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Impact of fungal and plant metabolites application on early development stages of pea powdery mildew

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Abstract

BACKGROUND: Pea powdery mildew incited by *Erysiphe pisi* represents a major constraint for pea crop worldwide. Crop protection is largely based on chemical control, although recently a renewed interest in the discovery of natural products as alternative to synthetic fungicides application has emerged. Thus, twelve bioactive plant and fungal metabolites belonging to different class of natural compounds were evaluated, together with a commercial fungicide, at different concentrations on detached pea leaves for their potential to inhibit spore germination and subsequent stages of fungal growth. Then, the most effective metabolites were also tested at different concentrations “*in planta*” under controlled conditions, in order to evaluate the level of control achieved by treatments before, concurrently and after pathogen inoculation. Pathogen development was macroscopically scored on whole plants as percentage of disease severity and area under the disease progress curve (AUDPC).

RESULTS: Cavoxin, inuloxin C and sphaeropsidin A strongly inhibited *E. pisi* germination and haustoria formation and reduced colony size. This effect was dose dependent. These results were further confirmed in whole plants by spraying the metabolites on plant leaves for preventive or curative control, which reduced fungal developmental of *E. pisi* at levels comparable with those obtained by application of the fungicide.

CONCLUSIONS: Cavoxin, inuloxin C and sphaeropsidin A have potential as alternatives to synthetic fungicides for the control of crop pathogens of economic importance such as powdery mildew.

KEYWORDS: *Erysiphe pisi*; *Pisum sativum*; biocontrol; fungal and plant metabolites

RUNNING TITLE: Plant and fungal metabolites for pea powdery mildew biocontrol

1 INTRODUCTION

Pea (*Pisum sativum* L.) is one of the important grain legume crops worldwide mainly used for consumption as green vegetables and dry seeds.¹ Pea is cultivated in 7.3 million hectares with a total production of 600 metric tons per year globally.² Productivity of pea is affected by many diseases and insect-pests. Among the various diseases affecting pea, powdery mildew, caused by *Erysiphe pisi*, is of great significance.³ The biological cycle of *E. pisi* includes germination of conidia (asexual spores) or ascospores (sexual spores) on host epidermis and direct penetration of epidermal cells by invagination of haustoria into epidermal cells for feeding, followed by subsequent epiphytic colony growth.⁴ *Erysiphe* species are obligate parasites feeding on alive plant cells, that cannot be cultured on artificial nutrient medias. Therefore bioassays for these pathogens are very complicated.

Owing to their economic importance, a large number of methods to control powdery mildew have been proposed, including cultural practices, the use of resistant varieties and fungicide application. Despite the substantial effort to generate resistant pea varieties, the use of synthetic chemical fungicides is the mainstay method for *E. pisi* control.⁵ However, their widespread application has caused many environmental concerns due to low specificity and slow biodegradability. These include pollution of soil and groundwater, negative impact on flora and fauna and risks to human and animal health. Furthermore, the high application costs and the ability of the pathogen to evolve resistance to chemical fungicides have prompted efforts to discover natural products as templates for development of fungicidal biochemicals with new molecular structures and mode of actions. Many plant pathogens, especially necrotrophic or hemibiotrophic fungi, produce novel antimicrobial compounds that can be a source of such useful metabolites.^{6,7,8} Similarly, active metabolites isolated from weeds and cultivated plants have a number of potentially useful biological activities.^{9,10} As spore germination, haustoria formation and subsequent colony growth are key phases for the initial development of biotrophic plant pathogens such as powdery mildews, an approach for their management could be the use of natural plant or fungal metabolites as inhibitors in these early stages of infection.

In this manuscript the potential of ten fungal and two plant toxins was tested as natural fungicides assaying their inhibitory effects on early stages of development of *E. pisi* in detached leaf bioassays. The three most effective metabolites were also tested in whole plants at several application times to determine their protective and/or curative effects against powdery mildew.

