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# Feruloyl-amides as natural antimicrobials for crop and food protection

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## Abstract

**Background** Plants have developed multiple chemical defence responses against pathogen attacks. The main mechanism of defence is based on a rapid transcriptional reprogramming of genes encoding biosynthetic enzymes that synthesize specific secondary metabolites. Increasing evidence indicates phenylamides (PAs) as an important group of bioactive compounds in food plants.

**Results** We synthesized a small collection of ferulic acid-derived phenylamides by chemoenzymatic approaches. The compounds were tested against fungal and bacterial pathogens to assess their antimicrobial potential. The treatment with the synthesized phenylamides showed modest inhibition of the fungal growth (up to 25%) and had no significant influence on spore germination, whereas some of the compounds gave a considerable inhibition of *Pyricularia oryzae* appressorium formation, up to 94%. They also exhibited in vitro antibacterial activity against six foodborne bacterial pathogens. Monitoring of six growth parameters (taking into account growth rate, time and absorbance) measured during 24 h incubation showed that the synthesized molecules, assayed at four concentrations between 12.5 and 100 mg/L, produced a stronger average antimicrobial effect against Gram-positive pathogenic strains than against Gram-negative ones.

**Conclusions** The obtained results evidenced that the effect of this class of compounds is mainly related to blocking fungal virulence mechanisms, mediated by a significant effect on appressorium maturation, rather than to mycelium growth inhibition. Together with the observed in vitro antibacterial activity against foodborne bacterial pathogens, we conclude that PAs are promising candidates for future developments in the agri-food sector.

**Keywords** Crop protection, Antimicrobial activity, Antifungal activity, Bacterial inhibition, Ferulic acid, Phenylamides, Bioactive compounds

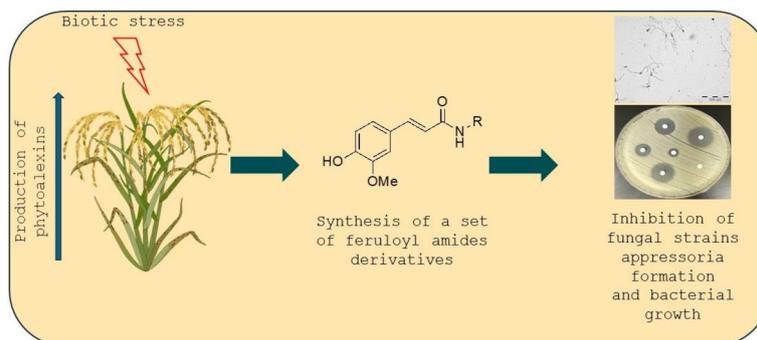
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## Graphical Abstract



## Background

Plants produce a plethora of specialized metabolites to protect themselves from biotic and abiotic stresses. Among these, phytoalexins are inducible antimicrobials synthesized in reaction to pathogen attack [1]. They are synthesized *de novo* by the induction of biosynthetic enzyme pathways, often in a tissue- and developmental stage-specific manner [2]. Phytoalexins display great variability in chemical structures among different plant species and are synthesized by diverse biosynthetic pathways [1, 2]. However, the multiplicity of defence metabolites different in structure may delay the development of tolerance against a specific compound in pathogens.

Phytoalexins known so far belong to the classes of phenylpropanoids, diterpenes, flavonoids, and phenylamides [3]. Phenylamides (PAs) are biosynthesized by coupling carboxylic acids with biogenic amines [4]. Representative acids are cinnamic (Cin), *p*-coumaric (Cou), caffeic (Caf), ferulic (Fer), and benzoic (Ben) acids, while amines are mainly tryptamine (Try), serotonin (Ser), tyramine (Tyr), agmatine (Agm), and putrescine (Put) [5].

In the past two decades, several studies have demonstrated that PAs accumulate to high levels in response to pathogen infection [4–6]. Their incorporation in the plant cell walls has been shown in many plant species, where they are cross-linked to various cell wall polymers via ester and ether linkages [5, 7–9]. Here, they exert a dual biological activity, both preventing pathogen penetration (by strengthening and thickening cell walls) and interfering with key microbial cellular structures and metabolism [5, 8].

In Solanaceae species, CouTyr and FerTyr accumulate at high concentrations after pathogen infection [10–13] and are incorporated into cell walls to form phenolic polymers, which prevent further invasions of microorganisms [14–16].

In grasses, PAs are induced by wounding and insect attack. Oat (*Avena sativa*) leaves accumulate avenanthramides after exposure to crown rust fungus and by treatment with elicitors [6, 17, 18]. Barley (*Hordeum vulgare*) shoots at the young seedling stage accumulate dimers of CouAgm and FerAgm (hordatines), which act as antifungal compounds [19]. Recently, dimers of FerAgm (muri namides A and B) have been found in wild *Hordeum* species [20].

Rice (*Oryza sativa* L.) produces numerous PAs endowed with significant antimicrobial activity against several bacterial and fungal pathogens such as *Burkholderia glumae*, *Xanthomonas oryzae*, *Pyricularia oryzae*, and *Bipolaris oryzae* [5, 21–23]. In addition, Morimoto and co-workers [5] monitored the accumulation of 25 phenylamides in rice leaves after the infection with *B. oryzae* and *X. oryzae*. Similarly, a metabolomics analysis found that PAs are closely related to immunity responses in rice infected by *P. oryzae* [24].

PAs are also well-known for their antibacterial activity [25]. In particular, hydroxycinnamic acid amides and their analogues have been reported to inhibit among others methicillin-resistant (MRSA), vancomycin-resistant (VRSA) *Staphylococcus aureus* [26, 27] and *Escherichia coli* O157:H7 strain [28].

On the other hand, the improper and continuous use of harmful agrochemicals may have significant impacts on both the environment and human health. Considering the urgency to reduce the employment of chemicals and the need to manage resistance to single-target site pesticides, in the last decade the agricultural sector has refocused its priorities on moving towards biological control methods, and naturally occurring substances. The biopesticides are often regarded much safer and less toxic alternative to synthetic pesticides [29]. Specialized plant metabolites, as discussed above, are produced as

chemical defence responses against pathogen attacks and act on multiple, often unknown, essential cellular targets. In addition, since Antimicrobial Resistance (AMR) has emerged as one of the greatest global concerns, the availability of new antimicrobials, synthesized from molecules naturally occurring in plants or animals, is one of the routes considered to fight the spreading of antimicrobial resistant microorganisms.

In the present paper, we report the results of our efforts aimed at investigating the effect of PAs as antimicrobial agents against a panel of plant fungal and food-borne bacterial pathogens, to address their possible application in crop protection or as post-harvest treatments. Nonetheless, direct isolation of PAs from natural matrices is quite impractical and troublesome because of their low concentration in plants and difficult extraction and purification procedures. To deal with these challenges, we synthesized a set of PAs by high-yielding chemical and biocatalytic approaches.

In the panel of bacteria, we included model foodborne pathogens. As regards the antifungal activity evaluation, we focused on *Pyricularia oryzae*, *Botrytis cinerea*, and *Fusarium culmorum*, major fungal pathogens of cultivated crops. We investigated the activity on mycelial growth as well as the effect on spore germination and in *P. oryzae* also appressorium maturation, a key step underpinning pathogen virulence.

## Methods

### Synthesis of the compounds

All reagents and solvents were purchased from commercial suppliers and used without further purification. Lipase B from *Candida antarctica* immobilized on acrylic resin (activity  $\geq 5,000$  U/g) was supplied by Sigma-Aldrich. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded with a Bruker AV600 ( $^1\text{H}$ , 600 MHz;  $^{13}\text{C}$ , 150 MHz) and a Bruker AMX-300 ( $^1\text{H}$ , 300 MHz;  $^{13}\text{C}$  75 MHz) spectrometers. Chemical shifts ( $\delta$ ) are expressed in ppm and coupling constants ( $J$ ) are in Hertz. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and all glassware was oven-dried. Isolation and purification of the compounds were performed by flash column chromatography on silica gel 60 (230–400 mesh) through isocratic or gradient elution with different ratios of cyclohexane/ethyl acetate mixture and Büchi Pump Manager (C-615 and C-601) equipment. Thin layer chromatography (TLC) analyses were performed by using commercial silica gel 60 F<sub>254</sub> aluminum sheets.

