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Inhibition of Spore Germination and Appressorium Formation of Rust Species by Plant and Fungal Metabolites

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Eight fungal and plant metabolites belonging to different classes of naturally occurring compounds, a 24-oxa[14]-cytochalasan as cytochalasin B (**1**), a trisubstituted isocoumarin as 6-hydroxymellein (**2**), a tetracyclic pimarane diterpene as sphaeropsidin A (**3**), a chalcone as cavoxin (**4**), a pentasubstituted benzofuranone as cyclopaldic acid (**5**), a bicyclic-sesquiterpene as inuloxin A (**6**), a epipolythiopiperazine as gliotoxin (**7**) and a cyclohexene epoxide as *epi*-epoformin (**8**), were tested for their effectiveness in reducing early stages of development of several major rust fungi from the genera *Puccinia* and *Uromyces*. Spore germination and appressoria formation were assessed on pre-treated detached leaves, under controlled conditions. Among the various metabolites evaluated, compounds **5** and **8** were the most effective in inhibiting fungal germination and penetration of all rust species studied at values comparable with those obtained by fungicide application, while compound **4** was phytotoxic to plant leaves at any concentration tested.

Keywords: *Puccinia*, *Uromyces*, Fungicidal activity, Fungal and plant metabolites, Biocontrol.

There are about 7000 species of rusts that attack an extremely wide range of crops worldwide and that are present in all areas where these crops are cultivated [1a]. Between then, *Puccinia* and *Uromyces* are the most important genera (with about 4000 and 600 species, respectively), which represent almost two-thirds of all known rust species, causing diseases on a wide host range including cereals and legumes [1b].

When infections start early in the season, yield components are severely affected by the infection and losses are important, ranging from 40 to more than 80% of crop production in susceptible material [2]. Owing to their economic importance, a large number of methods to control rusts have been proposed, with foliar fungicides and resistant cultivars being the most widely used. Although breeding for resistance is undoubtedly the most economical and environmentally friendly method of rust control, chemical treatments are often systematically established [3], even though fungicides have low specificity and are not easily biodegradable.

Recently, different approaches prompted the discovery of natural products as templates to develop potential biofungicides with new molecular structures and mode of actions. In fact, many plant pathogens, especially necrotrophic or hemibiotrophic fungi, are capable of producing phytotoxins of different nature interacting with fungal receptors responsible for fungal growth regulation and can be a source of many such useful metabolites [4,5]. Similarly, active metabolites isolated from weeds and cultivated plants have demonstrated a number of biological activities [6,7]. Considering that spore germination and fungal penetration are key phases for the initial development of biotrophic plant pathogens as rusts, a further approach proposed for their management could be the use of natural metabolites produced by fungi and plants as pathogen inhibitors in these pre-penetration stages.

Here we evaluate the potential of seven fungal and one plant toxin (Table 1) as natural fungicides testing their potential to inhibit spore germination and appressoria formation of six different rusts, including some of the most agronomically significant obligate biotrophs as leaf rust (caused by *P. triticina* Erikss (= *P. recondita* Roberge ex Desmaz. f. sp. *tritici*) and crown rust (*P. coronata* f. sp. *avenae* Eriks), that are respectively damaging on wheat (*Triticum aestivum* L.) and oats (*Avena sativa* L.) [8a], as well as *U. pisi* ([Pers.] D.C.)Wint., *U. ciceris-arietini* Jacz. in Boyer & Jacz., *U. viciae-fabae* (Pers.) J. Schröt. and *U. viciae-fabae* ex *Lens culinaris*, that are responsible for rust diseases on pea (*Pisum sativum* L.) [8b], chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.) and lentil (*Lens culinaris* Medik.) [2], respectively.

Table 1: Metabolites and controls tested for anti-rust effect.