2 MATERIAL AND METHODS

2.1 Plant and fungal bioactive metabolites

The phytotoxins used for the study (Fig. 1A; 1B, Table 1) belong to different classes of natural compounds, as follow: the sesquiterpenes inuloxins A and inuloxin C, and α -costic acid (**1-3**, Fig. 1A) were purified from the organic extract of *Inula viscosa* (L.) Ait.¹⁰ The diterpenes sphaeropsidin A and sphaeropsidin C (**4** and **5**, Fig. 1B) and cyclopaldic acid (**6**) were purified from the culture filtrates of *Diplodia cupressi* A.J.L. Phillips & A. Alves^{11,12} and *Seiridium cupressi* (Guba) Boesewinkel,¹³ respectively. Cavoxin (**7**) was isolated from the liquid culture of *Phoma cava* Schulzer,¹⁴ while epi-epoformin, (**8**) was purified from the organic extract of *Diplodia quercivora* Linaldeddu & A.J.L. Phillips.¹⁵ Cytochalasins A (**9**) and B (**10**) were isolated from the solid culture of *Pyrenophora semeniperda* (Brittleb. & D.B. Adam) Shoemaker,¹⁶ respectively (Fig. 1B, Table 1). Finally, gliotoxin (**11**) was isolated from the solid culture of *Neosartorya pseudofischeri* S. W. Peterson¹⁷ and 6-hydroxymellein (**12**) was isolated from the liquid culture of *Phoma chenopodiicola* Gruyter, Noordel. & Boerema (Fig. 1B, Table 1).¹⁸ Metabolites **1-3** were from plant origin, while metabolites **4 -12** were from fungal origin (Table 1).

2.2 Plant growth and pathogen multiplication

P. sativum subsp. *sativum* susceptible cv. Messire was used both for pathogen multiplication and assays. Plants were raised from seeds in pots (6 x 6 x 10 cm) filled with a potting mixture (sand/perlite, 1:1 vol/vol), then were grown under standard conditions of in growth chamber at $20 \pm 2^\circ\text{C}$ and 65% relative humidity under a photoperiod at 14 h light/10 h dark with light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density

supplied by high-output white fluorescent tubes. As its condition of biotrophic fungi requires, the pea powdery mildew (*E. pisi*) isolate CO-01 derived from mildew population collected at Córdoba (southern Spain) was maintained on Messire seedlings under conditions described above. Leaves with heavy sporulation were shaken one day before inoculation of selected accessions for the time course study to ensure a supply of young conidia (Fig. 2).

2.3 Detached leaves assays

For histological studies, detached leaves were used so that both inoculation density and incubation conditions were controlled precisely. Susceptible plants of cv. Messire were grown under controlled conditions mentioned above until the fifth leaf stage and then, several fourth-formed leaves were excised and placed, adaxial side up, on 4% technical agar in Petri dishes as described by Rubiales and Carver.¹⁹ Cut leaves were arranged in a randomized design with four replicates per treatment, each replicate having four leaves. Metabolites listed in Table 1 were tested at concentration of 10^{-2} , 10^{-3} , 10^{-4} , $0.5 \cdot 10^{-4}$ and $0.25 \cdot 10^{-4}$ M. Compounds were dissolved in MeOH (5%) and then brought up to the assay concentration with distilled water. The test solutions (50 μ L) were applied on the adaxial leaf side. Droplets (50 μ L) of distilled water and MeOH (5%) were applied as negative controls. Droplets (50 μ L) of commercial anti-powdery mildew fungicide (Nimrod Quattro® (ADAMA): bupirimate 25%, is the fungicide 5-butyl-2-ethylamino-6-methylpyrimidin-4-yl dimethylsulphamate at 0.8 M) were applied as positive control (0.2 g L^{-1}). The solvent was evaporated in a laminar flow cabinet, and then the Petri dishes were inoculated in a spore settling tower with 50 fresh conidiospores mm^{-2} (Fig. 2). Twenty-four hours after inoculation (h.a.i.), four leaves per plant, treatment and replicate were used to assess percentage of fungal spore germination, number of hyphae per colony and number of fungal tips per colony. Leaves were fixed by placing the adaxial surface upon filter paper moistened with 1:3 (v/v) glacial acetic acid: absolute ethanol. When the chlorophyll was eliminated, the leaves were transferred to filter paper moistened with tap water (minimum 2 h) to soften the tissue and then to filter paper moistened with lactoglycerol (1:1:1 v/v, lactic acid: glycerol: water). Fungal development was examined by light microscopy after staining the fungus with 0.25% Trypan blue, as

described by Barilli et al.²⁰ On every leaf fixed, germination frequency was assessed by scoring 100 conidiospores for the presence of a germ tube. The size of the colonies was also measured for another 35 randomly selected colonies in terms of number of hyphal tips and number of haustoria produced per colony. In addition, presence of necrosis indicating phytotoxic effect of the metabolites tested was also assessed macroscopically.

The effective concentration for 50% inhibition (EC₅₀) was calculated according to Alexander et al.²¹ using the inhibition rate of the fungal isolate in presence of each compounds. Spore germination for each of the replicate was converted to percent inhibition compared with the untreated control by: $100 - ([\text{percent germination of fungicide-compound}] / [\text{mean percent germination of control}])$. The concentration of compounds that effectively inhibited spore germination by 50% of the untreated control (EC₅₀) was determined by linear interpolation.