### Path A: a general procedure for the synthesis of compounds 3a, 3b, 3c, and 3e

Ferulic acid (4.12 mmol, 1 eq) and the selected amine (4.12 mmol, 1 eq) were dissolved in dry DMF (37.5 mL, 0.11 M) under  $\text{N}_2$  atmosphere. EDC·HCl (4.53 mmol, 1.1 eq), HOBT (4.53 mmol, 1.1 eq) and DIPEA (8.24 mmol, 2 eq) were added. The solution was stirred at room temperature for 24 h, then DMF was evaporated under reduced pressure. The crude was solubilized in ethyl acetate and washed with HCl 0.5 N, 5%  $\text{NaHCO}_3$ , water, and brine. The organic phases were dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated under reduced pressure. The crude products were purified by flash chromatography.

(3a) Purification by flash chromatography (cyclohexane/ethyl acetate 6:4) gave the desired product as a yellow oil in 89% yield.  $^1\text{H}$  NMR (600 MHz, acetone-*d*<sub>6</sub>)  $\delta$  8.04 (s, 1H, OH), 7.46 (d,  $J=15.6$  Hz, 1H, H3), 7.34–7.24 (m, 4H, Ar H2', Ar H6', Ar H3', Ar H5'), 7.24–7.19 (m, 1H, Ar H4'), 7.18 (d,  $J=1.9$  Hz, 1H, Ar H2), 7.06 (dd,  $J=8.2$ , 1.9 Hz, 1H, Ar H6), 6.85 (d,  $J=8.1$  Hz, 1H, Ar H5), 6.50 (d,  $J=15.6$  Hz, 1H, H2), 3.90 (s, 3H, OCH<sub>3</sub>), 3.56 (dd,  $J=13.0$ , 7.4 Hz, 2H, H1'), 2.87 (t,  $J=7.4$  Hz, 2H, H2').  $^{13}\text{C}$  NMR (150 MHz, acetone-*d*<sub>6</sub>)  $\delta$  165.6 (C1), 148.3 (Ar C4), 147.7 (Ar C3), 139.7 (C3), 139.6 (Ar C1'), 128.7 (Ar C3', Ar C5'), 128.3 (Ar C2', Ar C6'), 127.4 (Ar C5), 126.1 (Ar C4'), 121.7 (Ar C6), 119.1 (C2), 115.2 (Ar C5), 110.4 (Ar C2), 55.3 (OCH<sub>3</sub>), 40.7 (C1'), 35.7 (C2').

(3b) Purification by flash chromatography (cyclohexane/ethyl acetate 4:6) gave the desired product as a white solid in >99% yield.  $^1\text{H}$  NMR (300 MHz, acetone-*d*<sub>6</sub>)  $\delta$  8.06 (s, 1H, OH), 7.46 (d,  $J=15.6$  Hz, 1H, H3), 7.29 (t,  $J=5.8$  Hz, 1H, NH), 7.17 (d,  $J=1.9$  Hz, 1H, Ar H2), 7.11–7.03 (m, 3H, Ar H6, Ar H2', Ar H6'), 6.85 (d,  $J=8.1$  Hz, 1H, Ar H5), 6.80–6.75 (m, 2H, Ar H3', Ar H5'), 6.51 (d,  $J=15.6$  Hz, 1H, H2), 3.89 (s, 3H, OCH<sub>3</sub>), 3.57–3.45 (m, 2H, H1'), 2.77 (t,  $J=7.3$  Hz, 2H, H2').  $^{13}\text{C}$  NMR (150 MHz, acetone-*d*<sub>6</sub>)  $\delta$  165.7 (C1), 155.9 (Ar C4'), 148.3 (Ar C4), 147.7 (Ar C3), 139.6 (C3), 130.2 (Ar C2', Ar C6'), 129.6 (Ar C1), 127.4 (Ar C6), 121.7 (Ar C1'), 119.1 (C2), 115.2 (Ar C5, Ar C3', Ar C5'), 110.4 (Ar C2), 55.3 (OCH<sub>3</sub>), 41.1 (C1'), 34.9 (C2').

(3c) Purification by flash chromatography (cyclohexane/ethyl acetate 4:6) gave the desired product as a yellow solid in 80% yield.  $^1\text{H}$  NMR (300 MHz, acetone-*d*<sub>6</sub>)  $\delta$  10.05 (s, 1H, Ar NH), 7.95 (s, 1H, OH), 7.65 (d,  $J=7.9$  Hz, 1H, Ar H4'), 7.48 (d,  $J=15.6$  Hz, 1H, H3), 7.40 (d,  $J=8.1$  Hz, 1H, Ar H7'), 7.33 (t,  $J=5.8$  Hz, 1H, NH) 7.20 (d,  $J=2.3$  Hz, 1H, Ar H2'), 7.18 (d,  $J=1.9$  Hz, 1H, Ar H2), 7.14–7.08 (m, 1H, Ar H6'), 7.06 (dd,  $J=8.4$ , 1.5 Hz, 2H, Ar H6, Ar H5'), 7.02 (dd,  $J=7.0$ , 0.9 Hz, 1H, NH), 6.85 (d,  $J=8.1$  Hz, 1H, Ar H5), 6.52 (d,  $J=15.6$  Hz, 1H, H2), 3.89 (s, 3H, OCH<sub>3</sub>), 3.65 (td,  $J=7.3$ , 5.8 Hz, 2H, H1'), 3.02 (t,  $J=7.2$  Hz, 2H, H2').  $^{13}\text{C}$  NMR (150 MHz, acetone-*d*<sub>6</sub>)

$\delta$  165.7 (C1), 148.2 (Ar C4), 147.7 (Ar C3), 139.5 (C3), 136.9 (Ar C7a'), 127.7 (Ar C3a'), 127.4 (Ar C1), 122.5 (Ar C6), 121.7 (Ar C2'), 121.2 (Ar C6'), 119.3 (Ar C4'), 118.5 (C2, Ar C5'), 115.3 (Ar C5), 112.6 (Ar C3'), 111.3 (Ar C2), 110.4 (Ar C7'), 55.3 (OCH<sub>3</sub>), 39.9 (C1'), 25.6 (C2').

(**3e**) This compound was synthesized starting from Boc-protected putrescine. Purification by flash chromatography (cyclohexane/ethyl acetate 1:9) gave *tert*-butyl (*E*)-(4-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)butyl)carbamate (**Boc-3e**) as a white solid in 87% yield. <sup>1</sup>H NMR (300 MHz, acetone-*d*<sub>6</sub>)  $\delta$  8.07 (s, 1H, OH), 7.45 (d, *J* = 15.6 Hz, 1H, H3), 7.31 (t, *J* = 6.2 Hz, 1H, NH), 7.16 (d, *J* = 1.9 Hz, 1H, Ar H2), 7.06 (dd, *J* = 8.2, 1.9 Hz, 1H, Ar H6), 6.85 (d, *J* = 8.1 Hz, 1H, Ar H5), 6.50 (d, *J* = 15.6 Hz, 1H, H2), 5.97 (s, 1H, NHBoc), 3.90 (s, 3H, OCH<sub>3</sub>), 3.33 (q, *J* = 6.3 Hz, 2H, H1'), 3.11 (q, *J* = 6.3 Hz, 2H, H4'), 1.64–1.49 (m, 4H, H2', H3'), 1.41 (s, 9H, OC(CH<sub>3</sub>)<sub>3</sub>). <sup>13</sup>C NMR (150 MHz, acetone-*d*<sub>6</sub>)  $\delta$  165.6 (C1), 155.9 (COOC(CH<sub>3</sub>)<sub>3</sub>), 148.3 (Ar C4), 147.8 (Ar C3), 139.4 (C3), 127.4 (Ar C1), 121.6 (Ar C6), 119.2 (C2), 115.3 (Ar C5), 110.4 (Ar C2), 77.5 (OC(CH<sub>3</sub>)<sub>3</sub>), 55.3 (OCH<sub>3</sub>), 39.9 (C4'), 38.8 (C1'), 27.8 (C(CH<sub>3</sub>)<sub>3</sub>), 27.5 (C3'), 26.9 (C2').