Compounds	Name	Source	MW
Control	Water	water	
Control	Water + MeOH 5%	Methanol 5%	
Fungicide	Ortiva®	Azoxystrobin 25%	
1	Cytochalasin B	*Pyrenophora semeniperda* (fungus)	479
2	6-Hydroxymellein	*Phoma chenopodiicola* (fungus)	194
3	Sphaeropsidin A	*Diplodia cupressi* (fungus)	348
4	Cavoxin	*Phoma cava* (fungus)	320
5	Cyclopaldic acid	*Seridiun cupressi* (fungus)	238
6	Inuloxin A	*Inula viscosa* (plant)	248
7	Gliotoxin	*Neosartoria pseudofisheri* (fungus)	326
8	*epi*-Epoformin	*Diplodia quercivora* (fungus)	154

The phytotoxins used for the study belong to different classes of natural compounds: cytochalasin B (**1**), which is 24-oxa[14]-cytochalasans, and 6-hydroxymellein (**2**), which is a trisubstituted isocoumarine. Sphaeropsidin A (**3**) is a tetracyclic pimarane diterpene, and cavoxin (**4**) a chalcone. Cyclopaldic acid (**5**), inuloxin A (**6**) and gliotoxin (**7**) are a pentasubstituted benzofuranone, a bicyclic-sesquiterpene, belonging to the germacranolide subgroup, and an epipolythiopiperazine, respectively. Finally, *epi*-epoformin (**8**) is a cyclohexene epoxide (Figure 1).

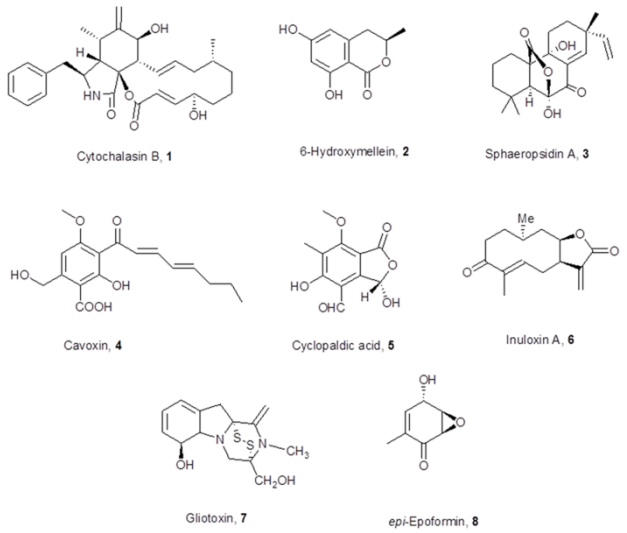

Figure 1: Structures of metabolites tested.

On each pathosystem studied at 24 hours after inoculation (h.a.i.) on detached leaves (Figure 2), the percentage of spore germination was high (up to 72%) in both water and methanol negative controls, with no significant differences between them (Figure 3). This suggests that methanol at 5%, necessary to dissolve the metabolites above cited in water, did not exert any fungitoxic activity on the pathogens tested. On the contrary, spore germination lower than 1% was observed in inoculated leaves treated with the positive control (Azoxystrobin 25%), as expected. In fact, this chemical is a well-known rust fungicide that prevents rust sporulation and also suppresses new subsequent fungal infections [9]. In general, all metabolites tested significantly impaired fungal spore germination at all concentrations tested (Figure 3). Percentages were lower than 22% for cereal rusts (*Puccinia* spp.), and 65% for legume rusts (*Uromyces* spp.), with large differences due to the diversity of metabolites and the concentration applied.

Metabolites **1** and **2** were highly effective at any concentration in reducing spore germination (values lower than 20%) of both cereal and legume rusts (Figure 3). Nevertheless, *U. pisi* germination was only reduced at rates higher than 10^{-3} M. Metabolite **1** is well known for its phytotoxic and cytotoxic activities [10a,b], while metabolite **2** is known for its phytotoxicity to weeds [11]. Spore germinations were generally lower than 15% when leaves were treated with metabolites **3** and **6** (Figure 3). As exceptions, *U. pisi* germination was highly hampered (< 5%) when metabolite **3** was applied at concentrations higher than 10^{-3} M, and inhibition was moderate at any concentration tested for *U. viciae-fabae*. *Puccinia* spp. spore germination was strongly reduced (< 2%) by metabolites **4** and **5** at all concentration tested (Figure 3), reaching values comparable with that achieved by treatment with the chemical. Nevertheless, *Uromyces* spp. spore germination achieved after treatment with metabolites **4** and **5** varied between 18 and 42%, depending on the fungal isolate and the concentration applied.

