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Evaluating methyl jasmonate for induction of resistance to *Fusarium oxysporum, F. circinatum* and *Ophiostoma novo-ulmi*

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Abstract

Damping off is probably the most common disease affecting seedlings in forest nurseries. In south-western Europe, the pitch canker and the Dutch elm disease cause relevant economic looses in forests, mostly in adult trees. The ability of the chemical plant elicitor methyl jasmonate (MeJA) to induce resistance in *Pinus pinaster* against *Fusarium oxysporum* and *F. circinatum*, and in *Ulmus minor* against *Ophiostoma novo-ulmi* was examined. In a first experiment, an aqueous solution of MeJA 5 mM was applied to *P. pinaster* seeds by immersion or spray, and different concentrations of MeJA (0, 0.1, 0.5, 1, 5 and 10 mM) were tested in seedlings before inoculations with *F. oxysporum* (10^5 and 10^7 spores mL^{-1}). In a second experiment, 6-months-old *P. pinaster* seedlings were sprayed with 0 and 25 mM of MeJA, and later challenged with mycelium of *F. circinatum*. Finally, 4-year-old *U. minor* trees were sprayed with 0, 50 and 100 mM of MeJA and subsequently inoculated with *O. novo-ulmi* (10^6 spores mL^{-1}). MeJA did not protect *P. pinaster* seeds and seedlings against *F. oxysporum*, probably because plants were too young for the physiological mechanisms responsible for resistance to be induced. Based on the morphological changes observed in the treated 6-months-old *P. pinaster* seedlings (reduction of growth and increased resin duct density), there is evidence that MeJA could have activated the mechanisms of resistance. However, 25 mM MeJA did not reduce plant mortality, probably because the spread of the virulent *F. circinatum* strain within the tree tissues was faster than the formation of effective defense responses. Based on the lack of phenological changes observed in the treated elms, there is no evidence that MeJA would cause induction of resistance. These results suggest that the use of MeJA to prevent *F. oxysporum* and *F. circinatum* in *P. pinaster* seedlings in nurseries and *O. novo-ulmi* in *U. minor* trees should be discarded.

Key words: *Pinus pinaster*; *Ulmus minor*; damping off; pitch canker; Dutch elm disease; traumatic resin ducts.

Resumen

Utilización de metil jasmonato para la inducción de resistencia ante *Fusarium oxysporum, F. circinatum* y *Ophiostoma novo-ulmi*

El "damping off" es una de las enfermedades más comunes en los viveros forestales. En árboles adultos del suroeste de Europa, el chancro resinoso y la grafiosis del olmo son enfermedades que están causando importantes pérdidas económicas en los bosques. Se ha estudiado la capacidad del metil jasmonato (MeJA), un elicitador químico de plantas, para inducir resistencia en *Pinus pinaster* ante *Fusarium oxysporum* y *F. circinatum*, y en *Ulmus minor* ante *Ophiostoma novo-ulmi*. En un primer experimento se aplicó una solución acuosa de MeJA 5 mM a semillas de *P. pinaster* mediante inmersión o pulverización de las mismas, y diferentes concentraciones de MeJA (0, 0.1, 0.5, 1, 5 and 10 mM) fueron pulverizadas en plántulas de *P. pinaster* antes de las inoculaciones con *F. oxysporum* (10^5 y 10^7 esporas mL^{-1}). En un segundo experimento, plántulas de *P. pinaster* de 6 meses de edad fueron pulverizadas con MeJA 0 y 25 mM, y posteriormente inoculadas con micelio de *F. circinatum*. Por último, brinzales de *U. minor* de 4 años de edad fueron pulverizados con MeJA a 0, 50 y 100 mM e inmediatamente inoculados con *O. novo-ulmi* (10^6 esporas mL^{-1}). El MeJA no protegió a las semillas ni a las plántulas de *P. pinaster* ante *F. oxysporum*, quizá debido a que las plántulas eran demasiado jóvenes para inducir los mecanismos fisiológicos responsables de la resistencia. Basándonos en los cambios morfológicos observados en las plántulas de 6 meses de *P. pinaster* (reducción del creci-

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miento e incremento de la densidad de los canales resiníferos), hay evidencia de que el MeJA pudo haber activado los mecanismos de resistencia. El MeJA a 25 mM no consiguió reducir la mortalidad probablemente porque la dispersión de *F. circinatum* en el interior de los tejidos fue más rápida que la formación de respuestas defensivas efectivas. Basándonos en la falta de cambios fenológicos de los olmos tratados, no hay evidencias de que el MeJA pueda haber causado una inducción de resistencia. Los resultados sugieren que el uso del MeJA para prevenir los patógenos *F. oxysporum* y *F. circinatum* en plántulas de *P. pinaster* en viveros y *O. novo-ulmi* en brinzales de *U. minor* debe ser descartado.