A solution of the above compound (0.55 mmol, 1 eq) and TFA 10% in DCM (5.5 mmol, 10 eq) was stirred at room temperature for 5 h. TFA was evaporated under reduced pressure and precipitation in diethyl ether gave **3e** as a white solid in 97% yield. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  9.44 (s, 1H, OH), 8.02 (t, *J* = 5.7 Hz, 1H, NH), 7.70 (s, 2H, NH<sub>2</sub>), 7.32 (d, *J* = 15.7 Hz, 1H, H3), 7.12 (d, *J* = 1.8 Hz, 1H, Ar H2), 6.99 (dd, *J* = 8.2, 1.8 Hz, 1H, Ar H6), 6.80 (d, *J* = 8.1 Hz, 1H, Ar H5), 6.44 (d, *J* = 15.7 Hz, 1H, H2), 3.81 (s, 3H, OCH<sub>3</sub>), 3.25–3.14 (q, *J* = 6.3 Hz, 2H, H1'), 2.81 (dd, *J* = 12.8, 6.5 Hz, 2H, H4'), 1.63–1.43 (m, 4H, H2', H3'). <sup>13</sup>C NMR (150 MHz, methanol-*d*<sub>4</sub>)  $\delta$  167.9 (C1), 148.5 (Ar C4), 147.9 (Ar C3), 140.8 (C3), 126.9 (Ar C1), 121.9 (Ar C6), 117.2 (OCH<sub>3</sub>), 115.1 (Ar C5), 110.1 (Ar C2), 55.0 (OCH<sub>3</sub>), 38.9 (C4'), 38.2 (C1'), 26.2 (C3'), 24.5 (C2').

### Synthesis of compound 3f

1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine (0.47 mmol, 0.9 eq) was added dropwise to a solution of **3e** (0.53 mmol, 1 eq) and TEA (1.59 mmol, 3 eq) in dry DCM (3.12 mL, 0.17 M) under N<sub>2</sub> atmosphere. The solution was stirred at room temperature for 4 h. The reaction mixture was diluted with DCM and washed with H<sub>2</sub>O. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and the solvent was evaporated under reduced pressure. Purification by flash chromatography (cyclohexane/ethyl acetate 5:5) gave **di-Boc-3f** as a pale white solid in 64% yield. <sup>1</sup>H NMR (600 MHz, acetone-*d*<sub>6</sub>)  $\delta$  11.52 (s, 1H, OH), 8.29–8.13 (m, 1H, NH-C), 7.92–7.74 (m, 1H,

NHBoc), 7.31 (d, *J* = 15.6 Hz, 1H, H3), 7.15 (t, *J* = 5.3 Hz, 1H, NH), 7.03 (d, *J* = 1.9 Hz, 1H, Ar H2), 6.92 (dd, *J* = 8.2, 1.9 Hz, 1H, Ar H6), 6.70 (d, *J* = 8.1 Hz, 1H, Ar H5), 6.37 (d, *J* = 15.6 Hz, 1H, H2), 3.75 (s, 3H, OCH<sub>3</sub>), 3.31 (dd, *J* = 12.7, 7.0 Hz, 2H, H4'), 3.23 (dd, *J* = 12.6, 6.6 Hz, 2H, H1'), 1.57–1.50 (m, 2H, H2'), 1.50–1.43 (m, 2H, H3'), 1.38 (s, 9H, OC(CH<sub>3</sub>)<sub>3</sub>), 1.30 (s, 9H, OC(CH<sub>3</sub>)<sub>3</sub>). <sup>13</sup>C NMR (150 MHz, acetone-*d*<sub>6</sub>)  $\delta$  165.6 (C1), 163.7 (C=NBoc), 156.1 (NHCOOC(CH<sub>3</sub>)<sub>3</sub>), 152.9 (NCOOC(CH<sub>3</sub>)<sub>3</sub>), 148.3 (Ar C4), 147.8 (Ar C3), 139.5 (C3), 127.4 (Ar C1), 121.6 (Ar C6), 119.3 (C2), 115.3 (Ar C5), 110.5 (Ar C2), 82.8 (C(CH<sub>3</sub>)<sub>3</sub>), 78.0 (C(CH<sub>3</sub>)<sub>3</sub>), 55.4 (OCH<sub>3</sub>), 40.2 (C4'), 38.7 (C1'), 27.6 (2xC(CH<sub>3</sub>)<sub>3</sub>), 27.3 (C3'), 26.7 (C2').

A solution of **di-Boc-3f** (0.326 mmol, 1 eq) and TFA 10% in DCM (3.26 mmol, 10 eq) was stirred at room temperature for 5 h. TFA was evaporated under reduced pressure and precipitation in diethyl ether gave **3f** as a brown solid in 94% yield. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  9.61–9.27 (s, 1H, OH), 8.02 (t, *J* = 5.7 Hz, 1H, CH<sub>2</sub>NH), 7.58 (t, *J* = 5.5 Hz, 1H, CONH), 7.32 (d, *J* = 15.7 Hz, 1H, H3), 7.12 (d, *J* = 1.8 Hz, 1H, Ar H2), 6.99 (dd, *J* = 8.2, 1.8 Hz, 1H, Ar H6), 6.80 (d, *J* = 8.1 Hz, 1H, Ar H5), 6.44 (d, *J* = 15.7 Hz, 1H, H2), 3.81 (s, 3H, OCH<sub>3</sub>), 3.22–3.16 (m, 2H, H4'), 3.16–3.09 (m, 2H, H1'), 1.56–1.40 (m, 4H, H2', H3'). <sup>13</sup>C NMR (150 MHz, methanol-*d*<sub>4</sub>)  $\delta$  168.0 (C1), 157.3 (C=NH), 148.5 (Ar C4), 147.9 (Ar C3), 140.8 (C3), 126.9 (Ar C1), 121.9 (Ar C6), 117.3 (C2), 115.1 (Ar C5), 110.1 (Ar C2), 55.0 (OCH<sub>3</sub>), 40.7 (C4'), 38.4 (C1'), 26.4 (C3'), 25.8 (C2').

The synthesis of compound **3d** was afforded in three steps, starting from anthranilic methyl ester.

To a solution of ferulic acid (2.57 mmol, 1 eq) in dry DCM (13 mL, 0.2 M) under N<sub>2</sub>, thionyl chloride (25.7 mmol, 10 eq) and dry DMF (64  $\mu$ L) were added. The solution was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure to obtain feruloyl chloride which was used for the next step without further purification.

To a solution of methyl anthranilate (5.14 mmol, 2 eq) in dry THF (1.3 mL), a solution of feruloyl chloride (2.57 mmol, 1 eq) in dry THF (2.57 mL) was added dropwise. The reaction was stirred at 10 °C for 30 min. The solution was diluted with DCM, washed with HCl 0.5 N, and 5% NaHCO<sub>3</sub>. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and the solvent was evaporated under reduced pressure. Purification by flash chromatography (cyclohexane/ethyl acetate 8:2) gave product methyl (*E*)-2-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)benzoate as a pale yellow solid in 65% yield. <sup>1</sup>H NMR (300 MHz, acetone-*d*<sub>6</sub>)  $\delta$  11.15 (s, 1H, NH), 8.90 (dd, *J* = 8.5, 0.9 Hz, 1H, Ar H3'), 8.08 (m, 1H, Ar H6'), 7.67 (d, *J* = 15.5 Hz, 1H, H2), 7.66–7.61 (m, 1H, Ar H5'), 7.46 (d, *J* = 1.9 Hz, 1H, Ar H2), 7.22 (dd, *J* = 8.2, 1.9 Hz, 1H,

Ar H6), 7.20–7.14 (m, 1H, Ar H4'), 6.91 (d,  $J=8.1$  Hz, 1H, Ar H5), 6.75 (d,  $J=15.5$  Hz, 1H, H2), 3.99 (s, 3H, COOCH<sub>3</sub>), 3.95 (s, 3H, OCH<sub>3</sub>). <sup>13</sup>C NMR (150 MHz, acetone-*d*<sub>6</sub>)  $\delta$  168.5 (COOCH<sub>3</sub>), 164.3 (C1), 148.9 (Ar C4), 147.9 (Ar C3), 142.4 (C3), 142.1 (Ar C2'), 134.3 (Ar C4'), 130.8 (Ar C6'), 126.9 (Ar C1), 123.0 (Ar C6), 122.2 (Ar C3', Ar C5'), 120.2 (C2), 119.0 (Ar C1'), 115.2 (Ar C5), 110.4 (Ar C2), 55.5 (OCH<sub>3</sub>), 52.0 (COOCH<sub>3</sub>).