The fungitoxic activity exerted by metabolite **5** was not dose-dependent, since spore germination did not decrease when the concentration increased. The phytotoxic [12a,b], fungicidal [12b,c], bactericidal [12d], insecticidal [4], as well as anticancer activities [12e,f] exerted by metabolites **3** and **5** have been largely tested. Metabolites **4** and **6** showed antifungal activity against plant pathogenic fungi such as *Colletotrichum* and *Phomopsis* [13], and seed germination inhibition of parasitic weeds such as *Orobanche crenata* Forssk. and *Cuscuta campestris* Yunck [7], respectively.

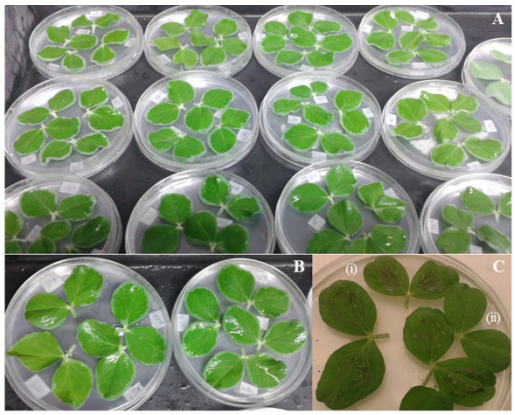

Figure 2: Petri dishes showing treated pea detached leaves inoculated with spores of *U. pisi* (A, B). Phytotoxic effect of cavoxin application (i) compared with MeOH 5% control (ii) at 24 hours after inoculation (h.a.i.).

Uromyces spp. spore germination was markedly inhibited in the presence of metabolites **7** and **8** at all concentration tested (Figure 3), not significantly different from the positive control (Azoxystrobin 25%). Metabolite **7** always operated in a dose-dependent way, confirming previous results [14]. In fact, we observed that spore germination was significantly reduced at rates lower than 20% on all rusts tested when the metabolite was applied at 10^{-4} M; then the effect increased at concentrations higher than 10^{-3} M. This metabolite recently showed the ability to inhibit strongly the growth of six human cancer cell lines [14]. Metabolite **8** has been tested against fungal pathogens of forestry and agronomic interest [15], showing a powerful growth-inhibitory antifungal activity operating at low micromolar concentrations, with which our results agreed.

The morphology and germ-tube formation of the rust spores treated with metabolites **2**, **5**, **6** and **7** were adversely affected, with the spores exhibiting abnormal surface appearances (probably indicative of denatured cellular material) and the germ-tubes, if present, were truncated or showed abnormal growth (data not shown). Furthermore, in spite of its antifungal activity, metabolite **4** was unique in showing macroscopic symptoms of phytotoxicity on plant hosts at all concentrations tested. Leaf surfaces exposed to cavoxin quickly turned brown and necrotic (Figure 2C).

As observed for spore germination, high percentages of appressoria forming over plant stomata (values up to 45%) were achieved for all rust species tested in both water and MeOH 5% (negative controls), with no significant differences between them. On the contrary, no appressoria formation was observed in all rust species treated with the positive control (Azoxystrobin 25%) (Figure 4), while most of the compounds strongly inhibited fungal appressoria formation when compared with the water control. This is very significant from a fungal control point of view, as the appressorium constitute the organ that allows host penetration and subsequent fungal expansion. Metabolites **2**, **4**, **5** and **8** were the most effective for controlling further steps of *Puccinia* spp. development, since no appressoria formation on plant stomata was observed, even at the lowest concentration applied (10^{-4} M). These results were comparable with those obtained with the chemical at the same concentration. Similarly, metabolites **1**, **5**, **6** and **8** were the most effective in reducing appressoria penetration in *Uromyces* spp. (Figure 4). Gliotoxin (**7**) also was very active in reducing appressoria formation in both *Puccinia* and *Uromyces* rusts, showing a fungitoxic effect that followed a linear equation where further increases in the doses applied led to further fungal growth inhibition levels.