2.4 Whole plant assays

To simulate the effect of the treatments with metabolites on the pathogen under real conditions, several experiments were carried out on whole young plants of the powdery mildew-susceptible pea cv. Messire under the controlled conditions mentioned in Section 2.2. Plants were arranged in a randomized design with five replicates per treatment, each replicate having three plants. Pea plants were inoculated at the third leaf stage by dusting the whole plants with fresh collected conidiospores of *E. pisi*. Fungal inoculation has already been described in Section 2.3. Metabolites cavoxin, inuloxin C and sphaeropsidin A were applied in whole plants at two concentrations found to be effective in Section 2.3, with no plant phytotoxicity. In order to study the protective effect of the metabolites, a set of plants was treated 5 days before fungal inoculation (DBI), and another set at the same time as fungal inoculation (0 DBI). In addition, to study the curative effect of the metabolites, a third set of plants was treated 2 days after fungal inoculation (DAI). Metabolites **2**, **4** and **7** were suspended in distilled water with MeOH (5%) and then sprayed over the canopy of the seedlings until run-off. For the negative controls, plants were untreated (Control), sprayed with distilled water (Water) and sprayed with distilled water with MeOH (5%). The commercial powdery mildew

fungicide (Bupirimato 25%, ADAMA) was applied as positive control at its recommended concentration of 0.2 g L⁻¹. When the fungus macroscopically appeared, disease severity was assessed daily as % of the whole plant covered by fungal mycelium, until its growth becomes stabilized. These data were used to calculate the area under the disease progress curve (AUDPC), using the following formula:

$$AUDPC = \sum_{i=1}^k \frac{1}{2} [(S_i + S_{i+1}) (t_{i+1} - t_i)]$$

where S_i is the fungal severity at assessment date i , t_i is the number of days after the first observation on assessment date i and k is the number of successive observations. Finally, the associated necrosis as plant phytotoxicity was assessed.

2.5 Statistics

For statistical analysis, average data were subjected to analysis of variance (ANOVA) to test the effect of each phytotoxins and treatment, after which residual plots were inspected to confirm data conformity to normality. Significant of differences between means was determined by calculating honestly-significant-difference (HSD) to test the differences between treatments for each phytotoxin evaluated. All statistical analyses were performed by R software.²²

3. RESULTS AND DISCUSSION

3.1 Chemical nature of fungal and plant metabolites tested

The phytotoxins used for the study belong to different classes of natural compounds as inuloxins A and C and α -costic acid (**1-3**, Fig. 1A) which are a three sesquiterpenoid belonging to germacrenolide, eudesmane and custunolide subgroup, respectively.¹⁰ Sphaeropsidins A and C (**4** and **5**, Fig. 1A) are a tetra- and tri-cyclic pimarane diterpenes,^{11,12} cyclopaldic acid and *epi*-epoformin (**6** and **8**, Fig. 1B), which are a

pentasubstituted benzofuranone¹³ and a cyclohexen oxide,¹⁵ respectively. Cytochalasins A and B (**9** and **10**, Fig. 1B) are two 24-oxa[14]-cytochalasans,¹⁶ and cavoxin and 6-hydroxymellein (**7** and **12**, Fig. 1B) are a chalcone¹⁴ and a tetra- and a tri-substituted benzoic acid and isocoumarine,¹⁸ respectively. Finally, gliotoxin (**11**, Fig. 1B) is a representative member of the epipolythiodioxopiperazine (ETP) class, which is well known for its potent cytotoxicity toward cancer cell lines.²³

Many of these metabolites are isolated as phytotoxins produced by fungi pathogenic to forest plants (Italian cypress and cork oak) as spaheropsidins A and C, cyclopaldic acid and *epi*-epoformin (**4-6** and **8**) but also showing other interesting biological activity. In fact, metabolite **4** showed bactericide activity as well as a significant anticancer activity associated with a new mode of action, while **5** and **8** showed insecticide and antirust activities. Cytochalasins A and B and 6-hydroxymellein (**9**, **10** and **12**) were isolated from fungi proposed as mycohercides for the control of noxious weeds such as cheatgrass (*Bromus tectorum*) and fat hen infesting very important agronomic crops,⁸ while cavoxin was reported as a phytotoxin of a fungus pathogenic for chestnut,¹⁴ and showed a strong antifungal activity against the causal agents of mold (*Aspergillus niger* and *Penicillium roqueforti*) in preserved food.²⁵ In addition, it was also formulated in a biofilm (PBS: polybutylene succinate) to improve food packaging.²⁵ Inuloxins A and C and α -costic acid (**1-3**) were isolated from an alleopathic plant showed phytotoxic activity against some parasitic weeds such as broomrapes (*Orobanche* and *Pelipanche*, spp.) and dodder (*Cuscuta campestris*).¹⁰ Finally gliotoxin (**11**) has antifungal activity.²⁶