To a solution of methyl (*E*)-2-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)benzoate (0.710 mmol, 1 eq) in methanol (1:1 with NaOH) a solution of NaOH 1 M (2.13 mL, 3 eq) was added. The reaction was stirred at room temperature for 2 h, acidified with HCl 1 M, and extracted with ethyl acetate. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and the solvent was evaporated under reduced pressure. **3d** was isolated without any further purification as a white solid in 92% yield. <sup>1</sup>H NMR (300 MHz, acetone-*d*<sub>6</sub>)  $\delta$  11.41 (s, 1H, COOH), 8.92 (dd,  $J=8.5, 0.8$  Hz, 1H, Ar H6'), 8.15 (dd,  $J=7.9, 1.5$  Hz, 1H, Ar H3'), 7.65 (d,  $J=15.4$  Hz, 1H, H3), 7.64 (m, 1H, Ar H4'), 7.44 (d,  $J=1.9$  Hz, 1H, Ar H2), 7.21 (dd,  $J=8.2, 1.9$  Hz, 1H, Ar H6), 7.18 (m, 1H, Ar H5'), 6.90 (d,  $J=8.2$  Hz, 1H, Ar H5), 6.70 (d,  $J=15.5$  Hz, 1H, H2), 3.94 (s, 3H, OCH<sub>3</sub>). <sup>13</sup>C NMR (150 MHz, acetone-*d*<sub>6</sub>)  $\delta$  169.8 (COOH), 164.4 (C1), 148.9 (Ar C4), 147.9 (Ar C3), 142.5 (C3), 142.2 (Ar C2'), 134.3 (Ar C4'), 131.4 (Ar C6'), 126.9 (Ar C1), 122.9 (Ar C6), 122.1 (Ar C3', Ar C5'), 120.1 (C2), 119.2 (Ar C1'), 115.2 (Ar C5), 110.6 (Ar C2), 55.5 (OCH<sub>3</sub>).

#### Path B: a general procedure for the synthesis of compounds **3a**, **3b**, **3c**, and **3e**

To a solution of 0.25 M of ethyl ferulate (1.25 mmol, 1 eq) in *tert*-amyl alcohol with 4 Å molecular sieves, the selected amine (0.5 M, 2.5 mmol, 2 eq) was added together with CaL-B acrylic resin (50 mg/mL). The obtained reaction mixture (final volume: 5 mL) was stirred at 200 rpm at 80 °C for 24 h. Then, the enzyme was removed by filtration and the solvent evaporated under reduced pressure. The crude products were purified by flash chromatography as described in path A and the desired products were isolated with the following yields: (**3a**) 41%, (**3b**) 23%, (**3c**) 21%, (**3e**) 6%.

Compound **3f** was synthesized from **3e** following the procedure described in Path A.

#### Antifungal activity of new compounds

In this study, four strains belonging to three different species were used: *Pyricularia oryzae* strain sensitive to quinone outside inhibitor (QoI) fungicides A252 and resistant to QoI PO21\_07, *Fusarium culmorum* Fc-UK (NRRL54111) [30] and *Botrytis cinerea* BC\_2A. The strains belong to a vast collection of monoconidial isolates maintained at the Laboratory of Plant Pathology,

University of Milan. The strains were maintained as single-spore isolates on malt-agar medium (MA: 20 g/L malt extract, Oxoid, U.K.; 15 g/L agar, VWR Life Science, U.S.A.) at 4 °C.

#### Inhibition of mycelium growth

The mycelium inhibition of the fungal strains by the tested compounds was evaluated as previously described [31]. Briefly, a 0.5 cm mycelium plug obtained from actively growing fungal colonies was transferred to MA medium plates supplemented or not with tested compounds in three biological replicates. The compounds were tested at a concentration of 500  $\mu$ M. Due to the low solubility of the tested molecules in water, they were dissolved in DMSO. Therefore, multiple controls were included: MA medium without any supplement (MA), and MA medium supplemented with DMSO at a final concentration of 1% v/v. The plates were incubated at 24 °C in the dark. The mycelium growth was measured 7 days after inoculation (DAI), and the inhibition of mycelium growth (%) was calculated by comparing the mycelium growth on solvent-containing medium and compound-supplemented plates.

#### *Pyricularia oryzae* spore germination and appressorium inhibition

*Pyricularia oryzae* PO21\_02 was inoculated on an MA medium and incubated in the growth chamber at 24 °C in the dark for 12–14 days. Then, 2 mL of sterile water were added to the mycelium and the formed conidia were scraped from the whole mycelium surface with the help of the glass spatula. The spore suspension was collected and filtered through two layers of sterile gauze into an Eppendorf tube to remove mycelium. The spore concentration was estimated using the Thoma hemacytometer and adjusted to a concentration of  $2 \times 10^4$  conidia/mL.

The tested compounds were dissolved in MeOH and were added to 100  $\mu$ L of conidial suspension to obtain a final concentration of 500  $\mu$ M and 1% MeOH. Conidia treated with 1% MeOH were considered control.

Twenty  $\mu$ L of conidial suspension in three replicates were applied on a microscopic cover slide in a wet chamber and incubated for 24 h at 24 °C in the dark. The germination of 100 randomly chosen conidia for each treatment and replica was determined. The conidia were assigned into germination classes: NG = not germinated, G = germinated, and A = germinated with appressorium.

#### *Botrytis cinerea* spore germination and germ tube elongation

*Botrytis cinerea* BC\_2A was inoculated on Czapek-Dox Yeast medium (CZY; 35 g/L Difco Czapek-Dox broth, BD, France; 2 g/L Difco yeast extract, Oxoid, U.K.; 15 g/L agar, VWR Life Science, U.S.A.) and incubated in the

growth chamber at 24 °C in the dark for 7–10 days. The spores were collected as described for *Pyricularia oryzae* and the concentration was adjusted to  $1 \times 10^4$  conidia/mL and were then diluted in 20% Potato dextrose broth (PDB; Difco Potato dextrose broth, BD, France).

The compounds were added to the spore suspension as described before. Twenty  $\mu\text{L}$  of conidial suspension in three replicates were applied on a microscopic cover slide in a wet chamber and incubated for 16 h at 15 °C in the dark. The germination of 100 randomly chosen conidia for each treatment and replica was determined. Moreover, the pictures of the germinating spores were taken using an Olympus BX 51 microscope equipped with an Olympus DP71 digital camera and the germination tube length of 20 randomly chosen spores for each treatment was measured using the Cell D software program (Olympus Soft Imaging Solutions GmbH, Germany).

#### Inhibition percentage calculations

The inhibition percentage of mycelium growth was calculated as  $I\% = (C - T)/C \times 100$ , where  $C$  = mycelium growth in the solvent medium and  $T$  = mycelium growth in the medium added to the tested compound.

For spore germination, the percentage of spore germination was calculated according to the formula: Germ (%) =  $\Sigma(G + A + ANM)/n \times 100$ , where  $n$  = total number of spores observed per replica. The inhibition of spore germination was calculated as  $IG (\%) = (\text{GermC} - \text{GermT})/\text{GermC} \times 100$ , where GermC was % germination in the control (solvent), and GermT was % germination in the treated sample.

Inhibition of appressoria formation was calculated as  $IA (\%) = (\text{GermT} - \text{AT})/\text{GermT} \times 100$ , where AT was the percentage of spores with appressoria in the treated sample.

#### Antibacterial activity of new compounds

The antibacterial activity of compounds **3a–3f** was evaluated against foodborne pathogens *Enterococcus faecalis* ATCC 700802 (V583), *Escherichia coli* ATCC 8739, *Listeria monocytogenes* DSM 20600, *Pseudomonas aeruginosa* DSM 939, *Staphylococcus aureus* DSM 799, *Salmonella enterica* subsp. *enterica* ATCC 13311.