Palabras clave: *Pinus pinaster; Ulmus minor;* "damping off"; chancro resinoso; grafiosis del olmo; canales resiníferos traumáticos.

Introduction

Plants protect themselves against a diversity of attackers through constitutive and inducible defense strategies. Constitutive defenses are structural or chemical compounds permanently present in the tree and represent the first lines of protection. Inducible defenses are activated by plants upon perception of a foreign challenge, and occur at the site of the initial attack (local defence), in distant parts of the plant or throughout the entire plant (systemic defence) (Eyles *et al.*, 2010). Several types of systemic induced resistance have been characterized in detail, such as pathogen-induced systemic acquired resistance (SAR), systemic induced resistance by plant growth-promoting rhizobacteria or fungi (SIR), and wound or herbivore induced resistance (Pieterse and Van Loon, 2007; Eyles *et al.*, 2010). These types of resistance are initiated by different elicitors and partially controlled by distinct signaling pathways, but all share the characteristic of having a broad spectrum of effectiveness (Pieterse and Van Loon, 2007).

It is of practical interest to determine if elicitor molecules released, during the early stages of the plant–pathogen interaction could be directly applied to plants in order to suppress the effects of fungal diseases of plants. Exogenous applications of salicylic acid (SA) and carvacol to *Ulmus minor* successfully enhanced the resistance of trees to the fungal pathogen *Ophiostoma novo-ulmi* (Martín *et al*., 2008b, 2010). Also, foliar sprays of SA or of benzothiadiazole to *Pinus radiata* significantly decreased plant infections by *Diplodia pinea* or by *Phytophthora cinnamomi*, respectively (Reglinski *et al*., 1998; Ali *et al.*, 2000). Within forestry, both elm and pine trees are appropriate hosts for testing active elicitor molecules, since previous research on these species reported several types of induced resistance to be operative (Solla and Gil, 2003; Bonello *et al*., 2006; Gordon *et al*., 2010; Martín *et al*., 2010; Kim *et al*., 2010). Worldwide, damping off caused by *Fusarium oxysporum* is probably one the most severe disease affecting seedlings in forest nurseries (Machón *et al.*, 2006). In south-western Europe, the pitch canker and the Dutch elm disease, caused by *F. circinatum* and *O. novo-ulmi* respectively, are amongst the problems causing higher impact in forests (Martín *et al*., 2008a, b; Vivas *et al*., 2012) and none of the suggested control strategies have been effective, either for technical, economical, or environmental limitations. In view of this, the study of disease control methods based on the direct application of natural molecules on trees is gaining interest by researchers and foresters (Holopainen *et al.*, 2009).

There is growing evidence that exogenous applications of methyl jasmonate (MeJA) can enhance the levels of certain defensive compounds of plants and in consequence be used to trigger the defense mechanisms of trees (Moreira *et al.*, 2009; Eyles *et al.*, 2010). MeJA or (*Z,E*)-methyl 3-oxo-2-(2-pentyl) cyclopentane acetate is one of the mayor physiological active forms of jasmonates, and the most commonly studied elicitor in conifer species (Holopainen *et al.*, 2009). This compound is usually mixed with the surfactant Tween 20 at 0.1% (Hubber *et al*., 2005; Zeneli *et al*., 2006; Moreira *et al.*, 2009) and directly applied by spraying or by brushing the plant. An aqueous solution of MeJA has been used to artificially induce defense responses of trees, through increasing the synthesis of terpenoid, phenolic and alkaloid compounds (Heijari *et al.*, 2005; Zeneli *et al*., 2006) or by promoting the formation of traumatic resin ducts (Martin *et al.*, 2002; Hudgins *et al*., 2004). Exogenous MeJA has been successfully used to enhance the resistance of trees against several insects and pathogens, i.e. of *Picea abies* against *Pythium ultimum* and *Ceratocystis polonica* (Kozlowski *et al.*, 1999; Zeneli *et al*., 2006; Krokene *et al*., 2008), or of *P. sylvestris* and *P. pinaster* against *Hylobius*

abietis (Heijari *et al.*, 2005; Moreira *et al.*, 2009). The purpose of this study was to test if exogenous applications of MeJA can induce resistance in (i) *P. pinaster* seeds and seedlings against *F. oxysporum*, (ii) *P. pinaster* seedlings against *F. circinatum*, and (iii) *U. minor* trees against *O. novo-ulmi*.