3.2 Inhibition of powdery mildew spore germination

On the pea-powdery mildew pathosystem studied on detached leaves, the percentage of fungal spore germination was higher than 80% in all the negative controls (control untreated, water and MeOH 5%), with no significant differences between them (Table 2). This suggests that either water or methanol at 5% (necessary to dissolve the metabolites above mentioned in water) did not modify normal spore development and did not exert any fungitoxic activity on the pathogen tested. On the contrary, spore germination lower than 15% was observed in inoculated leaves treated with the fungicide as positive control, as expected. In

fact, this chemical is a well-known powdery mildew fungicide that prevents powdery mildew sporulation as well as suppresses new subsequent fungal infections.²⁷ Most of the metabolites tested significantly reduced powdery mildew spore germination (Table 2), with large differences between effects of the different compounds. Metabolites **1**, **2** and **3** were effective in reducing spore germination, with an effect that was dose dependent (Table 2). Both metabolites **1** and **2** are well known for their seed germination inhibition of parasitic weeds as *Orobanche crenata* Forssk. and *Cuscuta campestris* Yunck,¹⁰ while metabolite **3** showed antimicrobial activity against *Penicillium roqueforti* Thom. and *Aspergillus niger* P.E.L. van Tieghem.²⁸ On *E. pisi*, metabolite **2** was particularly effective when applied at concentrations higher than 10^{-3} M, with a reduction on spore germination comparable to the fungicide. Metabolites **3**, **4** and **6** showed lower antifungal activity, although still significant. Metabolite **5** was less effective, with only some slight but significant inhibition on germination at 10^{-2} M. Metabolite **7** reduced spore germination at all concentrations, reaching reductions similar to the fungicide when applied at 10^{-2} M (Table 2), confirming previous results on plant pathogenic fungi as rusts,²⁹ *Colletotrichum* and *Phomopsis*.³⁰ Effects on *E. pisi* spore germination by metabolites **8** to **12** were moderate to low, not comparable to the fungicide at any concentration.

The effective concentration for 50% inhibition (EC₅₀) was calculated to compare the fungal sensitivity to the twelve natural compounds and the chemical on the basis of the spore germination inhibition (Table 3). The chemical fungicide is a 5-butyl-2-ethylamino-6-methylpyrimidin-4-yl dimethylsulphamate belonging to the DMI (De-Methylation Inhibitors) group of ergosterol-biosynthesis-inhibiting (EBI) fungicides, which showed systemic curative and protectant actions. Because DMI fungicides are powerful inhibitors that operates during the early stages of powdery mildew development (Fungicide Resistance Action Committee [FRAC] group 8), an assay based on spore germination is likely to be a better method for determining sensitivity of powdery mildew to natural compounds compared with the synthetic fungicide. The EC₅₀ value for the fungicide was significantly lower (range 0.092-1.458, average 1.03 gL⁻¹) than those reported for natural metabolites (Table 3). Regarding the natural metabolites tested, differences in sensitivity were found for *E. pisi* to metabolites **2** (range 1.022 - 2.532, mean EC₅₀ = 1.86), **4** (range 0.985 - 2.751, mean EC₅₀ = 1.78) and **7** (range 1.678 – 2.548, mean EC₅₀ = 1.98), which were comparable with the fungicide (Table 2).

3.3 Inhibition of powdery mildew colony development

As observed for spore germination, high number of hyphal tips per colony (values up to 3) and haustoria per colony (up to 2.5) were achieved by the fungus in the untreated negative controls (water and 5% MeOH), with no significant differences between them (Supplementary Table 1). On the contrary, no or strongly reduced colony development was observed in inoculated leaves treated with the positive control, while several compounds strongly inhibited both number of hyphal tips and haustoria per colony (Supplementary Table 1). This is very significant from a fungal control point of view, as the metabolites enhanced a penetration resistance mechanism which mainly inhibits the fungal infection process after the fungus has formed secondary haustoria, resulting in a lower subsequent fungal expansion. In particular, metabolites **2**, **4** and **7** were the most effective for controlling further steps of *E. pisi* development, since values lower than 1.5 hyphal tips per colony were observed, even at the lowest concentration applied ($0.25 \cdot 10^{-4}$ M) (Supplementary Table 1). The same tendency was observed for haustoria formation, where values were lower than 1 for metabolite **2** when applied at concentration of 10^{-3} and 10^{-2} M, and for metabolites **4** and **7** at any concentration applied. These results were comparable with those obtained with the chemical at the same concentration. No phytotoxicity was observed 24 h after inoculation with any metabolite and at any concentrations tested. As a consequence, inuloxin C, sphaeropsidin A and cavoxin are promising compounds, as their general high efficacy reducing fungal development in the pea-*E. pisi* pathosystem included in the study under *in vitro* conditions. Nevertheless, larger time frames between metabolite application and fungal growth measurements need to be taken into account in further assays in order to evaluate their long-lasting efficacy. For these reasons, *in planta* assays have been performed under controlled conditions.