Effective concentration for 50% inhibition (EC_{50}) was also calculated for each rust isolate to test their sensitivities to the eight natural compounds and the chemical on the basis of the spore growth inhibition (Table 2). The chemical fungicide azoxystrobin belongs to the quinone outside inhibitor (QoI) class of fungicides, which inhibit cellular respiration. Because QoI fungicides are powerful inhibitors of spore germination (Fungicide Resistance Action Committee [FRAC] group 11), an assay based on this parameter is likely to be a better method for determining sensitivity of rusts to natural compounds compared with the chemical. In general, EC_{50} values for the fungicide were significantly lower than those reported for natural metabolites (Table 2). Only sensitivity to metabolite **4** is comparable with the fungicide in *P. triticina* and *P. coronata*. In our study, we also found no variation in sensitivity to the chemical fungicide between the rust isolates used ($P = 0.06879$), with values that ranged from 0.080 to 1.05 g L^{-1}.

Regarding the natural metabolites tested, we did not find significant difference between the mean of EC_{50} values for *U. pisi*, *U. viciae-fabae* and *U. ciceris-arietini* ($P > 0.05$) (Table 2). On the contrary, differences in sensitivity were found for *U. viciae-fabae* ex *L. culinaris* to metabolites **5** (range 1.250 – 2.11, mean EC_{50} = 1.795) and **7** (range 1.671 – 1.704, mean EC_{50} = 1.679), as well as for *P. triticina* and *P. coronata* to metabolites **4** (range 0.455 – 0.669, mean EC_{50} = 0.499 and range 0.333 – 1.0, mean EC_{50} = 0.583, respectively) and **8** (range 0.667 – 2.166, mean EC_{50} = 1.644 and range 0.333 – 2.334, mean EC_{50} = 1.308, respectively) (Table 2). Though peaking at different levels, the EC_{50} displayed a continuous and unimodal distribution in pooled population (data not shown), with a range from 0.170 g L^{-1} in the most sensitive isolate (*U. ciceris-arietini* to **8**) to 3.389 g L^{-1} in the least sensitive isolate (*U. viciae-fabae* to **2**).

Cyclopaldic acid (**5**) and *epi*-epoformin (**8**) were the most promising compounds tested, since their general high efficacy, together with the absence of phytotoxic effects, was proved for all rust pathogens included in this study. In contrast, 6-hydroxymellein (**2**) and cavoxin (**4**) were more active against *P. triticina* and *P. coronata* than against the other genera tested (*Uromyces* spp.). This apparent selective activity towards *Puccinia* indicates that these compounds may have relatively limited fungicidal activity against non-target fungi. This is in agreement with Schrader *et al.* [13], who showed that cavoxin was highly efficient against *in vitro Phomopsis* development, but not toward other test fungi such as *Botrytis cinerea* and *Fusarium oxysporum*. In addition, application on leaves of cavoxin solutions (at any concentration tested) led to severe visible damage on both cereal and leguminous plants. Hence, these results, together with the moderate effect exerted on *Uromyces* fungal growth impairment, made cavoxin unsuitable for in planta tests. To our knowledge, this is the first report of a phytotoxic effect of cavoxin application.

At 48 hours after inoculation (h.a.i.), results obtained on the pea-*U. pisi* pathosystem showed that the fungitoxic activity performed by the metabolites studied was generally maintained (data not shown). In particular, the presence of metabolites **5** and **8** reduces spore germination at values lower than 20% at any concentration tested.

Rust fungi are regularly exposed to fungicides, especially when no genetic resistance is available. When rust control has focused on the deployment of cultivars carrying dominant resistance genes, mainly in the case of cereals, fungicides contribute about equally to genetics in the control of the disease [3]. As a result, evolved resistance to rust fungicides has emerged as one of the most serious issues threatening food security leading to the application of general

