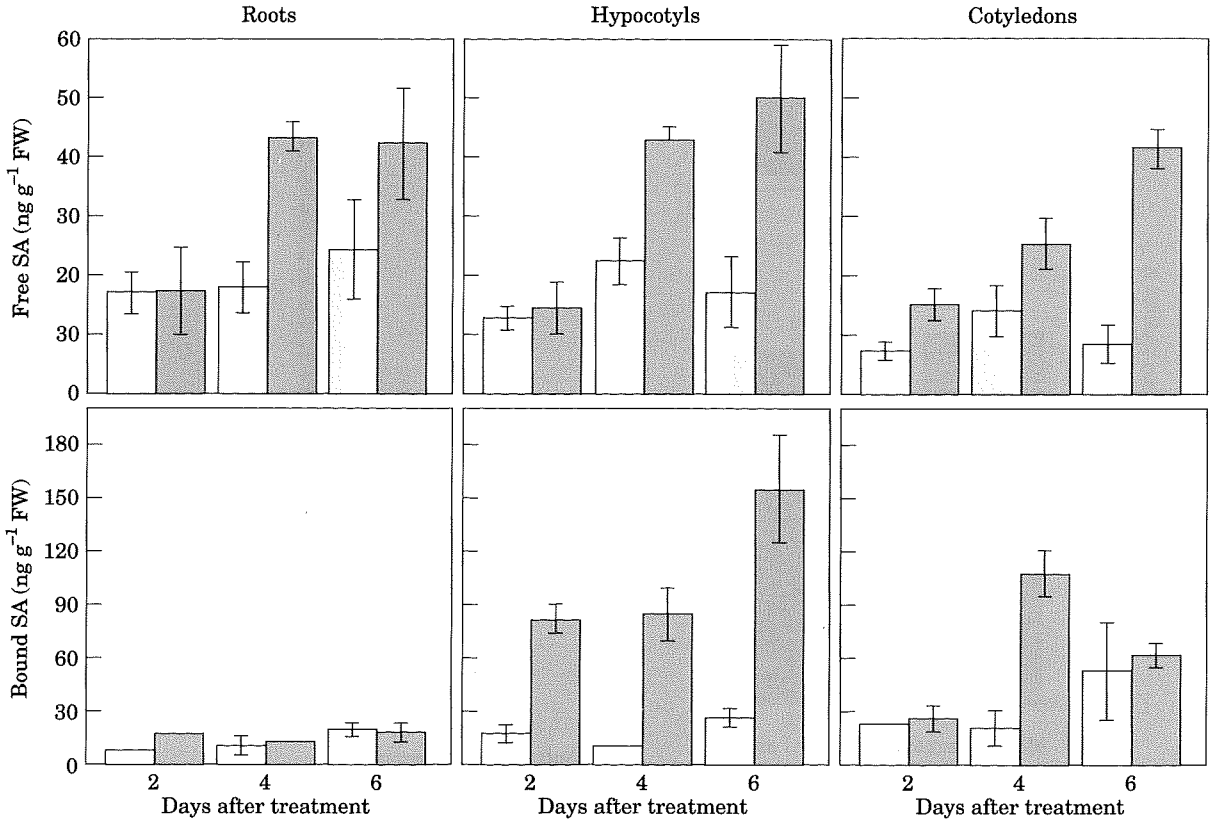

FIG. 5. Salicylic acid level in different parts of spruce seedlings after treatment with MeJA (25 μl/100 l^{-1} of air). Data represent means of three independent experiments, each consisting of 250 seedlings (±SD). ▨, control; ■, MeJA.

cultures [*13*] and have been proposed to be important in the defence of plants against herbivores or pathogens [*11, 12, 19, 26*]. Results presented here indicate that MeJA, one of the major physiological active forms of jasmonates, can protect spruce seedlings against the soil-borne pathogen *Pythium ultimum*. Previous results have shown that JA and MeJA applied to potato leaves can induce systemic resistance against *Phytophthora infestans* [*5*]. Similarly, local treatment of rice leaves with JA induced partial resistance against *Magnaporthe grisea* in the upper leaves [*22*]. The evidence that MeJA acts via host plant mechanisms is based on two observations. Firstly, growth and development of *Pythium ultimum in vitro* is not inhibited by MeJA (Fig. 3). Fungitoxicity tests on artificial media should however be considered with some caution, as the fungus is under different conditions than when growing on its host. However, observations of *Pythium* growth *in vivo* in those treated seedlings that damped off showed no particular features indicating developmental alterations that could be attributed to MeJA. Secondly, a search for possible effects of MeJA on the physiology of spruce showed a stimulation of chitinase activity and SA accumulation (Figs 4 and 5). In many plants, these reactions are considered as markers for induced defence reactions [*20*].

However, it is not certain that chitinase acts against *Pythium* infections in spruce as discussed in a previous study [*15*]. Hyphal cell walls of oomycetes are mainly constituted by β-glucans [*24*], but hydrolysis also yields small amounts of glucoseamine which could derive from chitin or glycoproteins. Chérif *et al.* [*4*] were able to demonstrate the presence of chitin in the innermost layers of the cell wall of *Pythium ultimum*. Such a location makes it highly unlikely that the increase in chitinase activity alone could account for the increase in resistance against *Pythium*. Also, *Pythium* is not blocked by the high levels of chitinase constitutively expressed in roots where the fungus penetrates [*15*].

Taken together, this indicates that the MeJA-induced increase in chitinase in cotyledons represents a physiological response induced by MeJA, that is not directly linked to increased resistance against *Pythium ultimum*. Possibly, chitinase alone or together with other induced but yet uncharacterized defence reactions could be a potential barrier against other invaders of spruce seedlings.

The absence of increase in jasmonic acid after inoculation is surprising. It might be possible that other derivatives of the octadecanoic pathway change during infection. Phytodienoic acid, a biologically active precursor of jasmonic acid, has recently been shown to increase during mechanical stimulation of bean and *Bryonia* leaves [*25*].

Future work should now be directed towards understanding the physiological and molecular basis for MeJA-induced resistance in spruce seedlings.

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