Pathogens were cultivated in Brain Heart Infusion (BHI, Bioline Italiana, Milan, Italy) for 24 h at 37 °C. After the incubation, bacterial cell density was adjusted to  $0.3 \pm 0.05$  (ca.  $10^8$  cfu/mL) in sterile saline solution.

Dry powder compounds **3a–3f** were dissolved in 50% DMSO:water solution as received. Then, to minimize DMSO carryover, these solutions were diluted to 1 mg/mL (1000 ppm) in plate count broth (PCB) and further diluted in PCB to 100, 50, 25 and 12.5 ppm. Each concentration was inoculated with 5% of bacterial cell

suspension. PCB without compounds and cells was used as blank while PCB supplemented with compounds without cells was considered to measure broth absorbance background.

Bacterial growth was monitored by measuring optical density every 10 min with the Varioskan Flash (Thermo Fischer Scientific) spectrofluorimeter at a wavelength of 600 nm up to 24 h at 37 °C as previously reported [32, 33]. Each antimicrobial assay was performed in triplicate. Growth curves were analyzed through the SkanIt™ software (Thermo Fischer Scientific) to calculate average and maximum growth rate ( $\mu_{\text{av}} \text{ABS h}^{-1}$ ,  $\mu_{\text{max}} \text{ABS h}^{-1}$ ) [34], time to max rate, time to change and the half-run absorbance value.

For the molecule showing the strongest effect, the doubling time (DT, h), defined as the time needed to doubling the OD value starting from the beginning of the exponential growth, as reported by Scher et al., was also calculated [35].

#### Statistical analysis

The statistical analyses were performed using R software, version 4.4.0. [36] in the R Studio, version 2024.09.0.375 [37]. The percentage data of mycelium growth inhibition and *P. oryzae* spore germination and appressorium formation were square root arcsine transformed and together with *B. cinerea* germ tube length data were submitted to ANOVA, followed by Tukey's post hoc test for multiple comparison ( $P = 0.05$ ), using the TukeyC package [38].

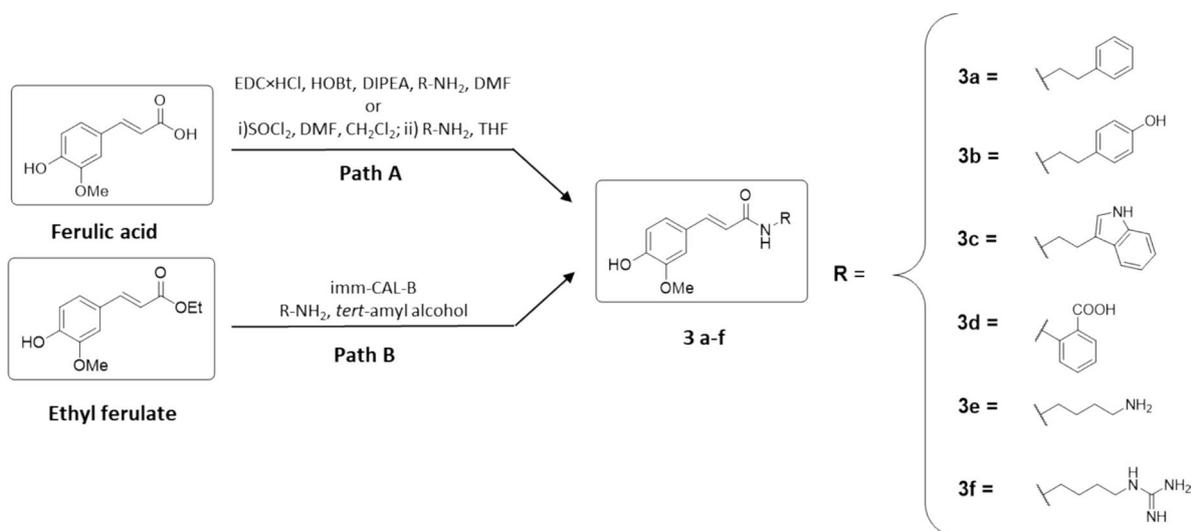
## Results and discussion

### Synthesis

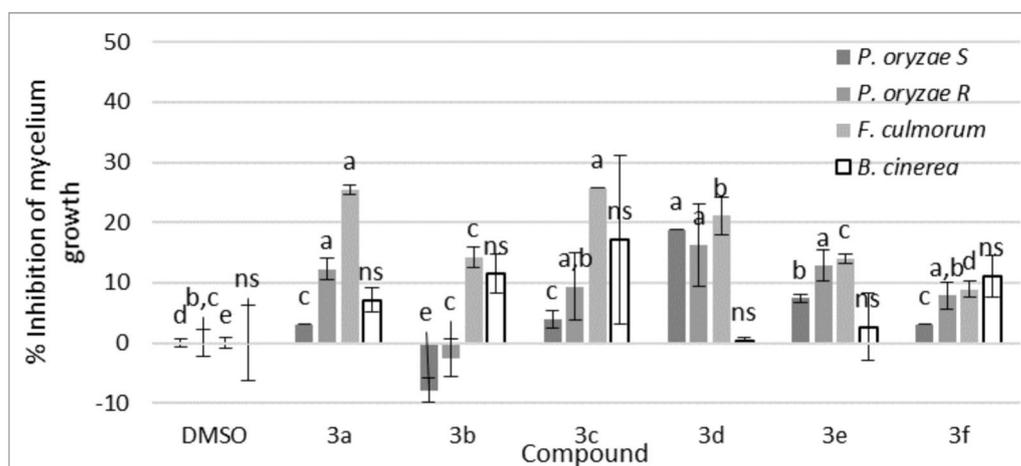
Most phenylamides are not commercially available. Since they are present only in small amounts in natural matrices and their purification from plants could be troublesome, we prepared a small collection of natural PAs by an efficient synthetic strategy, in order to produce them in adequate quantity to carry out biological activity studies.

Ferulic acid was selected as a representative cinnamic acid, due to its high natural abundance (Fig. 1). The synthesis of FerPAs was approached using a traditional amidation protocol (Fig. 1, Path A). Ferulic acid and the desired amine were stirred at room temperature for 24 h under nitrogen atmosphere in dry DMF and DIPEA, using HOBt and EDC·HCl as coupling agents to obtain **3a**, (FerPhe), **3b** (FerTyr), **3c** (FerTry), **3e** (FerPut), and **3f** (FerAgm). Avenantramide **3d** (FerAnt) was synthesized by reaction of feruloyl chloride with methyl anthranilate in dry THF, followed by the hydrolysis of the ester group (Fig. 1, Path A). All the desired amides **3a–f** were isolated in excellent yield (from 80% to quantitative).

In parallel, with the aim of developing a more sustainable protocol, we explored an enzymatic approach



**Fig. 1** Synthetic pathways for the obtention of compounds 3a-f



**Fig. 2** Activity of compounds against mycelium growth of *P. oryzae* QoI-sensitive (S, PO-A252) and resistant (R, PO21\_07) strains, *F. culmorum* FC-UK and *B. cinerea* BC-2A. 1% DMSO was used as a solvent for the compounds. Error bars represent the standard deviation. The different letters indicate statistically significant differences among means ( $P > 0.05$ ) calculated by the Tukey post hoc test. ns = not significant

based on the use of the cheap and commercially available immobilized lipase B from *Candida antarctica* (Imm-CaL-B) in the unconventional green solvent *tert*-amyl alcohol (Fig. 1, Path B).

To a 0.25 M solution of ethyl ferulate in *tert*-amyl alcohol, the selected amine and imm-CaL-B were added and stirred at 80 °C for 24 h. Compounds 3a-c and 3e-f were obtained in modest to good isolated yields (6–41%). Product 3d was not isolated in the tested conditions, most likely due to the substrate specificity of CaL-B.

### Evaluation of antifungal activity

The antifungal activity on mycelium growth was evaluated both on wild-type (QoI-S) and azoxystrobin-resistant (QoI-R) strains of *P. oryzae*. Additionally, the activity was tested on two other pathogens: *B. cinerea*, responsible for grey mold disease, and *F. culmorum*, which causes Fusarium head blight in wheat and barley.