Materials and methods

Fungal pathogens and inoculum preparation

The pathogen *F. oxysporum* (Fo-4P) used for the first experiment was isolated in 2005 from a diseased seedling growing in a commercial nursery located in Soria, central Spain. The isolate was selected because previous research confirmed its high virulence on *Pinus sylvestris* (Machón *et al.*, 2006). The isolate was long-term maintained on Komada (K) medium (Komada, 1975). Inoculum was prepared by subculturing the fungus in PDA and then placing four pieces of mycelium in sterile flasks containing potato dextrose broth (PDB) liquid medium under the dark. The flasks were shaken for 7 days at room temperature, and the suspensions filtered and adjusted to 10^5 and 10^7 spores mL^{-1} water.

The *F. circinatum* isolate (Fc7-1) used for the second experiment was isolated in 2005 from a stem canker of a *P. pinaster* tree in Asturias, northern Spain. Information about its virulence on *P. pinaster* seedlings is available (Vivas *et al.*, 2012). Long term storage of the strain was carried out on PDA in the fridge, for periods no longer than 3 months. Inoculum was prepared by growing during 7 days the fungus into Petri dishes containing PDA, at 25 ± 1 °C under the dark.

The *O. novo-ulmi* ssp. *americana* isolate PM-SP used for the third experiment was selected because of its rapid in vitro growth rate (4.7 mm per day on 2% malt extract agar at 20 °C). The pathogen was isolated in 2002 from an *U. minor* tree growing in Majorca island (Spain), maintained on 2% Oxoid malt extract agar (MEA) in Petri dishes at 4 °C in the dark, and was subcultured at 3-month intervals. The inoculum consisted of a spore suspension prepared in Tchernoff's liquid medium, adjusted with water to 10^6 spores mL^{-1} (Martín *et al*., 2008a).

Experiment 1

Plant material consisted of *P. pinaster* seeds (assay 1) and seedlings (assay 2), which originated from a single tree located in Cangas del Morrazo, north-west Spain. All seeds were surface sterilized in 30% H_2O_2 for 30 min, and then 10-times rinsed with sterile distilled water. In a first assay using seeds, these were divided into three groups and the following treatments were performed: (i) immersion of seeds during 10 minutes in an aqueous solution of MeJA; (ii) spraying of seeds with an aqueous solution of MeJA; and (iii) immersion during 10 minutes in water and subsequent spraying with water (untreated control). The aqueous solution of MeJA (Sigma-Aldrich, Germany) was adjusted to 5 mM and contained 0.1% (v/v) of Tween 20 (Panreac, Spain). One hundred fifty seeds per treatment were individually sown in 250 mL cylindrical pots containing sterilized soil (peat and sand, 1:1, v/v), and again subdivided into three groups; seeds were (i) inoculated by pipeting 5 mL of a spore suspension of *F. oxysporum* (10^7 spores mL^{-1}) onto the ground, (ii) inoculated by pipeting 5 mL of *F. oxysporum* (10^5 spores mL^{-1}) onto the ground, or (iii) irrigated with 5 mL of sterile water (control). Pots were daily watered (~2 mL) and kept at room temperature. The germination of each seed was daily assessed, and mortality of seedlings was recorded once a week during 5 weeks.

In a second assay using seedlings, about a thousand seeds were sown and maintained as previously described. Four weeks after sowing, half of the germinated seedlings were treated with aqueous solutions of MeJA, at concentrations of 0 (control), 0.1, 0.5, 1, 5 and 10 mM. All solutions contained 0.1% (v/v) Tween 20 and were applied by spraying the whole plant. Five weeks after sowing, the other half of the seedlings was treated in the same manner. Four and five weeks after sowing, seedlings were about 1-2 and 2-3 weeks old, respectively, coinciding in time with the susceptibility window of *P. pinaster* to *F. oxysporum* (Fig. 1). The susceptibility curve of *P. pinaster* to *F. oxysporum* was obtained previously, with seedlings (n = 30) being inoculated during 6 weeks at 10^7 spores mL^{-1} (Fig. 1). For each of the MeJA concentrations, spraying was performed in separate rooms in order to avoid MeJA evaporation and a possible contamination of the control plants. One day after treatments, all seedlings were placed in the same room. Inoculations with *F. oxysporum* were performed by pipeting 5 mL of a spore suspension onto the stems (Alves-Santos *et al.*, 2007) at two spore concentrations (10^5 and 10^7 spores mL^{-1}) and at two inoculation dates (1 and 7 days after treatments). In consequence, the assay consisted of a complete factorial design including 6 MeJA solutions x 2 plant

