Environmental Microbiology (2015) **17**(4), 1103–1118 doi:10.1111/1462-2920.12514

Novel aspinolide production by *Trichoderma arundinaceum* with a potential role in *Botrytis cinerea* antagonistic activity and plant defence priming

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Summary

Harzianum A (HA), a trichothecene produced by *Trichoderma arundinaceum*, has recently been described to have antagonistic activity against fungal plant pathogens and to induce plant defence genes. In the present work, we have shown that a *tri5* gene-disrupted mutant that lacks HA production overproduces two polyketides, aspinolides B and C, which were not detected in the wild-type strain. Furthermore, four new aspinolides (D–G) were characterized. These compounds confirm that a terpene-polyketide cross-pathway exists in *T. arundinaceum*, and they may be responsible for the antifungal activity and the plant sensitization effect observed with the *tri5*-disrupted mutant. In addition, the molecular changes involving virulence factors in the phytopathogenic fungus *Botrytis cinerea* 98 (Bc98) during interaction with *T. arundinaceum* were investigated. The expression of genes involved in the production of botrydial by Bc98 was relatively repressed by HA, whereas other virulence genes of this pathogen were induced by the presence of *T. arundinaceum*, for example *atrB* and *pg1* which encode for an ABC transporter and endopolygalacturonase 1 respectively. In addition, the interaction with Bc98 significantly repressed the production of HA by *T. arundinaceum*, indicating that a bidirectional transcriptional regulation is established between these two antagonistic fungi.

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Introduction

Trichoderma is well known for the ability of some of its strains to act as important biocontrol agents against phytopathogenic fungi (Harman *et al.*, 2004; Lorito *et al.*, 2010). It has been shown that in interaction with plants, some *Trichoderma* strains can act as biofertilizers, as inducers of plant defence responses, and also can increase tolerance to abiotic stresses (Shoresh *et al.*, 2010; Hermosa *et al.*, 2012). Many *Trichoderma* strains produce primary or secondary metabolites and enzymes with diverse industrial interest. Among these metabolites, the trichothecenes have attracted attention in recent years since they are mycotoxins, with detrimental effects on animals and human health as well as plants (Rocha *et al.*, 2005; Desjardins, 2006; McCormick *et al.*, 2011). Although knowledge about how trichothecenes interact with plants is limited, it has generally been thought that they are phytotoxic and suppress the defence response in host plants (Masuda *et al.*, 2007). Some trichothecenes, such as T-2 toxin produced by *Fusarium sporotrichioides*, induce the formation of lesions and the generation of hydrogen peroxide, as well as the accumulation of salicylic acid (SA), callose deposition, activation of defence-related mitogen-activated protein kinases (MAPKs) and expression of defence genes (Nishiuchi *et al.*, 2006). *Trichoderma* species *T. brevicompactum* and *T. arundinaceum* produce two different trichothecenes, trichodermin and harzianum A (HA) respectively. While trichodermin has strong *in vivo* phytotoxic activity (Tijerino *et al.*, 2011), HA does not. Thus, HA would be a model of a trichothecene produced by a fungal strain that has significant biocontrol activity (Malmierca *et al.*, 2012; 2013), and as such can be used to study the role of trichothecenes in the interaction with phytopathogenic fungi and plants.

HA has a 2′,4′,6′-octatrienedioic acid esterified on the 4β hydroxyl group of trichodermol (4β hydroxyl,

12,13-epoxytrichothec-9-ene) (Fig. 1). HA biosynthesis begins with cyclization of farnesyl diphosphate (FPP) by a terpene cyclase, encoded by the *tri5* gene, to give rise to trichodiene. This gene may be a key point in the terpene biosynthetic pathway and in the regulation of the intracellular FPP pool (Malmierca *et al*., 2013). Thus, a modification in its level of expression and/or in the level of trichodiene synthase activity may have a systemic effect on *T. arundinaceum* metabolism.