None of the compounds showed noteworthy inhibition of the mycelium growth (Fig. 2). *F. culmorum* seemed to be the most sensitive fungal pathogen, but the inhibition was just moderate for compounds 3a, 3c and 3d (25.46%, 25.69%, and 21.10% respectively).

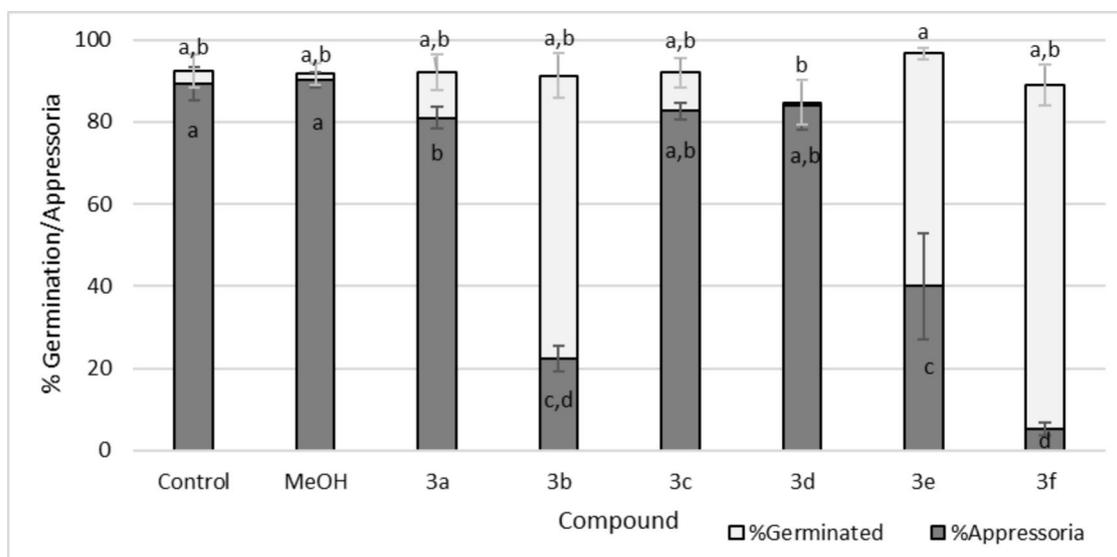
**Table 1** Inhibition of spore germination and appressorium formation of *P. oryzae* by the tested compounds

Compound	Structure	<i>P. oryzae</i>	
		Germination inhibition %	Appressorium inhibition %
3a		N.I. <sup>a</sup>	11.96 ± 2.88
3b		N.I.	75.55 ± 3.52
3c		N.I.	10.14 ± 2.26
3d		10.75 ± 5.69	N.I.
3e		N.I.	58.62 ± 13.45
3f		N.I.	94.03 ± 1.74

Mean % inhibition with standard deviation are listed. <sup>a</sup>N.I. = inhibition < 10%

The antifungal activity of the synthesized compounds was evaluated also on spores of *P. oryzae*. The results are reported in Table 1, Fig. 3 and Supplementary Figure S1. None of the compounds was effective on germination, all showing a germination inhibition  $\leq 10\%$ . However, the situation was different when we considered the inhibition of the appressorium development. The best results were obtained by compounds **3b** and **3f** with a 75% and 94% inhibition of appressoria formation, respectively. Also compound **3e** showed a significant activity with an inhibition rate of 59%. The comparison between compounds **3a** and **3b** suggested that the hydroxy group on

the aromatic ring has a relevant impact on the activity. When the terminal amine group of **3e** was replaced with a guanidino group, as in **3f**, the activity improved considerably, suggesting that the basicity of the guanidino group could enhance the inhibition of the appressorium formation. Considering the importance of ferroptosis for appressorium development [33], we evaluated the effect of the tested compounds on iron availability. None of them showed chelating activity, suggesting that other mechanisms are involved in appressorium inhibition (Supplementary Figure S2).



**Fig. 3** Activity of compounds on the germination (light grey) and appressorium formation (dark grey) of *P. oryzae* spores. A solution of 1% methanol in water (MeOH) was used as a solvent for the compounds. Control represents water. Error bars represent the standard deviation. The different letters indicate statistically significant differences among means ( $P > 0.05$ ) calculated by the Tukey post hoc test

Last, the activity of the compounds on germination and germ tube length of *B. cinerea* was evaluated (Table 2). Similarly to *P. oryzae*, none of the compounds inhibited spore germination, as >97% of spore germination was observed for all treatments. Only a minimum reduction of germ tube growth of *B. cinerea* was observed for compound **3a**, which was not statistically different from the solvent (MeOH). On the contrary, compounds **3c** and **3d** statistically enhanced the growth of the *B. cinerea* germ tube (Supplementary Figure S1).

#### Evaluation of antibacterial activity

The in vitro antibacterial activity of the prepared phenylamides against six foodborne pathogens was also evaluated. The effect of the addition of these molecules to microbial broth medium, in the range of 12.5–100 mg/L, was assessed by calculating the following parameters for each strain and at each concentration; (i) the average and (ii) maximum growth rate, (iii) the time to reach the max growth rate and (iv) the time to change absorbance value from baseline value. The absorbance values measured after 12 h incubation (v) (reading n° 72) were also compared. In addition, for the most active compound, (vi) the doubling time (DT, h) was calculated.

The antibacterial effect of phenylamides was evaluated by comparing the growth parameters retrieved from the growth kinetics of control samples not supplemented with the compounds with those supplemented with the organic molecules (Table 3, Supplementary Table 1).

The strongest antibacterial effect was shown by compound **3d** followed by **3b** with 54 and 32 values differentiating from their controls, respectively (Table 3).

Compound **3d** was thus selected for more detailed analysis. Figures 4 and 5 show the absorbance growth curves of *E. faecalis* ATCC 700802 (V583), *S. enterica* subsp. *enterica* ATCC13311 (5A) and *L. monocytogenes* DSM 20600 (5B) in the presence of different concentrations of compound **3d**.

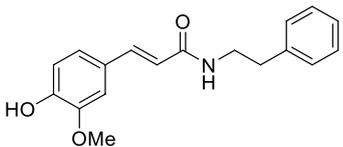
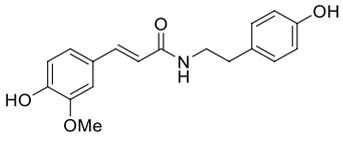
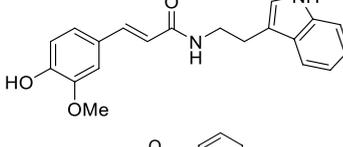
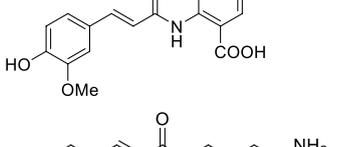
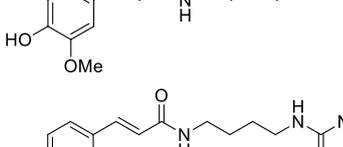
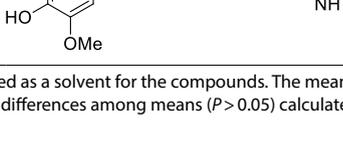
It is interesting to note that Gram-positive strains were more sensitive than Gram-negative ones. For those strains, it is possible to define multiple antibacterial effects on the growth kinetics. With regards to the Gram-negative bacteria, only *E. coli* ATCC 8739 was specifically affected by the addition of compound **3a** compared to other strains, as demonstrated by the high number of growth parameters (13) affected by the addition of phenylamides (Table 3).

In the case of *E. faecalis* ATCC 700802, compound **3d** caused a reduction in the growth rates and delay in increasing absorbance values above the baseline (time to change).

A stronger activity was found against *L. monocytogenes* DSM 20600 (Fig. 5, panel B) which was unable to grow when treated with **3d** at concentrations 100 and 50 mg/L.