3.4 Studies performed “*in planta*”

Inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**) were also tested in whole-plant studies under controlled conditions. Metabolites were sprayed on pea susceptible plants at the two lowest concentration found to be

effective under *in vitro* conditions (10^{-3} and 10^{-2} M) at three different time points to fungal inoculation. Then, visible disease was macroscopically evaluated. Treatment with metabolites **2**, **4** and **7** provided a significant reduction in the percentage of final disease severity (DS%) (values < 45%) and AUDPC (values < 288) ($P < 0.001$) at any time of application compared with the negative controls (DS > 90%; AUDPC > 455) (Table 4), with no significant differences between compounds in each time of application considered. The detailed microscopic study performed *in vitro* showed that this protection provided by the metabolites acted both before and after fungal penetration to plant leaves. No symptoms of phytotoxicity were observed on plants treated with metabolites **2** or **4**, confirming previous results of microscopic assessment, while some necrotic spots were observed on leaves treated with metabolite **7** at 5 days before inoculation (DBI). This is not surprising, as some phytotoxic effects on pea plants have been observed in a study conducted against rust fungi.³¹ Authors observed brown lesions on leaves 48 h after their treatment with cavoxin. This is probably the reason because we did not microscopically find any symptom, as leaves for histological studies were removed earlier in our study (24 hours), when the metabolite did not still affect the plant tissues. The protective effect reached with all metabolites significantly improved when they were applied at the same time as fungal inoculation (0 DBI) compared when they were applied 5 DBI ($P < 0.01$), similarly to the effects of the commercial fungicide (Table 4, Fig. 4, 5, 6). Here we also found that significantly reduced symptoms compared with untreated controls were strongly maintained when all metabolites were applied 2 days after fungal inoculation (2 DAI) (curative effect) (Table 4, Fig. 4, 5, 6). In fact, fungal development measured as diary evaluation of disease severity (DS%), as well as the AUDPC, were significantly decreased in plants treated with natural compounds compared with the controls (Table 4, Fig. 4, 5 y 6), with a consequent delay in fungal growth and sporulation. In particular, results on plants treated with metabolites **4** and **7** were comparable with those provided by the fungicide (Table 4, Supplementary Fig. 1). A study on the effects at early and late stages (early aborted colonies and/or primarily mycelial growth impairment) of fungal infection should be considered for further study.

A fully compatible reaction not associated with necrosis was observed in all treatments (Supplementary Fig. 1). As a consequence, the observed resistance after treatment with metabolites was

never complete, as in the case of all experiments performed under controlled conditions, where high inoculum loads were used in situations favoring pathogen development. However, treatment at least at 10^{-3} M with any metabolite tested effectively reduced by 50% the disease severity, with this reduction being higher when applied at 0 DBI and 2 DAI. This reduction could be attributed to the toxic effect on the fungus, as all of them showed fungistatic activity against the conidiospores. Information on the mode of action is crucial for the successful development and adoption of any control strategy. Such information is critical to optimize application methods and for correct timing of field sprays.

Biotrophic fungi such as powdery mildew are regularly exposed to fungicides, especially when no genetic resistance is available.⁵ When pathogen control focuses on the deployment of cultivars carrying dominant resistance genes, fungicides contribute about equally to genetics in the control of the disease. As a result, resistance to chemicals evolved by fungal isolates has emerged as one of the most complicated issues threatening food security, leading to the application of general integrated disease management strategies such as dose limitation, mixtures and the search for alternative molecules.

In the present study, twelve fungal metabolites from different sources belonging to different classes of natural compounds were tested with general success for their fungitoxic activity against a disease of agronomic importance as pea powdery mildew. In *in vitro* studies, inuloxin C, sphaeropsidin A and cavoxin were promising compounds, all reducing the early developmental stages of *E. pisi*. At concentrations 10^{-3} M and higher the fungal inhibition was comparable with that of positive control. Furthermore, additional studies performed *in planta* also showed that the compounds, when applied at the same moment of fungal infection (0 DBI) or later (5 DAI), inhibited fungal growth at pre- and post-penetration stages with consequent preventive and curative effects against powdery mildew infection similar to those achieved by positive control. As cavoxin was phytotoxic to pea, this metabolite is unsuitable for further studies. The effect of against other biotrophic fungi has also been previously proven against leguminous and cereal rusts.²⁹ In particular, this metabolite was highly effective against two isolates belonging to *Puccinia* spp. and four isolates belonging to *Uromyces* spp. Thus, inuloxin C and sphaeropsidin A warrant further attention to gain a better understanding of its biological properties for practical applications in agriculture

against powdery mildew and other possible pathogens of agronomic interest. It is also important to note that sphaeropsidin A is produced in good yield (560 mg L^{-1}) from the liquid culture filtrates of *D. cupressi*, and is easily crystallized as white needles (250 mgL^{-1}).¹¹ Scale-up of production in large fermenters to produce large amounts of sphaeropsidin A could make the compound economical for use in agriculture. Similarly, inuloxin C was obtained in dried *I. viscosa* plants as pure oil (80 mgkg^{-1}).¹⁰ This is a common Mediterranean basin plant that it is easy to collect, transport and process, reducing that cost of obtaining abundant amounts of inuloxin C.