It was previously shown by our group that pretreatment of tomato plants with the HA-producing *T. arundinaceum* wild-type strain resulted in a strong induction of tomato defence-related genes upon infection with the phytopathogen *Botrytis cinerea*, while transformants with a disrupted *tri4* (trichodiene oxygenase) gene had reduced biocontrol activity and no plant sensitization ability. Taking into account that the difference between the wild-type strain and the *tri4*-disrupted transformant was the HA production, a role in the induction of plant defence genes was attributed to this trichothecene (Malmierca *et al*., 2012). In a more recent study, a *tri5* gene-disrupted transformant (TaΔTri5) that is totally blocked in HA biosynthesis was also shown to retain significant antifungal activity, which was attributed to the production of alternative compounds as a result of blocking HA production (Malmierca *et al*., 2013).

In a first approach to characterize the role of HA production in the *T. arundinaceum* environmental interactions, a bidirectional interaction between *T. arundinaceum* and the fungal phytopathogen *B. cinerea* 98 (Bc98) was proposed. Thus, *T. arundinaceum* produces extracellular hydrolytic enzymes and trichothecenes that inhibit the growth of Bc98 while *B. cinerea* represses the expression of *tri* genes in *T. arundinaceum* (Malmierca *et al*., 2012). In addition, *B. cinerea* produces during infection a huge number of pathogenicity factors (including proteins and toxins) that are involved in disease development but are not essential for fungal survival or reproduction (Idnurm and Howlett, 2001; Elad *et al*., 2004). Most of the genes contributing to *B. cinerea* pathogenicity factors can be classified into four different groups based on the function in which they are involved: (i) cuticle and cell wall degradation [e.g. endopolygalacturonases (endoPG)]; (ii) appressoria formation; (iii) toxin production (e.g. botrydial = BOT) and (iv) intracellular signalling.

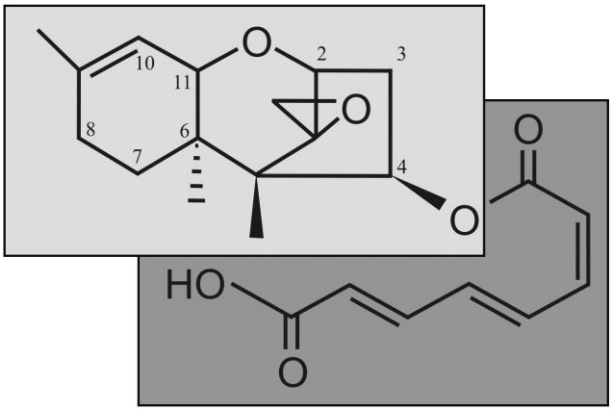


Fig. 1. Structure of HA showing the core of trichodermol (squared in light grey) with the polyketide derived 2′,4′,6′-octatrienedioyl (squared in dark grey) side-chain esterified on the 4β hydroxyl group.

In an attempt to gain a global view of the effect of *T. arundinaceum* on *B. cinerea* pathogenicity, we focused our attention in this work first on the metabolomics characterization of the TaΔTri5 mutant, to identify those compounds differentially produced by the mutant with respect to the wild-type strain, and their function in both *T. arundinaceum* antifungal activity and in interactions with other environmental organisms. Then, the effect of HA production by *T. arundinaceum* on the regulation of expression of several *B. cinerea* genes which are involved in different stages of disease development, and the Bc98 influence on the *tri* gene expression and HA biosynthesis in *T. arundinaceum* IBT 40837 were studied. The ability of the *tri5*-disrupted transformant to elicit defence responses in tomato plants allowed us to investigate the relationship between the induction of plant defence marker genes linked to the salicylic signalling pathway and the synthesis of new metabolites by the TaΔTri5 mutant.

Results

Metabolomics characterization of the TaΔTri5 mutant: identification of aspinolides in Ta37 and TaΔTri5 broths

To study the chemical signalling involved in the antagonistic effect of *T. arundinaceum* on *B. cinerea*, we focused our attention on the metabolomics characterization of the TaΔTri5 mutant, in order to identify those compounds differentially produced with respect to the wild-type strain.

A comparative qualitative thin layer chromatographic (TLC) study of the organic extracts obtained from the interaction and non-interaction zones (Fig. 2) in the confrontation assays between Ta37 and TaΔTri5 against Bc98 revealed significant differences in the metabolite profiles of these two *Trichoderma* strains.