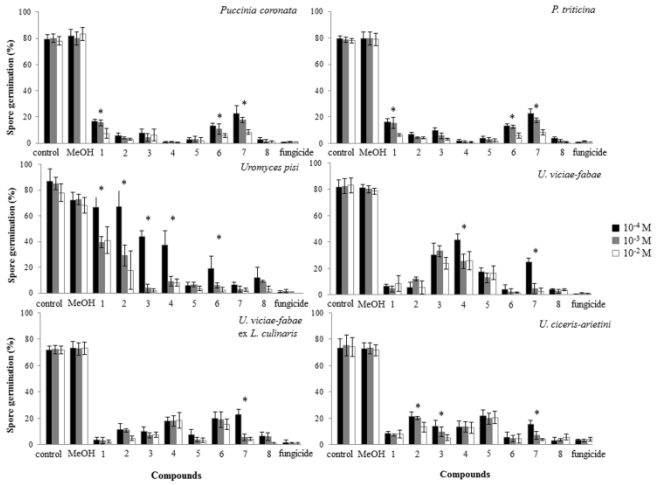

Figure 3: Inhibition of *Puccinia* spp. and *Uromyces* spp. spore germination in Petri dish assays at 24 hours after inoculation (h.a.i.) on negative (water and MeOH 5%) controls, treated leaves with fungal and plant metabolites (**1-8**) and positive control (fungicide Azoxystrobin 25%). The compounds were tested in a concentration range of 1 x 10^{-4} M (black column), 1 x 10^{-3} M (grey column) and 1 x 10^{-2}M (white column). The experiment was repeated four times, and the standard deviation calculated. For each metabolite, the asterisk indicates significant differences in fungal growth due to the concentration tested ($p < 0.01$).

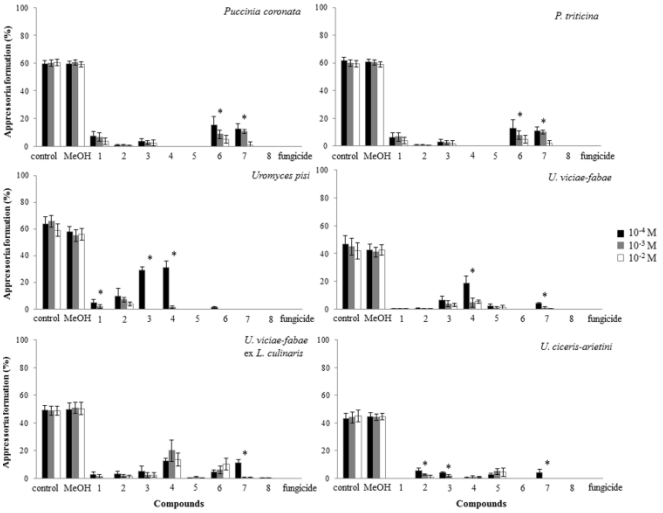

Figure 4: Inhibition of *Puccinia* spp. and *Uromyces* spp. appressoria formation in Petri dish assays at 24 hours after inoculation (h.a.i.) on negative (water and MeOH 5%) controls, treated leaves with fungal and plant metabolites (**1-8**) and positive control (fungicide Azoxystrobin 25%). The compounds were tested in a concentration range of 1 x 10^{-4} M (black column), 1 x 10^{-3} M (grey column) and 1 x 10^{-2}M (white column). The experiment was repeated four times, and the standard deviation calculated. For each metabolite, the asterisk indicates significant differences in fungal growth due to the concentration tested ($p < 0.01$).

integrated disease management strategies such as dose limitation, mixtures, and the search of alternative compounds. Here, for the first time, eight fungal metabolites from different sources and belonging to different classes of natural compounds were tested with general success for their fungitoxic activity against several rusts of agronomic importance.

Between them, application of metabolites **5** and **8** were the most promising, reducing the early developmental stages of *Uromyces* spp. and *Puccinia* spp. at values comparable with those obtained by application of chemicals. Thus, it seems that these compounds would deserve further attention to understand better their biological

Table 2: Effective concentration for 50% inhibition (EC_{50}) of different metabolites tested on spore growth inhibition of six rust isolates. Values per rust isolate followed by different letters differ significantly at $P = 0.05$.