In addition, at 25 mg/L the achieved maximum optical density was 0.16 compared to 0.318 (at the same reading) of the control samples. Since the starting exponential absorbance value (0.098) produced after 2.5 h incubation

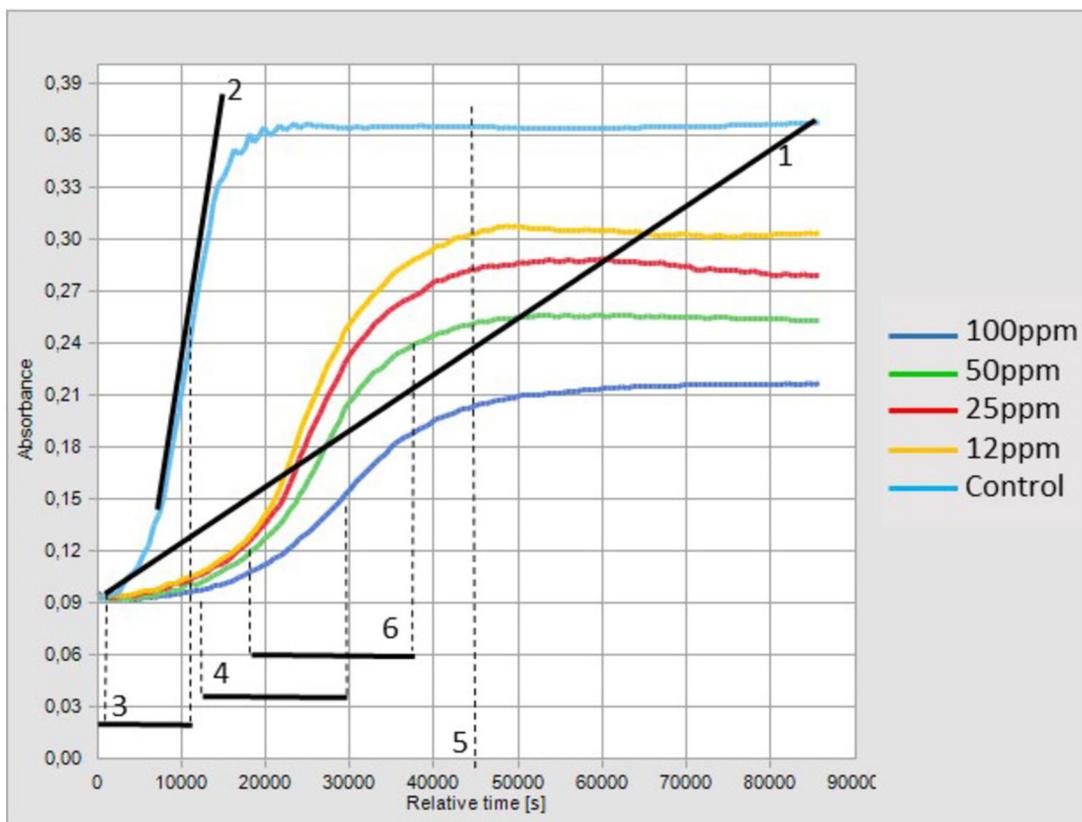
**Table 2** Effect of the tested compounds on germ tube length of *B. cinerea*

Compound	Structure	<i>B. cinerea</i> Germ tube length ( $\mu\text{m}$ )
MeOH		115.3 $\pm$ 76.9 b,c
3a		99.2 $\pm$ 24.9 c
3b		125.6 $\pm$ 58 b,c
3c		202.3 $\pm$ 67.2 a
3d		191.5 $\pm$ 86.8 a
3e		167.3 $\pm$ 79.8 a,b
3f		127.8 $\pm$ 42.8 b,c

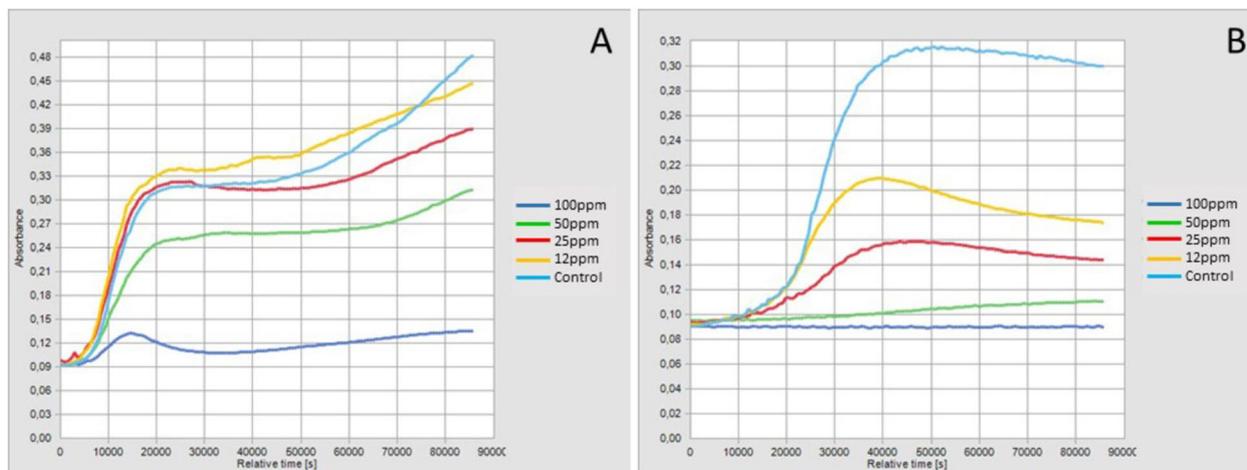
A solution of 1% methanol in water (MeOH) was used as a solvent for the compounds. The mean length of the germ tube with standard deviation in brackets is listed. The different letters indicate statistically significant differences among means ( $P > 0.05$ ) calculated by the Tukey post hoc test

**Table 3** Number of growth parameters of six pathogenic bacterial strains affected by the addition of phenylamides in the culture medium after 24 h at 37 °C

Bacterial strain	3a	3b	3c	3d	3e	3f
Gram-negative						
<i>E. coli</i> ATCC 8739	13	5	4	4	0	1
<i>P. aeruginosa</i> DSM 939	1	2	1	3	0	0
<i>S. enterica</i> ATCC 13311	5	7	2	7	0	1
Gram-positive						
<i>E. faecalis</i> ATCC 700802	2	4	3	11	9	5
<i>L. monocytogenes</i> DSM 20600	1	9	5	18	1	2
<i>S. aureus</i> DSM 799	1	6	4	11	7	9
Total	23	33	19	54	17	18



**Fig. 4** Absorbance growth curve of *E. faecalis* ATCC 700802 (V583), in the presence of different concentrations of compound **3d**. Growth parameters: 1, average growth rate; 2, max growth rate; 3, time to max rate; 4, time to change; 5, absorbance value after 12 h of incubation; 6, doubling absorbance time



**Fig. 5** Microbial growth kinetics of *S. enterica* subsp. *enterica* ATCC13311 (**A**) and *L. monocytogenes* DSM 20600 (**B**) grown by automatic 24 h absorbance reading when incubated with molecule **3d** at different concentrations

**Table 4** Values of different growth parameters deriving from the absorbance growth curve of six bacterial pathogens supplemented with different concentrations of the compound **3d**