In order to study and characterize these compounds, the Ta37 and TaΔTri5 strains were grown in twelve 150 mm Petri dishes containing 100 ml of malt extract agar (MEA) medium. The organic extracts obtained were submitted to column chromatography, and the fractions purified by high-performance liquid chromatography (HPLC) and analysed by extensive ^{1}H-nuclear magnetic

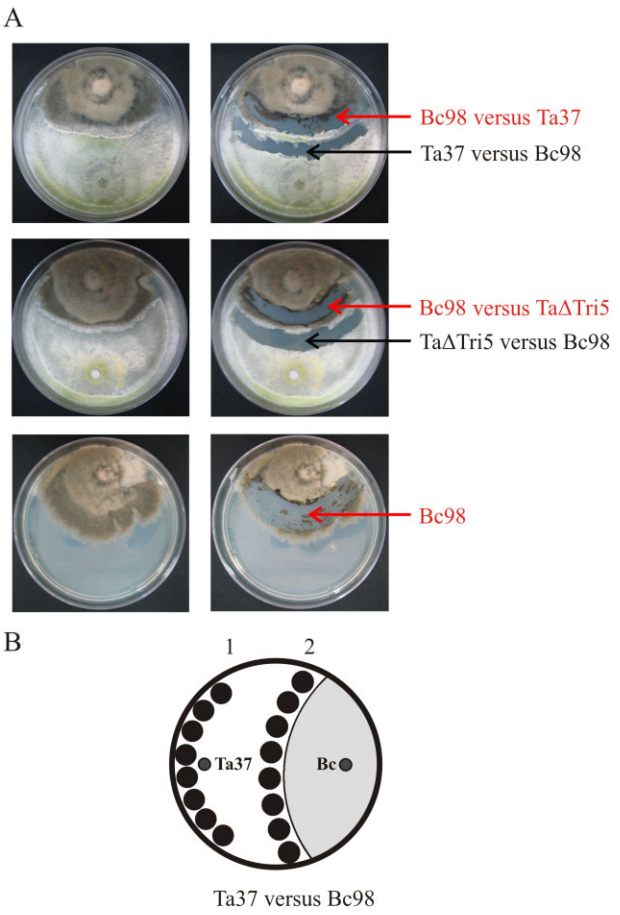


Fig. 2. (A) Photographs of confrontation assays of Bc98 against Ta37 and TaΔTri5 strains. The areas from which the RNAs were extracted are indicated. (B) Schematic representation indicating the regions of the confrontation plates of Ta37 from which HA was extracted and quantified, where 1 is the '*non-interaction*' zone and 2 is the '*interaction*' zone.
*Note that the Ta37 (an HA producer) strain showed a higher ability to overgrow Bc98 than TaΔTri5 (an HA non-producer mutant).

resonance (NMR) and ^{13}C-NMR experiments. The study of fractions obtained from the TaΔTri5 solid culture medium yielded two major compounds whose spectroscopic data were coincident with those reported for aspinolide B (**1**) and C (**2**) (Fuchser and Zeeck, 1997). In addition, small amounts of three related metabolites, aspinolide D (**3**), aspinolide E (**4**) and aspinolide G (**6**), were isolated and characterized from the TaΔTri5 mutant (Table 1). The Ta37 MEA culture medium contained, in addition to HA (Corley *et al.*, 1994; Malmierca *et al.*, 2012), small amounts of aspinolide D (**3**) and aspinolide F (**5**) (Table 1).

The same production profiles of these aspinolides were found when the *Trichoderma* strains were grown in potato dextrose agar (PDA) medium (see Experimental procedures), indicating that at least in the studied media, there is a consistent correlation with the metabolites identified regardless of the medium.