	Uromyces pisi		***U. viciae-fabae***		***U. ciceris-arietini***		***U. viciae-fabae* ex *L. culinaris***		***Puccinia triticina***		***P. coronata***	
Compound	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
1	1.86-2.33	2.19a	1.83-2.33	2.11a	0.83-3.00	2.06a	1.83-2.67	2.24a	2.11-2.30	2.21a	1.67-2.17	1.97a
2	2.07-2.31	2.23a	1.76-3.39	2.20a	1.17-1.92	1.70a	1.67-2.33	1.90ab	1.67-2.29	1.98ab	1.67-2.17	1.98a
3	0.83-2.33	1.72a	1.88-2.13	1.99a	1.83-2.00	1.94a	1.67-2.17	1.85ab	1.67-2.33	2.00a	1.68-1.79	1.71a
4	1.82-2.32	2.13a	2.18-2.56	2.31a	1.67-2.22	2.04a	1.71-2.00	1.87ab	0.46-0.67	0.50c	0.33-1.00	0.58c
5	1.64-2.67	2.00a	1.78-2.33	2.14a	1.67-3.00	2.37a	1.25-2.11	1.80b	1.67-2.29	1.94ab	1.00-2.23	1.77a
6	1.67-1.86	1.74a	1.70-2.27	1.99a	0.17-2.24	1.65a	1.33-2.11	1.84b	1.86-2.23	2.10a	1.67-2.30	1.94a
7	1.67-1.87	1.73a	2.01-2.33	2.18a	1.67-1.86	1.77a	1.67-1.70	1.68b	2.01-2.30	2.10a	1.87-2.13	2.06a
8	0.33-2.45	1.70a	1.70-2.33	1.92a	0.17-1.93	1.48a	1.78-2.19	1.93ab	0.67-2.17	1.64b	0.33-2.33	1.31b
fungicide	0.20-0.79	0.68b	0.82-1.05	0.99b	0.08-0.88	0.71b	0.19-0.97	0.77c	0.10-0.53	0.36c	0.10-0.80	0.42c

properties for practical applications in agriculture against rust and other possible biotrophic pathogens of agronomic interest. Nevertheless, for further assays, larger timeframes between metabolite application and fungal growth measurements will be taken into account in order to evaluate the long lasting efficacy of the biofungicides. In addition, further experiments testing their efficacy in preventing the disease inducing systemic acquired resistance (SAR) responses on the host plant and/or reduction of fungal growth after plant penetration as a curative effect are in progress.

Table 3: Collection code and origin of rust species of *Puccinia* and *Uromyces* used in antifungal assays (specimens deposited at the IAS-CSIC, Córdoba, Spain).

Collection code	Specimen	Host species	Origin
PS-03	*Puccinia triticina*	*Triticum aestivum*	Cádiz, Spain
CO-04	*P. coronata* f.sp. *avenae*	*Avena sativa*	Córdoba, Spain
UPC-04	*Uromyces pisi*	*Pisum sativum*	Córdoba, Spain
UVFC-11	*U. viciae-fabae*	*Vicia faba*	Córdoba, Spain
UVFC-12	*U. viciae-fabae* ex *L. culinaris*	*Lens culinaris*	Valladolid, Spain
UCAC-01	*U. ciceris-arietini*	*Cicer arietinum*	Cuenca, Spain

Experimental

Fungal and plant bioactive metabolites: Structures of metabolites involved in the study are shown in Figure 1. Cytochalasin B (**1**) was isolated from the solid culture of *Pyrenophora semeniperda* (Brittleb. & D.B. Adam) Shoemaker, while 6-hydroxymellein (**2**) and cavoxin (**4**) were isolated from the liquid culture of *Phoma chenopodiicola* Gruyter, Noordel. & Boerema and *Phoma cava* Schulzer, respectively. Sphaeropsidin A (**3**), cyclopaldic acid (**5**) and *epi*-epoformin, (**8**) were purified from the culture filtrates of *Diplodia cupressi* A.J.L. Phillips & A. Alves, *Seiridium cupressi* (Guba) Boesewinkel, and *Diplodia quercivora* Linaldeddu & A.J.L. Phillips, respectively. Inuloxin A (**6**) was purified from the organic extract of *Inula viscosa* (L.) Ait. Finally, gliotoxin (**7**) was isolated from the solid culture of *Neosartorya pseudofischeri* S. W. Peterson.