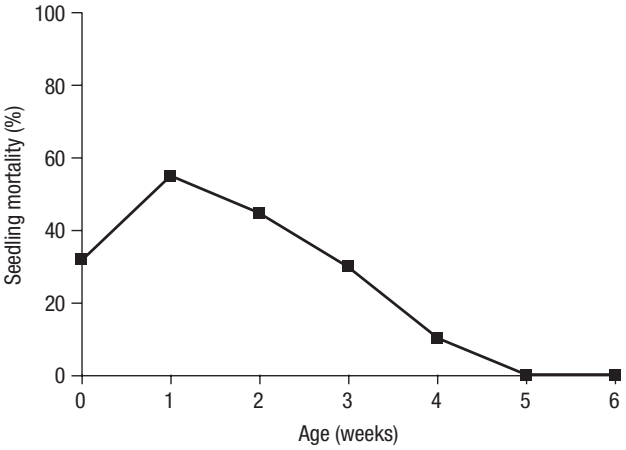

Figure 1. Susceptibility curve of *Pinus pinaster* seedlings to *Fusarium oxysporum* depending on plant age. Each value represents plant mortality 5 weeks post inoculation, if inoculations (10^7 spores mL^{-1}) were perfomed when the seedlings had the indicated age in weeks (n = 30 seedlings).

ages x 2 *F. oxysporum* spore concentrations x 2 inoculation dates, i.e. 48 treatments. Each treatment included 20 seedlings as replicates. Plant death and growth height was weekly recorded during 8 weeks. After this period, fungal re-isolations were carried out onto Komada medium to confirm the presence of the pathogen. Samples from the non-inoculated seedlings were cut from the centre of the stem using a manual microtome and immediately photographed. Transverse sections were approximately 20 µm in thickness, and in each section the number of resin ducts was counted. The resin canal system was characterised through the *resin duct density* (# mm^{-2}), i.e. resin ducts per unit area, and the *relative duct area* (%), obtained by dividing the area occupied by the ducts in the section by the total area of the section (Moreira *et al*., 2008). The *root length* (cm) and the *root surface* (cm^2) of non-inoculated seedlings were obtained using WinRhizo Pro v.2007d (Régent Instruments Inc., Quebec, Canada) software (Solla *et al.*, 2011). Plant tissues were separately placed inside paper bags, oven-dried at 65 °C for 48 hours and weighed.

Experiment 2

Plant material consisted of *P. pinaster* seedlings obtained from the same tree used in experiment 1. Seeds were surface sterilized and individually sown as previously described. When seedlings were about 6 months old (25th of April), the pots were divided into four groups, and the following treatments were applied: (i) spraying of seedlings with water and, 1 month later, inoculation with *F. circinatum*; (ii) spraying with an aqueous solution of MeJA (25 mM) and, 1 month later, inoculation with *F. circinatum*; (iii) spraying with MeJA (25 mM); and (iv) spraying water (control treatment). The MeJA dose was selected according to Moreira *et al*. (2009). Each treatment consisted of 45 plants distributed among two blocks. The aqueous solutions contained Tween 20 at 0.1% (v/v), and inoculations consisted of placing mycelium of *F. circinatum* into a wound made in the stem. Mycelium was scraped off the PDA agar surface with a sterile scalpel, and immediately used to make a 1-mm-long slit wound into the succulent stem tissue, 5 cm above the ground level (Correll *et al.*, 1991; Vivas *et al*., 2012). Plants from treatments iii and iv were wounded without placing any mycelium of the pathogen. Plant death was recorded once a week during eight weeks. Dead seedlings were removed weekly and fungal re-isolations were carried out onto FSM medium (Aegerter and Gordon, 2006). Eight weeks after inoculation, all remaining seedlings were harvested and cultured, and non-inoculated seedlings were assessed for height growth, the number of resin ducts, root parameters and dry weight as described before.

Experiment 3

The experiment included 42 ramets of the *U. minor* clone UPM171, used because of its high susceptibility to *O. novo-ulmi* (Martín *et al*., 2008a). The clone was propagated in 2004 by root cuttings at the Forest Breeding Centre in Puerta de Hierro (Madrid, Spain) and the ramets were grown in 30 L pots containing perlite and peat (1:1, v/v), and irrigated to field capacity when required. Trees were placed outside under a shading mesh providing 25% of full sunlight throughout the experiment. When the first treatments were applied, the trees were 4 years old and 1.1-2.6 m in height. On 17 April 2008, ramets were divided into three groups, and the main trunk of the 14 trees per group were then sprayed with an aqueous solution of MeJA at concentrations of 0 (control), 50, and 100 mM, respectively. All solutions contained 0.1% (v/v) Tween 20. Two months later, seven trees per group were inoculated with *O. novo-ulmi* into the sap stream through a blade wound made at the base of the trunk and seven trees per group were inoculated with water. Dieback symptoms shown

Table 1. Mortality of *Pinus pinaster* seedlings (%) being their seeds treated with 5 mM methyl jasmonate (MeJA) through immersion or spray, or with water (control) and subsequently inoculated with spore suspensions of *Fusarium oxysporum* or water. Different letters indicate significant differences of mortality values within lines (abc) and within columns (xy) ($P < 0.05$)

		Conditioning treatments		
		MeJA immersion	**MeJA spray**	**Water (control)**
Challenging inoculations	10^7 spores mL^{-1}	93 a x	69 b x	32 c x
	10^5 spores mL^{-1}	70 a xy	50 ab x	39 b x
	Water (control)	55 a y	33 ab x	0 b y

by the trees were evaluated at 120 days and at one year after inoculations. Bud break of trees was studied from March to May 2009 following Martín *et al.* (2008b). Bud break date was defined as the day when half of the buds had their scales open. Plant height was measured on dormant trees before the treatments and at the end of the 2008 and 2009 growing seasons, thus obtaining the apical growth of the trees.