ASSOCIATED CONTENT

Additional information may be found in the online version of this article

Notes

The authors declare no competing financial interest.

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Table 1 Metabolites and controls tested for pea powdery mildew inhibition.

Compound	Name	Source	Molecular weight
Control	Water	Water	
Control	Water and metanol 5%	Methanol 5%	
Fungicide	Nimrod Quattro® (ADAMA)	Bupirimate 25% (p:v) (5-butyl-2-ethylamino-6-methylpyrimidin-4-yl dimethylsulphamate at 0.8 M)	
1	Inuloxin A	<i>Inula viscosa</i> (plant)	248
2	Inuloxin C	<i>Inula viscosa</i> (plant)	248
3	α -Costic acid	<i>Inula viscosa</i> (plant)	234
4	Sphaeropsidin A	<i>Diplodia cupressi</i> (fungus)	348
5	Sphaeropsidin C	<i>Diplodia cupressi</i> (fungus)	332
6	Cyclopaldic acid	<i>Seridium cupressi</i> (fungus)	238
7	Cavoxin	<i>Phoma cava</i> (fungus)	320
8	<i>epi</i> -Epoformin	<i>Diplodia quercivora</i> (fungus)	154
9	Cytochalasin A	<i>Pyrenophora semeniperda</i> (fungus)	477
10	Cytochalasin B	<i>Pyrenophora semeniperda</i> (fungus)	479
11	Gliotoxin	<i>Neosartoria pseudofisheri</i> (fungus)	326
12	6-Hydroxy-mellein	<i>Phoma chenopodicola</i> (fungus)	194

Table 2 Percentage of spore germination achieved by twelve metabolites at different concentrations (0.25 10^{-4} , 0.5 10^{-4} , 10^{-4} , 10^{-3} and 10^{-2} M) tested on pea detached leaves inoculated with powdery mildew. Experiment was performed at 24 hours after inoculation (hai). Values, per column and treatment, followed by different letters differ significantly at $P = 0.05$.