The high-resolution mass spectroscopy (HRMS) of aspinolide D (**3**) and E (**4**) indicated a molecular formula of $C_{14}H_{21}O_5$ and $C_{14}H_{22}O_6$ respectively (Table 2). They presented similar spectroscopic constants to those displayed by aspinolides B and C. The principal differences observed in the ^{1}H-NMR and ^{13}C-NMR spectra were, for aspinolide D, the absence in their ^{1}H-NMR of the signal corresponding to the geminal proton to the hydroxyl group at C-4, and for aspinolide E, the absence of the signals corresponding to the olefinic protons of the butenoyl ester at C-8 (Supporting Information Fig. S1). From these data, the structures indicated in Fig. 3 for compounds **3** and **4** were inferred. The structures and stereochemistry were consistent with the observed correlations in their heteronuclear multiple bond correlation (HMBC) and NOESY-D1 experiments (Supporting Information Tables S1 and S2) and heteronuclear single quantum coherence (HSQC) (data not shown).

Aspinolides F (**5**) and G (**6**) showed a molecular formula of $C_{16}H_{22}O_8$ and $C_{15}H_{22}O_7$ respectively, as deduced from their HRMS (Table 2). They presented ^{1}H-NMR signal patterns similar to those displayed for aspinolide C, with the absence of characteristic signals corresponding to the olefinic protons of the double bond at carbons C6-C7. Additionally, both ^{1}H-NMR and ^{13}C-NMR spectra showed signals characteristic for an acetyl (δ 1.93 and 2.00 ppm) and methoxy (δ 3.31 ppm) groups, for compounds **5** and **6** respectively (Supporting Information Fig. S1), which on the basis of their gradient heteronuclear multiple bond correlation (gHMBC) correlations (Supporting Information Table S1) were assigned at C-7 carbon, for compounds **5** and **6** (Fig. 3). Furthermore, signals at δ 4.36 and 4.39 ppm were assigned to the hydroxyl group at C-4 for both compounds. The structures and stereochemistry were inferred from the observed correlations in their HMBC, NOESY-D1 experiments (Supporting Information Tables S1 and S2) and HSQC (data not shown).

Table 1. Comparison of the amounts of aspinolides isolated from cultures of Ta37 and TaΔTri5 (mg) (for structures of aspinolides see Fig. 3).

Compound	Ta37	TaΔTri5
Aspinolide B (**1**)	nd	112.8
Aspinolide C (**2**)	nd	191.9
Aspinolide D (**3**)	0.8	1.0
Aspinolide E (**4**)	nd	1.2
Aspinolide F (**5**)	7.9	nd
Aspinolide G (**6**)	nd	8.53

nd, not detected.

Table 2. Physical and spectroscopic constants (^{1}H-NMR ^{13}C-NMR and HRMS) for aspinolides D–G (**3–6**).

Aspinolide D (3): White amorphous solid; t_r = 28 min, ethyl acetate: hexane (27:73), flow = 3.0 ml min^{-1}; $[\alpha]_D^{20} = -24.1°$ (c = 0.23 in $CHCl_3$); ^{1}H NMR (400 MHz, [D_1]-$CHCl_3$) δ = 7.00 (dq,^3J (H-H) = 6.9, 15.5 Hz, 1H; H-3′), 5.84 (dq,^3J (H-H) = 1.7, 15.5 Hz, 1H; H-2′), 5.72 (ddd,^3J (H-H) = 0.7, 2.0, 16.0 Hz, 1H; H-6), 5.48 (ddd,^3J (H-H) = 2.3, 8.6, 16.0 Hz, 1H; H-7), 5.12 (dq,^3J (H-H) = 6.3, 9.3 Hz, 1H; H-9), 4.98 (dd,^3J (H-H) = 8.9, 1H; H-8), 4.50 (m, 1H; H-5), 2.47 (m, 1H; H-2), 2.05 (m, 3H; H-2, H-3, H-4), 1.89 (dd,^3J (H-H) = 1.7, 6.9 Hz, 3H; H-4′), 1.69 (m, 1H; H-4), 1.56 (m, 1H; H-3), 1.31 ppm (d,^3J (H-H) = 9.3 Hz, 3H; H-10); ^{13}C NMR (125 MHz, [D_1]-$CHCl_3$) δ = 176.20, 165.50, 145.76, 136.75, 126.61, 122.21, 79.14, 72.60, 68.01, 36.44, 35.81, 18.20, 18.05, 16.74 ppm; IR ν_{max} 3492(OH), 2920, 2342, 1715(CO), 1153 cm^{-1}; HRESIMS (+): calcd for $C_{14}H_{21}O_5$ $[M + H]^+$ 269.1389, found 269.1393.