Statistical analysis

Data were analyzed using Statistica v7.0 (Stat Software Inc., Tulsa, OK, USA). To compare *germination*, *incidence* and *mortality* of pines (dependent binomial variables) among *MeJA treatments* and *pathogen inoculations* (factors), a Generalized Logit Model (GLZ) was used. In the second assay of the first experiment, mortality of pines was analyzed by two steps; first among dates of MeJA treatments and dates of inoculation, and then among MeJA concentrations and *F. oxysporum* spore suspensions. The *time to germination* was used as a covariate (continuous predictor). To compare *growth height* and *morphological parameters* of pines and *dieback* of elms (dependent continuous variables) among *MeJA treatments* and *pathogen inoculations* (factors), a General Linear Model was used. Individual means were separated by Fisher´s least significant difference (LSD) test ($P = 0.05$).

Results

Experiment 1

Germination rates of *P. pinaster* seeds immersed or sprayed with MeJA (first assay) were significantly increased (~10%) if compared to those of controls. Inoculations with *F. oxysporum* significantly reduced, in about 20%, the germination rates of untreated seeds for both spore concentrations tested ($P < 0.01$). Germination rates of inoculated seeds immersed in MeJA, inoculated seeds sprayed in MeJA and inoculated untreated seeds were 64, 51 and 41%, respectively, the first and third rates differing significantly ($P < 0.05$). Five weeks post inoculation, conditioning treatments with MeJA did not protect seeds against challenging inoculations with *F. oxysporum*, and mortality of seedlings was significantly higher if seeds were immersed in MeJA than if seeds were not treated ($P < 0.05$) (Table 1). Some other non-inoculated seeds especially those immersed in MeJA resulted in plant chlorosis, tip necrosis, closure of cotyledons and plant mortality (Fig. 2a).

In the second assay, final mortality of *P. pinaster* seedling sprayed with MeJA significantly varied depending on the date in which treatments were performed (Table 2). If MeJA treatments were performed 4 or 5 weeks after sowing, overall plant mortalities were 59 and 45% respectively. The date at which *F. oxysporum* was inoculated did not cause significantly different mortality rates of plants (50 or 57% if plants were inoculated 1 or 7 days after MeJA treatments; Table 2). Final mortality of seedlings depended of the concentration of MeJA and the dose of *F. oxysporum* used, but

Table 2. Test of all effects to compare mortalities of 10-weeks-old *Pinus pinaster* seedlings among two dates of methyl jasmonate treatments (4 and 5 weeks after sowing) and two dates of *Fusarium oxysporum* inoculations (1 and 7 days after treatments). A Generalized Logit Model was performed, and time to germination was used as a continuous predictor

Effect	**d.f.**	**Wald statistic**	***P*-value**
Date of methyl jasmonate treatment (DMeJA)	1	4.81	0.02
Date of *F. oxysporum* inoculation (DFO)	1	1.10	0.29
DMeJA x DFO	1	0.12	0.72
Time to germination	1	1.59	0.20

(a)

(b)

(c)

Figure 2. Side-effects caused by methyl jasmonate (MeJA) in a 5-weeks-old *Pinus pinaster* seedling (a, plant on the left), after immersing its seed in MeJA 5 mM. Note the difference with the non treated 5-weeks-old seedling, on the right. Apical resinosis and reduction of height growth of a 8-month-old *P. pinaster* seedling (b, plant on the left), 8 weeks after spraying the plant with MeJA 25 mM. Note the difference with the non treated seedling, on the right. Leaf necrosis in a 4-year-old *Ulmus minor*, 5 weeks after spraying the tree with MeJA 100 mM (c).

the conditioning treatments did not significantly protect the seedlings against the pathogen (Fig. 3). Moreover, seedlings treated with MeJA at 5 and 10 mM and subsequently inoculated with *F. oxysporum* showed higher mortality values than seedlings treated with the aqueous solution and subsequently inoculated with *F. oxysporum* (Fig. 3). No mortality was observed in the non-inoculated MeJA treated seedlings, but treatments at doses above 1 mM showed clear phytotoxicity, similar as the one described for the first assay. The percentage of infected seedlings (incidence, data not shown) showed the same trend as mortality, and re-isolation of the pathogen was possible in every inoculated seedlings. The number of constitutive resin ducts per transversal section of plant stems ranged from 4 to 6, and similar values of resin duct densities and relative duct areas among treated and untreated seedlings were obtained (~13 ducts mm^{-2} and ~2.5%, respectively). Any of the above or belowground plant parameters were affected by the MeJA treatments ($P > 0.05$; data not shown).