Metabolite 1: Inuloxin A						Metabolite 2: Inuloxin C					
Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M	Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M
Negative control	100a	100a	100a	100a	100a	Negative control	100a	100a	100a	100a	100a
Water	95.3a	89.2a	87.8a	94.5a	95.4a	Water	95.7a	91.6ab	93.5a	92.3a	98a
MeOH	95.7a	91.2a	86.7a	88.7a	89.9a	MeOH	95.8a	88.9ab	88.2ab	91.6a	95.5a
Phytotoxin	63.9b	47.9b	59.5b	59.2b	53.6b	Phytotoxin	84.4a	70.9b	65.2b	28bc	25.9bc
Positive control	11.7c	11.5c	9.9c	14.4c	12.8c	Positive control	16.2b	9.0c	11.9c	10.7c	12c
Metabolite 3: α-Costic acid						Metabolite 4: Sphaeropsidin A					
Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M	Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M
Negative control	100a	100a	100a	100a	100a	Negative control	100a	100a	100a	100a	100a
Water	96.2a	90.1a	97.5ab	97.8a	96.5a	Water	95.2a	89.2a	87.8a	94.5a	95.4a
MeOH	91ab	85.7ab	91.6ab	97a	95.3a	MeOH	95.7a	91.2a	86.7a	88.7a	89.9a
Phytotoxin	82.5b	72.3b	70.9b	72.1b	61.2b	Phytotoxin	63.9b	47.9b	48.5b	43.9b	31.3b
Positive control	10c	14c	12.3c	10.7c	14c	Positive control	11.7c	12.7c	9.9c	14.4c	16.8c
Metabolite 5: Sphaeropsidin C						Metabolite 6: Cyclopaldic acid					
Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M	Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M
Negative control	100a	100a	100a	100a	100a	Negative control	100a	100a	100a	100a	100a
Water	96.8a	98.3a	96a	94.6a	93.5a	Water	88.3a	89.3ab	88.7a	98.3a	97.2a
MeOH	93.1a	89.5a	95.6a	96.6a	96.2a	MeOH	90a	93ab	88a	85ab	92a
Phytotoxin	85.2a	89.2a	91.9a	92.2a	74.9b	Phytotoxin	56.5b	66.1b	52.7b	55.7b	72b
Positive control	15.3b	15b	14b	8.3b	4.7c	Positive control	13b	12.7c	14b	11c	12c
Metabolite 7: Cavoxin						Metabolite 8: epi-epoformin					
Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M	Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M
Negative control	100a	100a	100a	100a	100a	Negative control	100a	100a	100a	100a	100a
Water	89.3a	95.6a	91.7a	91.6a	89.7a	Water	88a	87.7ab	88ab	88a	92.7ab
MeOH	94.3a	91.3ab	90.4a	88.2a	89a	MeOH	91a	87ab	85ab	85a	84.4b
Phytotoxin	58.3b	53.9b	51.1b	30.6b	21.7b	Phytotoxin	78.8a	68.8b	63.5b	54.2b	86.3b
Positive control	13c	9.3c	13.7c	14.7c	12b	Positive control	11.3b	9.3c	15.3c	11c	9.6c
Metabolite 9: Cytochalasin A						Metabolite 10: Cytochalasin B					
Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M	Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M
Negative control	100a	100a	100a	100a	100a	Negative control	100a	100a	100a	100a	100a
Water	89.3a	95.4ab	87.7a	97.3a	87.3a	Water	90.2ab	91.1ab	94.7a	96.7ab	87.5a
MeOH	92.2a	88ab	89.7a	90.7a	89a	MeOH	89.3ab	92ab	94.7a	89.8ab	90.7a
Phytotoxin	86a	76b	77.3a	77.7a	71.8b	Phytotoxin	72b	73.7b	85.3a	81.8b	77.3a
Positive control	13.6b	13.4c	14.3b	12.1b	10.4c	Positive control	15c	11c	13b	12.4c	11.6b
Metabolite 11: Gliotoxin						Metabolite 12: 6-Hydroxymellein					
Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M	Treatment	0.25 10^{-4} M	0.5 10^{-4} M	10^{-4} M	10^{-3} M	10^{-2} M
Negative control	100a	100a	100a	100a	100a	Negative control	100a	100a	100a	100a	100a
Water	90a	94.3a	87.2a	95.2a	96.8a	Water	95.5a	91.9a	96.4a	90.9a	88.9a
MeOH	89ab	92.8a	86.7ab	87ab	93.5b	MeOH	91.9ab	90.9a	82.6ab	89.6a	88.9a
Phytotoxin	68b	55.1b	62.6b	68.1b	79.9b	Phytotoxin	78.3b	76.9a	66.6b	64.7b	57.7b
Positive control	12.3c	13.2c	14.4c	13.8c	11.7c	Positive control	13c	15.66b	13.1c	15c	12.9c

Table 3 Effective concentration for 50% inhibition (EC_{50}) of different metabolites (gL^{-1}) tested on spore growth inhibition of pea powdery mildew. Values followed by different letters differ significantly at $P = 0.05$.

Compound	EC_{50} Spore germination (%)	
	Range	Mean
1	2.138 – 4.344	3.52ab
2	1.022 – 2.532	1.86c
3	2.253 – 3.963	3.08ab
4	0.985 – 2.751	1.78c
5	2.233 – 5.001	3.74ab
6	2.106 – 6.702	4.65a
7	1.678 – 2.548	1.98c
8	2.683 – 5.888	4.31ab
9	1.957 – 10.08	4.58a
10	3.246 – 5.121	3.94ab
11	2.402 – 3.713	3.11ab
12	2.002 – 2.762	2.47bc
Positive control	0.092 – 1.458	1.03c

Table 4 Disease severity (DS%) and Area Under the Disease Progress Curve (AUDPC) of *Erysiphe pisi* development on plants of pea cv. Messire. DS% is expressed as the percentage of disease severity covering the whole plant 12 days after infection (DAI). Metabolites **2**, **4** and **7**, negative (untreated, water and MeOH 5%) and positive (bupirimate 25%) controls were applied to plants at different times to fungal inoculation: 5 days before inoculation (DBI), 0 DBI and 2 days after inoculation (DAI). Compounds were tested at a concentration of 10^{-3} and 10^{-2} M.