Aspinolide E (4): White amorphous solid; t_r = 30 min, ethyl acetate: hexane (50:50), flow = 1.0 ml min^{-1}; $[\alpha]_D^{20} = -14.5°$ (c = 0.24 in $CHCl_3$); ^{1}H NMR (500 MHz, [D_1]-$CHCl_3$) δ = 5.65 (dd, ^3J (H-H) = 1.5, 15.9 Hz, 1H; H-6), 5.54 (ddd, ^3J (H-H) = 2.3, 8.4, 15.9 Hz, 1H; H-7), 5.06 (dq, ^3J (H-H) = 6.3, 9.3, 1H; H-9), 4.90 (dd, ^3J (H-H) = 8.7 Hz, 1H; H-8), 4.50 (br.s, 1H; H-5), 3.64 (br.d, ^3J (H-H) = 11.1 Hz, 1H; H-4), 2.46 (dd, ^3J (H-H) = 9.4, 15.6 Hz, 1H; H-2), 2.31 (t, ^3J (H-H) = 7.4 Hz, 2H; H-2′), 2.29 (m, 1H; H-3), 2.06 (dd, ^3J (H-H) = 11.6, 15.6 Hz, 1H; H-2), 1.77 (m, 1H; H-3), 1.65 (m, 2H; H-3′), 1.31 (d, ^3J (H-H) = 6.3 Hz, 3H; H-10), 0.95 ppm (t, ^3J (H-H) = 7.4 Hz, 3H; H-4′); ^{13}C NMR (125 MHz, [D_1]-$CHCl_3$) δ = 175.47, 172.74, 131.22, 127.80, 78.68, 75.16, 72.91, 71.87, 36.20, 32.44, 27.32, 18.39, 16.70, 13.64 ppm; IR ν_{max} 3418 (OH), 2925, 1728 (CO), 1157 cm^{-1}; HRESIMS (+): calcd for $C_{14}H_{22}O_6Na$ $[M + Na]^+$ 309.1314, found 309.1327.

Aspinolide F (5): Colourless oil; t_r = 15 min, ethyl acetate: hexane (60:40), flow = 3.0 ml min^{-1}; $[\alpha]_D^{20} = +32.3°$ (c = 0.68 in $CHCl_3$); ^{1}H NMR (400 MHz, [D_1]-$CHCl_3$) δ = 7.02 (dq,^3J (H-H) = 6.9, 15.5 Hz, 1H; H-3′), 5.82 (dq,^3J (H-H) = 1.7, 15.5 Hz, 1H; H-2′), 5.72 (ddd, ^3J (H-H) = 1.7, 9.7, 10.7 Hz, 1H; H-7), 5.13 (dd,^3J (H-H) = 10.0 Hz, 1H; H-8), 4.54 (dq,^3J (H-H) = 6.1, 10.2 Hz, 1H; H-9), 4.36 (br.t,^3J (H-H) = 5.0 Hz, 1H; H-4), 3.43 (dd,^3J (H-H) = 10.7, 17.5 Hz, 1H; H-6), 2.63 (ddd,^3J (H-H) = 2.5, 12.7, 16.5 Hz, 1H; H-2), 2.39 (m, 3H; H-2, H-3, H-6), 2.10 (m, 1H; H-3), 1.93 (s, 3H; MeCO-C7), 1.89 (dd,^3J (H-H) = 1.7, 6.9 Hz, 3H; H-4′), 1.19 ppm (d,^3J (H-H) = 6.1, 3H; H-10); ^{13}C NMR (125 MHz, [D_1]-$CHCl_3$) δ = 206.70, 169.47, 169.16, 165.04, 146.95, 121.49, 74.04, 73.90, 70.56, 69.47, 41.51, 27.77, 27.26, 20.8, 18.17, 16.98 ppm; IR ν_{max} 3459 (OH), 1727 (CO), 1244 cm^{-1}; HRESIMS (+): calcd for $C_{16}H_{22}O_8Na$ $[M + Na]^+$ 365.1212, found 365.1214.