Experiment 2

Eight weeks post inoculation, the *P. pinaster* seedlings inoculated with *F. circinatum* showed higher mortality rates than the non-inoculated control seedlings (58 *vs* 0%; $P < 0.01$). Mortality of seedlings treated with MeJA was 0%, and mortality of seedlings treated with MeJA and subsequently inoculated with *F. circinatum* was 60%, thus the challenging treatment did not show any positive effect against the pathogen tested. By the end of the experiment, all inoculated seedlings that had survived showed leaf symptoms (incidence of 100%), and re-isolation of the pathogen was always possible. The exogenous application of MeJA in non-inoculated seedlings produced apical resinosis (Fig. 2b) and significantly reduced above and belowground plant growth ($P < 0.05$; Table 3). Internally, exogenous application of MeJA significantly increased the average number of resin ducts per transverse section ($P < 0.01$) and marginally increased the relative conductive area of resin ducts ($P = 0.056$) in relation to the control plants (Table 3).

Experiment 3

At day 120 post inoculation, the plants inoculated with *O. novo-ulmi* showed higher dieback symptoms

Table 3. Allometric parameters of 8-months-old *Pinus pinaster* seedlings treated with 25 mM methyl jasmonate (MeJA) in comparison with untreated control seedlings. Within lines, different letters indicate significant differences among values ($P < 0.05$)

Treatment	MeJA (25 mM)		Water (control)	
Total height growth (cm)	0.7	a	1.5	b
Root length (cm)	252.2	a	365.3	b
Root surface (cm^2)	56.2	a	77.6	b
Total dry weight (g)	1.0	a	1.4	b
Resin duct density (# mm^{-2})	15.1	a	14.3	b
Relative duct area (%)	0.19	a	0.18	a

(20.3 ± 6.7 %; mean ± SE) than water-inoculated plants (3.4 ± 2.3 %) ($P < 0.05$). Again, the MeJA treatments did not show any positive effect against *O. novo-ulmi* inoculation with respect to the control plants (Table 4). Furthermore, the treatment 100 mM of MeJA was slightly toxic to the trees, causing leaf necrosis and some wilting (Fig. 2c). One year after inoculation, the trees inoculated with *O. novo-ulmi* and treated with MeJA 0 mM showed a reduction of dieback symptoms with respect to the previous year (Table 4). On the contrary, the trees inoculated with *O. novo-ulmi* and treated with MeJA 50 and 100 mM notably increased their dieback symptoms ($P = 0.03$; Table 4). No significant effects of the MeJA treatments on the time to bud burst and tree growth was observed ($P > 0.15$).

Table 4. Dieback symptoms (% of the total crown) shown by *Ulmus minor* trees treated with methyl jasmonate (MeJA) at different concentrations and subsequently inoculated with *Ophiostoma novo-ulmi*. Within lines, different letters indicate significant differences among values ($P < 0.05$)

Days after inoculation	MeJA (100 mM)		MeJA (50 mM)		Water (control)	
120	17.5	a	16.3	a	20.3	a
365	61.4	a	40.3	a	8.7	b