Time	Treatment	Metabolite 2		Metabolite 4		Metabolite 7	
		DS%	AUDPC	DS%	AUDPC	DS%	AUDPC
5 DBI	Negative control	95a	522a	95a	522a	96a	522.5a
	Water	90a	470a	90a	465a	90a	465a
	MeOH 5%	90a	535a	90a	535a	90a	505a
	Positive control	30c	169c	28c	174c	30c	171d
	10^{-3} M	43.3b	288b	44b	249.1b	44.3b	251.6b
	10^{-2} M	35.8c	198c	35.9c	195.6c	42.3b	213.6c
0 DBI	Negative control	95a	492.5a	95a	522.5a	95a	521.5a
	Water	90a	455a	90a	457a	90a	455a
	MeOH 5%	95a	457a	95a	462a	95a	467a
	Positive control	0d	0c	0c	0c	0c	0d
	10^{-3} M	30.8b	115.1b	22.9b	109.9b	29.1b	133.9b
	10^{-2} M	22.2c	91.9b	21b	91.4b	28.6b	107.9c
2 DAI	Negative control	90a	510a	90a	510a	90a	510a
	Water	90a	485a	90a	485a	90a	485a
	MeOH 5%	90a	465a	90a	465a	90a	465a
	Positive control	0c	0c	0b	0b	0c	0b
	10^{-3} M	17b	86.6b	8b	31.35b	12bc	54.1b
	10^{-2} M	14.2b	80.9b	7.7b	29.6b	9.4c	44.4b

Fig. 1A Structures of the plant (1-3) and fungal (4 and 5) terpenes tested against pea powdery mildew.

Fig. 1B Structures of the fungal (6-12) metabolites belonging to different classes of natural compounds tested against pea powdery mildew.

Fig. 2 Seedling of *Pisum sativum* cv. Messire placed in a spore settling tower for *E. pisi* multiplication (A) and pathogen sporulation eight days after plant inoculation (B). Petri dishes with pea cv. Messire detached leaves treated with phytotoxins at different concentrations (C).

Fig. 3 Diary disease severity (DS%) development of *Erysiphe pisi* on plants of pea cv. Messire. DS% is expressed as the percentage of disease severity covering the whole plant. Metabolites **2**, **4** and **7**, negative (untreated, water and MeOH 5%) and positive (fungicide) controls were applied to plants at different times to fungal inoculation: (1) five days before inoculation (5 DBI), (2) 0 DBI and (3) two days after inoculation (2 DAI). Compounds were tested at a concentration of 10^{-3} and 10^{-2} M.

Fig. 4 Powdery mildew symptoms on plants of pea cv. Messire treated with metabolite 2 (Inuloxin) C. Disease Severity (DS%) is expressed as the percentage of canopy covered by mildew 9 days after infection (DAI). With daily assessments of DS, Area Under the Disease Progress Curve (AUDPC) was calculated. Metabolite 2 (Inuloxin C), negative (untreated, water and MeOH 5%) and positive (fungicide) controls were applied to plants at different times to fungal inoculation: (1) five days before inoculation (5 DBI), (2) 0 DBI and (3) two days after inoculation (2 DAI). Compounds were tested at a concentration of 10^{-3} and 10^{-2} M.

Metabolite 2 (Inuloxin C)

Fig. 5 Powdery mildew symptoms on plants of pea cv. Messire treated with metabolite **4** (Sphaeropsidin A). Disease Severity (DS%) is expressed as the percentage of canopy covered by mildew 9 days after infection (DAI). With daily assessments of DS, Area Under the Disease Progress Curve (AUDPC) was calculated. Metabolite **4** (Sphaeropsidin A), negative (untreated, water and MeOH 5%) and positive (fungicide) controls were applied to plants at different times to fungal inoculation: **(1)** five days before inoculation (5 DBI), **(2)** 0 DBI and **(3)** two days after inoculation (2 DAI). Compounds were tested at a concentration of 10^{-3} and 10^{-2} M.

Fig. 6 Powdery mildew symptoms on plants of pea cv. Messire treated with metabolite **7** (Cavoxin). Disease Severity (DS%) is expressed as the percentage of canopy covered by mildew 9 days after infection (DAI). With daily assessments of DS, Area Under the Disease Progress Curve (AUDPC) was calculated. Metabolite **7** (Cavoxin), negative (untreated, water and MeOH 5%) and positive (fungicide) controls were applied to plants at different times to fungal inoculation: **(1)** five days before inoculation (5 DBI), **(2)** 0 DBI and **(3)** two days after inoculation (2 DAI). Compounds were tested at a concentration of 10^{-3} and 10^{-2} M.

Supplementary Material

Supplementary Fig. 1 Symptoms of *Erysiphe pisi* on susceptible pea plants cv. Messire on negative controls (water and MeOH 5%), positive control (fungicide), and treated with inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**). Disease was scored 9 days after inoculation.

Supplementary Table 1 Effect on *Erysiphe pisi* hyphal tips development and haustoria formation per colony in Petri dish assays at 24 hours after inoculation (24 h.a.i.) on negative (untreated control, water and MeOH 5%) controls, treated leaves with inuloxin C (**2**), sphaeropsidin A (**4**) and cavoxin (**7**) tested at different concentrations ($0,25 \cdot 10^{-4}$, $0,5 \cdot 10^{-4}$, 10^{-4} , 10^{-3} and 10^{-2} M) and positive control (fungicide Bupirimato 25%).