Aspinolide G (6): Amorphous solid; t_r = 23 min, ethyl acetate: hexane (50:50), flow = 3.0 ml min^{-1}; $[\alpha]_D^{20} = -5.17°$ (c = 0.13 in $CHCl_3$); ^{1}H NMR (500 MHz, [D_1]-$CHCl_3$) δ = 7.04 (dq,^3J (H-H) = 6.9, 15.5 Hz, 1H; H-3′), 5.88 (dq,^3J (H-H) = 1.6, 15.5 Hz, 1H; H-2′), 5.02 (dd, ^3J (H-H) = 9.4, 1H; H-8), 4.50 (dq,^3J (H-H) = 6.1, 10.2 Hz, 1H; H-9), 4.39 (br.s, 1H; H-4), 3.97 (m, 1H; H-7), 3.31 (s, 3H; MeO- C7), 3.29 (dd,^3J (H-H) = 10.4, 17.7 Hz, 1H; H-6), 2.61 (m, 1H; H-2), 2.40 (m, 3H; H-2, H-3, H-6), 2.11 (m, 1H; H-3), 1.91 (dd,^3J (H-H) = 1.7, 6.9 Hz, 3H; H-4′), 1.19 ppm (d,^3J (H-H) = 6.1 Hz, 3H; H-10); ^{13}C NMR (125 MHz, [D_1]-$CHCl_3$) δ = 208.79, 169.36, 165.39, 146.10, 122.07, 77.20, 75.32, 74.29, 70.62, 57.87, 41.02, 27.82, 27.28, 18.14, 17.09 ppm; ; IR ν_{max} 3446(OH), 2918,1720 (CO), 1644 (CO) cm^{-1}; HRESIMS (+): calcd for $C_{15}H_{22}O_7Na$ $[M + Na]^+$ 337.1263, found 337.1272.

As far as we know, this is the first report that describes the production of aspinolides by *Trichoderma* strains. Aspinolides D–G (**3–6**) have not been previously reported in the literature.

Analysis of the antifungal activity of aspinolides C and B

In order to determine if aspinolides could play a role in the antifungal activity of the TaΔTri5 strain, antibiograms against Bc98 using concentrations of aspinolide C and B from 1 to 10 mg ml^{-1} were carried out (Fig. 4). A clear inhibition area was obtained for all assayed concentrations of aspinolide C.

The minimal inhibitory concentration (MIC) of aspinolide C against this fungus was calculated to be 0.54 mg ml^{-1}. Aspinolide B has no activity against Bc98.

Similar results to that observed with Bc98 were obtained when other fungal strains were assayed. Thus, the MIC of aspinolide C against *B. cinerea* Bc05.10 (= Bc05.10) and *F. sporotrichioides* were 0.34 mg ml^{-1} and 0.21 mg ml^{-1} respectively. Aspinolide B did not show any activity against these two strains.