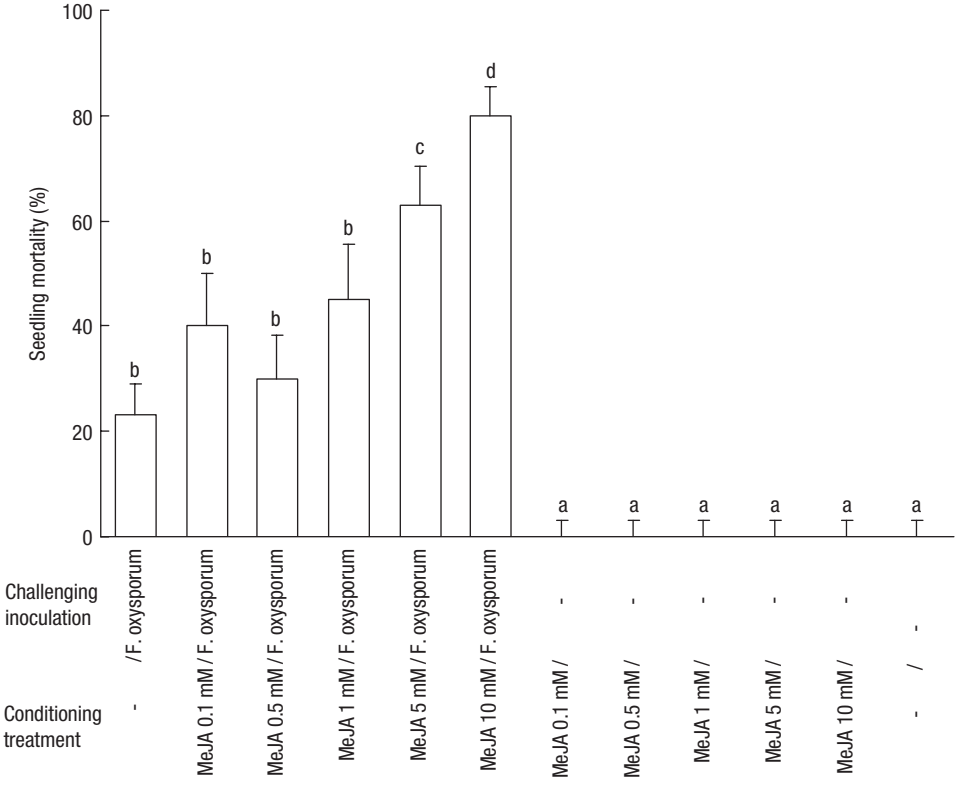

Figure 3. Mortality of *Pinus pinaster* seedlings sprayed with different concentrations of methyl jasmonate (MeJA) and subsequently inoculated with a spore suspension of *Fusarium oxysporum* (10^7 spores mL^{-1}). Vertical bars indicate standard errors and different letters show significant differences at $P < 0.001$.

Discussion

Exogenous applications of MeJA did not protect *P. pinaster* seed and seedlings against *F. oxysporum* probably because plants were too young for the physiological mechanisms responsible for resistance to be operative. Plant resistance can be described on several mechanistic levels, and ontogenetic disease resistance, also known as age-related resistance (Panter and Jones, 2002; Develey-Rivière and Galiana, 2007), refers to resistance to a pathogen that changes with the developmental stage of the host, with resistance usually increasing with age. Differences in resistance to the same pathogen among young seedlings and mature trees have been reported previously (Solla *et al*., 2005; Aegerter and Gordon, 2006). From an ecological point of view, resistance to pathogens is a strong selective force and a competitive ability oj young trees; as a result, there could be a trade-off between growth and expression of quantitative defences (Bonello *et al*., 2006; Walters and Heil, 2007). Such interaction may be especially evident in young seedlings where growth during an early establishment phase is likely to be an important component of competitive ability. Resin production is the most familiar and visible component of pine defense (Davis *et al*., 2002; Kim *et al*., 2010). It was recently observed that the relative resin production of *P. nigra* was much lower in 1- than in 2-year-old seedlings, suggesting that the younger trees allocated a lower proportion of the carbon budget to resin synthesis (Wainhouse *et al*., 2009). Traumatic resin ducts, easily induced in conifers in response to MeJA (Martin *et al.*, 2002; Hudgins *et al*., 2004; Huber *et al*., 2005) have never been reported in less than 1-year-old seedlings, in accordance to our observations.

In the first assay, although germination rates of seeds were higher if previously immersed in MeJA, the conditioning treatments did not finally protect seeds against *F. oxysporum*. Elicitor-induced changes in plant resistance can occur within hours or days after treatments, but their lasting effect could be also short. In the second assay, phytotoxicity was observed in seedlings treated at doses above 1 mM MeJA, and no protection occurred at lower doses. Phytotoxicity and plant mortality after exogenous application of 100 mM MeJA has been previously reported for older *P. sylvestris* and *P. pinaster* seedlings (Heijari *et al.*, 2005; Moreira *et al*., 2009). Our findings do not give support to the idea of adding MeJA to irrigation water in nurseries as a method for protection against pests and pathogens, as suggested by Huber *et al*. (2005).