Growth parameter	Bacterial strain	Control	Concentration (mg/L)			
			100	50	25	12
Average growth rate $\Delta$ Abs/h	<i>E. coli</i> ATCC 8739	0.027	<b>0.005</b>	0.019	0.027	0.028
	<i>P. aeruginosa</i> DSM 939	0.060	0.055	0.056	0.058	0.058
	<i>S. enterica</i> ATCC 13311	0.013	<b>0.009</b>	<b>0.009</b>	0.011	0.012
	<i>E. faecalis</i> ATCC 700802	0.007	0.007	0.008	0.009	0.010
	<i>L. monocytogenes</i> DSM 20600	0.011	<b>nd</b>	<b>0.001</b>	<b>0.003</b>	<b>0.004</b>
	<i>S. aureus</i> DSM 799	0.011	<b>nd</b>	<b>0.002</b>	<b>0.002</b>	<b>0.003</b>
Max growth rate $\Delta$ Abs/h	<i>E. coli</i> ATCC 8739	0.129	<b>0.069</b>	<b>0.098</b>	0.101	0.109
	<i>P. aeruginosa</i> DSM 939	0.334	<b>0.167</b>	0.322	0.329	0.292
	<i>S. enterica</i> ATCC 13311	0.099	<b>0.042</b>	<b>0.071</b>	<b>0.078</b>	<b>0.087</b>
	<i>E. faecalis</i> ATCC 700802	0.119	<b>0.020</b>	<b>0.039</b>	<b>0.043</b>	<b>0.049</b>
	<i>L. monocytogenes</i> DSM 20600	0.071	<b>nd</b>	<b>0.004</b>	<b>0.018</b>	<b>0.032</b>
	<i>S. aureus</i> DSM 799	0.078	<b>0.007</b>	<b>0.032</b>	<b>0.052</b>	0.068
Time to max growth rate hh:mm	<i>E. coli</i> ATCC 8739	02:00	01:50	02:30	02:20	02:30
	<i>P. aeruginosa</i> DSM 939	10:20	<b>11:20</b>	10:40	10:40	10:50
	<i>S. enterica</i> ATCC 13311	02:50	<b>03:50</b>	02:40	02:20	02:40
	<i>E. faecalis</i> ATCC 700802	03:10	<b>08:20</b>	<b>07:20</b>	<b>07:00</b>	<b>06:10</b>
	<i>L. monocytogenes</i> DSM 20600	06:50	<b>nd</b>	<b>07:50</b>	<b>08:10</b>	06:30
	<i>S. aureus</i> DSM 799	02:20	02:40	02:10	01:30	01:40
Time to change hh:mm	<i>E. coli</i> ATCC 8739	01:35	01:51	01:27	01:24	01:23
	<i>P. aeruginosa</i> DSM 939	03:18	<b>04:05</b>	03:04	03:05	03:00
	<i>S. enterica</i> ATCC 13311	02:25	<b>03:43</b>	02:26	02:14	02:03
	<i>E. faecalis</i> ATCC 700802	02:02	<b>nd</b>	<b>06:18</b>	<b>05:51</b>	<b>05:39</b>
	<i>L. monocytogenes</i> DSM 20600	06:12	<b>nd</b>	<b>nd</b>	<b>08:51</b>	06:22
	<i>S. aureus</i> DSM 799	01:52	<b>nd</b>	<b>02:52</b>	01:49	01:42
Half run absorbance OD600nm	<i>E. coli</i> ATCC 8739	0.43	<b>0.27</b>	0.35	0.43	0.49
	<i>P. aeruginosa</i> DSM 939	1.12	<b>0.86</b>	1.06	1.12	1.14
	<i>S. enterica</i> ATCC 13311	0.32	0.25	0.33	0.35	0.33
	<i>E. faecalis</i> ATCC 700802	0.37	<b>0.20</b>	<b>0.25</b>	<b>0.28</b>	0.30
	<i>L. monocytogenes</i> DSM 20600	0.31	<b>0.09</b>	<b>0.10</b>	<b>0.16</b>	<b>0.21</b>
	<i>S. aureus</i> DSM 799	0.40	<b>0.10</b>	<b>0.16</b>	<b>0.21</b>	<b>0.25</b>
Doubling time hh:mm	<i>E. coli</i> ATCC 8739	01:10	<b>02:00</b>	01:30	01:20	01:20
	<i>P. aeruginosa</i> DSM 939	01:50	<b>02:30</b>	02:00	01:40	01:40
	<i>S. enterica</i> ATCC 13311	01:30	<b>03:40</b>	<b>02:00</b>	01:30	01:30
	<i>E. faecalis</i> ATCC 700802	02:00	<b>09:10</b>	<b>07:30</b>	<b>06:20</b>	<b>05:50</b>
	<i>L. monocytogenes</i> DSM 20600	04:50	<b>nd</b>	<b>nd</b>	<b>nd</b>	<b>05:40</b>
	<i>S. aureus</i> DSM 799	02:10	<b>nd</b>	<b>nd</b>	<b>03:00</b>	02:10

Values related to possible antimicrobial effect are in bold

nd not determined

was never doubled, it was not possible to calculate the doubling time.

A different pattern of inhibition was found for *S. aureus* DSM 799 whose growth curve was progressively flattened with the increase of phenylamides concentration. In fact, after 2.5 h incubation, none of the absorbance values of supplemented broth was close to those of

control samples, as reported in Table 4 for the reading n° 72 (half run).

It is well known that ferulic acid leads to irreversible changes in membrane structure and properties resulting in ruptures and pore formation with consequent leakage of intracellular compounds [39]. A similar mechanism of antimicrobial activity was demonstrated, by scanning

electron microscopy, in some *Xanthomonas oryzae* strains whose cell membrane resulted irreversibly deformed in presence of ferulic amide Ac6c derivatives [40]. However, a recent study, carried out using *Escherichia coli* as a model microorganism, showed that feruloyl and coumaroyl amides inhibited purine and pyrimidine biosynthesis [41].

In absence of specific experiments, we hypothesize that the main mechanism of antimicrobial inhibition of phenylamides here assayed resulted from interference with membrane functions even though other mechanisms could be involved.

## Conclusions

Phenylamides are specialized metabolites included in the defence arsenal of plants, commonly characterized by natural occurrence and low toxicity. To gain a deeper understanding of the role of these compounds in plant defence a small set of phenylamides was prepared and tested to evaluate the activity against both fungi and bacteria relevant in the agri-food sector.

None of the synthesized phenylamides significantly affected the fungal growth or spore germination, whereas some of them (**3b**, **3e**, **3f**) considerably inhibited the appressorium formation in *P. oryzae*. This result confirms that the effect of compounds is mainly related to fungal infection rather than to mycelial growth inhibition.

The substitution pattern on both the aromatic rings of the acid and amino moieties plays a role in modulating the fungal appressorium formation.

As far as the antibacterial activity is concerned, all molecules affected the normal growth kinetics of the strains considered in this work. The strongest antimicrobial activities were observed for compound **3d** followed by **3b**. Interestingly, the most active compounds against appressorium formation of *P. oryzae*, **3e** and **3f**, were the least active against the food-borne bacteria.

Even though toxicity and allergenic data of these molecules still need to be produced, the results of this work open the possibility of using these molecules in a large spectrum of applications to preserve crops in pre- and postharvest and foodstuffs from microbial contamination.

## Abbreviations

Agm	Agmatine
AVA	Avenanthramide
Ben	Benzoic
Boc	<i>tert</i> -Butoxycarbonyl
Caf	Caffeic
Cal-B	<i>Candida antarctica</i> Lipase B
Cin	Cinnamic
Cou	<i>p</i> -Coumaric
CZY	Czapek-Dox Yeast medium
DAI	Days after inoculation
DCM	Dichloromethane

DIPEA	Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EDC HCl	<i>N</i> -(3-Dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide hydrochloride
Fer	Ferulic
HOBt	Hydroxy benzotriazole
MA	Malt-agar medium
MeOH	Methanol
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
PA	Phenylamide
PCB	Plate count broth
PDB	Potato dextrose broth
Put	Putrescine
Qol	Quinone outside inhibitor
Ser	Serotonin
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Try	Tryptamine
Tyr	Tyramine
VRSA	Vancomycin-resistant <i>Staphylococcus aureus</i>
<i>B. cinerea</i>	<i>Botrytis cinerea</i>
<i>B. oryzae</i>	<i>Bipolaris oryzae</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. oryzae</i>	<i>Pyricularia oryzae</i>
<i>S. enterica</i>	<i>Salmonella enterica</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>X. oryzae</i>	<i>Xanthomonas oryzae</i>

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40538-025-00736-8>.

Supplementary material 1

Supplementary material 2

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## Author contributions

A.K., S.P., A.P., S.D.: Conceptualization; D.D., S.G., J.L.E.S., D.C., L.P.: Formal analysis, experiments conduction, investigation; A.P., M.C., P.C., F.B., S.D.: Supervision; A.K., S.P., S.D.: Writing — original draft. S.P., A.P., P.C., F.B., S.D.: Methodology, Data curation; All authors: Review and editing.

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## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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