Plant growth and rust multiplication: The experiments were performed with wheat (*Triticum aestivum* L.) cv. Meridiano, oats (*Avena sativa* L.) cv. Araceli, pea (*Pisum sativum* L.) cv. Messire, faba bean (*Vicia faba* L.) cv. Baraca, lentil (*Lens culinaris* Medik.) cv. Pardina and chickpea (*Cicer arietinum* L.) cv. Blanco Lechoso, which were susceptible to *Puccinia triticina*, *P. coronata*, *Uromyces pisi*, *U. viciae-fabae*, *U. viciae-fabae* ex *L. culinaris* and *U. ciceris-arietini*, respectively. Plants were raised from seeds in pots (6 x 6 x 10 cm) filled with a potting mixture (sand/perlite, 1:1 v/v) in a growth chamber at 20 ± 2ºC and 65% relative humidity under a photoperiod of 14 h light/10 h dark with light intensity of 150 µmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes. Twelve-days-old seedling plants of each species were inoculated with their respective fungal isolates, preserved at -80ºC, for spore multiplication. Rust isolates (listed in Table 3) were from the IAS-CSIC fungal collection. Plants were inoculated following the procedure of Barilli *et al.* [8b]. Fresh urediospores were collected from leaves the same day of the bioassay. Every set of plants per isolate was maintained apart from the others in distinct growth chambers.

Antifungal assays and microscopic assessments: For histological studies, detached leaves were used so that both inoculation density and incubation conditions were controlled precisely. Plants of all species were grown under the controlled conditions mentioned above until the fifth leaf stage and then, several fourth-formed leaved were excised and placed, adaxial side up, on 4% technical agar in Petri dishes. Cut leaves were arranged in a randomized design with 4 replicates per treatment, each replicate having 4 leaves. Metabolites listed in Table 1 were tested at concentrations of 10^{-2}, 10^{-3} and 10^{-4} M. Compounds were dissolved in MeOH (5%) and then brought up to the assay concentration with distilled water. The test solutions (50 µL) were applied on the adaxial leaf side. Droplets (50 µL) of distilled water and MeOH (5%) were applied as negative controls. Droplets (50 µL) of commercial anti-rust fungicide (Azoxystrobin 25%, Syngenta) were applied as a 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 0.5 mg fresh uredospores per plate. Plates were transferred during 24 h to a cabinet at 20 ± 2ºC in complete darkness and high relative humidity, and then were returned to growth chamber conditions (Figure 2). Twenty-four h after inoculation (h.a.i.), 4 leaves per plant, treatment and replications were used to assess percentage of fungal spore germination and appressoria formation. In order to monitor the long-lasting effect of metabolites, an additional 4 leaves were collected at 48 h.a.i. only on *U. pisi*-infected pea leaves. Leaves were stained according to Sillero and Rubiales method [16], and the different stages of the infection process were assessed using a phase contrast Leica DM LS microscope at x 20 and x 40 magnifications (Leica Microsystems, Wetzlar, Germany). On every leaf fixed at 24 and 48 h.a.i., germination frequency was assessed by scoring 100 uredospores for the presence of a germ tube. An additional 100 germinated spores were examined to determine the percentage of appressoria formed over plant stomata, indicating right fungal penetration. The effective concentration for 50% inhibition (EC_{50}) was calculated according to the method of Alexander *et al.* [17], using the inhibition rate of each isolate in the presence of each compound. Spore germination for each of the replicates was converted to percent inhibition compared with the untreated control by: 100 – ([percent germination of fungicide-compound] / [mean percent germination of control]). The concentration of compounds that effectively inhibited spore germination by 50% of the untreated control (EC_{50}) was determined for each isolate by linear interpolation.

Statistics: For statistical analysis, percentage data were transformed to arcsine square roots (transformed value = 180/π x arcsine [√(%/100)]) to normalize data and stabilize variances throughout the data range. Transformed data were subjected to analysis of variance (ANOVA) using Statistix 8 (Analytical Software, Tallahassee, FL, USA), after which residual plots were inspected to confirm data

conformity to normality. Significance of differences between means was determined by calculating least significant difference (LSD).

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