We postulate four non-exclusive hypotheses to explain why exogenous applications of MeJA did not protect *P. pinaster* seedlings against *F. circinatum*. First and as mentioned before, the protective and lasting effect of MeJA on pines would depend on the host's age. Positive results using MeJA were only reported using seedlings above one year old (Heijari *et al*., 2004; Huber *et al*., 2005; Moreira *et al*., 2009) or mature trees (Zeneli *et al*., 2006). Second, the dose and the timing were probably not appropriate to protect the seedlings accordingly. SIR is contingent on the type of treatment and dose to which a tree is subjected (Bonello *et al*., 2006). In other words, the expression of SIR can be sustained or transiently expressed depending on the damage level resulting from the induction event. Moreover, changes involving cell division and differentiation such as traumatic resin duct formation are slow processes (Bonello *et al.*, 2006) and probably need more than one month to occur. As a third hypothesis, the inoculation method was probably too severe to allow the treatment to be effective. Initial stages of fungal infection usually include the deposition and attachment of spores to aerial parts of the host plants, spore germination and subsequent formation of germ tubes that direct their growth to natural openings or wounds of plants. The inoculation method used here created an optimal infection court, allowing direct infection of the plant through a wound practiced deep into the xylem. At the time of inoculation, the seedlings were rather succulent, and the inoculum density of the pathogen high, while in nature the pitch canker disease starts at low pathogen inoculum density. Finally, we postulate that the high virulence nature of the pathogen used allowed the resistance threshold of the plants to be easily surpassed. In the same way that constitutive defences are not always enough to protect trees against attack by microbes or herbivores, in many circumstances inducible defences are not enough too. At the earliest stages of pathogen infection, SIR responses are predicted to rapidly and systemically increase concentrations of compounds involved in defence. However, if the pathogen is able to grow despite the deployment of localized defensive responses, the infection will progress, and the plant will become increasingly diseased. Elicitor compounds affect the synthesis of chemical compounds in plants, but this will result in constraints in carbon allocation and ultimately with reduce plant

growth or even stop the shoot elongation after the elicitor treatment (Heijari *et al.*, 2005), as observed here. MeJA increased the resin duct density of our seedlings, but despite the general effectiveness of traumatic ducts to contain and reduce damages caused by insects and pathogens (Phillips and Croteau, 1999), *F. circinatum* is able to tolerate the resin and even stimulate its production on pine trees (Davis *et al.*, 2002; Kim *et al.*, 2010). Thus, even if an increase of resin duct density was observed, *F. circinatum* would be able to surpass this inducible defense strategy. Among the families of *P. virginiana* examined in Barrows-Broaddus and Dwinell (1984), the high-to-moderately susceptible family had the largest ducts, and the least susceptible family had the smallest. The pitch canker fungus appears to frequently use the resin ducts as portals for vertical spread of the pathogen beyond the inoculation point (Barrows-Broaddus and Dwinell, 1984), thus large and numerous ducts seems to be a disadvantage to the host.

In addition to inherent genetic resistance to *F. circinatum*, systemic induced resistance has been reported to occur in *P. radiata* in California (Gordon *et al.*, 2010). However, the year-round susceptibility of pines to *F. circinatum* (Kuhlman *et al.*, 1982), together with the erratic results obtained here and the rapid spread of the pathogen within the host (Barrows-Broaddus and Dwinell, 1984) suggest that the use of MeJA for its control is not practical.

Concerning the biotroph *O. novo-ulmi*, a number of investigations explored the possibility of inducing resistance in elm trees threatened by Dutch elm disease, with variable results in terms of inducing agents (bacteria and fungi), range of effects, and applicability to disease management (Solla and Gil, 2003; Scheffer *et al.*, 2008). Normally, any stress factor causing a reduction of the normal growth of elms, a delayed budbreak, or an alteration of earlywood or latewood formation will generate resistance (Brener and Beckman, 1968; Martín *et al.*, 2008b). Delayed budbreak or decreases of plant growth resulting from differential allocations of carbon to defence rather than growth were not observed in the MeJA-treated elms. Based on the lack of phenological changes and on the increased mortality observed in our treated plants, there is no evidence that MeJA had cause SIR on elms. The expression of induced plant defenses is mediated by complex signaling networks in which the plant jasmonates (MeJA) and salicylates (SA) play key roles. In general, JA-mediated signaling pathways are implicated in the regulation of defences against herbivores and necrotroph pathogens, while the SA pathway is associated with defences against biotrophic pathogens (Glazebrook, 2005). There are many exceptions to this basic framework, but signaling pathways controlled by jasmonates are required for host resistance to some pathogens, but not to all of them (Glazebrook, 2005; Kusumoto *et al.*, 2007). Thus, it could be expected that the role of the MeJA molecule would greatly vary among different pathosystems, e.g. foliar application of MeJA failed to enhance host resistance against *Phytophthora cinnamomi* in several *Eucalyptus* spp. (McComb *et al.*, 2008).

Conclusion

There is a real need for careful, long-term experiments on the use of induced resistance with trees to provide robust information, not just on understanding the systemic mechanisms of resistance, but also on effectiveness of disease control. While extensive research has examined plant and conifer SIR responses to attack by herbivores and pathogens, equivalent information for angiospermous tree species is lacking. This is the first work reporting the effect of MeJA on *U. minor* and *P. pinaster* seeds, and the first approach to test MeJA against three asomicetes previously not used for this purpose. Based in our results the use of MeJA to prevent damping-off and pitch canker in nurseries of *P. pinaster* or Dutch elm disease on elm trees should be discarded.

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