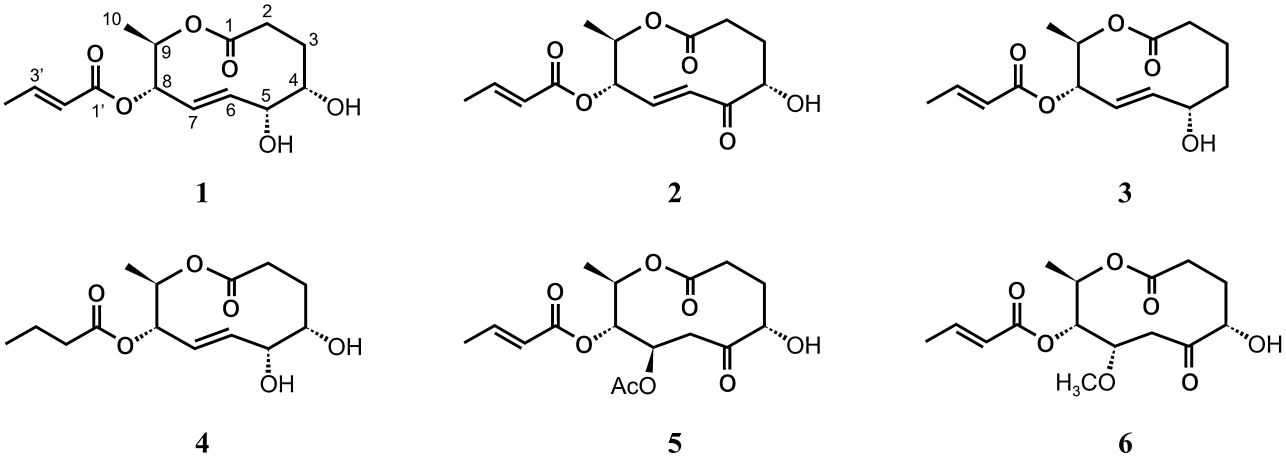


Fig. 3. Chemical structures of isolated aspinolides B–G (numbers **1–6**) from Ta37 and TaΔTri5 strains.

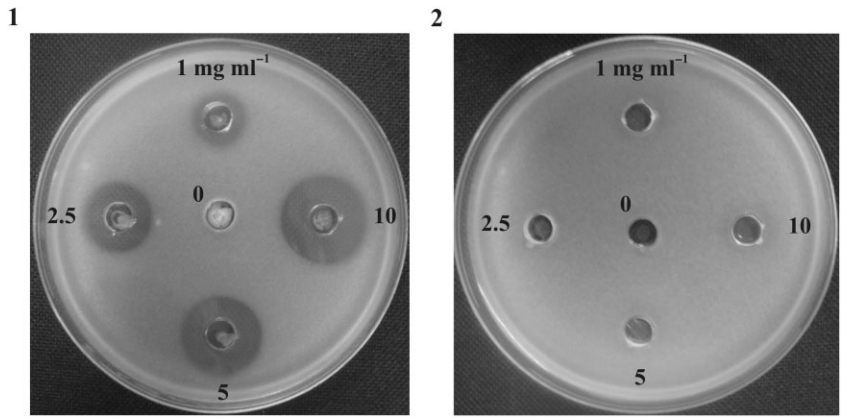


Fig. 4. Antibiogram assay of aspinolides C and B against Bc98. Concentrations used are indicated on each hole in mg ml^{-1}. Dilutions of (1) aspinolide C and (2) aspinolide B were carried out in acetone and ethanol respectively. In the center of the plates (0), acetone or ethanol were loaded to show that these solvents do not affect the Bc98 growth.

Production of HA regulates expression of Botrytis *virulence genes*

The effect of the loss of HA production, as a result of the *tri5* gene disruption, on the expression of several Bc98 genes linked to virulence of this phytopathogen was analysed. The expression of *BcBOT1*, *BcBOT2*, *BcBOT3* and *BcBOT5* involved in the biosynthesis of BOT (Pinedo *et al*., 2008), *sod1* encoding a superoxide dismutase (Rolke *et al*., 2004), *atrB* encoding an ABC transporter (Schoonbeek *et al*., 2001), *bmp1* encoding a mitogen-activated protein kinase (Zheng *et al*., 2000) and *pg1* encoding an endopolygalacturonase (endoPG1) (Ten Have *et al*., 1998), was analysed by quantitative polymerase chain reaction (qPCR) during interaction in dual cultures of Bc98 against the Ta37 wild-type strain or TaΔTri5 mutant (see Fig. 2). We found that expression of genes involved in BOT biosynthesis was induced in confrontation assays against the mutant lacking HA production (Fig. 5) in comparison with the expression observed when the confrontation assays were against the Ta37 wild-type strain, with relative values of gene expression ranging between 2.731 ($P = 0.031$)-fold for *BcBOT5* and 14.231 ($P = 0.000$)-fold for *BcBOT1* (Fig. 5C). Among the other virulence genes analysed, *atrB* was strongly induced in the presence of both wild-type and the *tri5* mutant strain, with no significant differences between them. The *pg1* gene was also induced by the presence of either of the two *T. arundinaceum* strains used but at a slightly higher level when Bc98 was confronted with Ta37 [4.235 ($P = 0.000$)-fold] than with TaΔTri5 [2.267 ($P = 0.048$)-fold]. The expression of two other virulence genes examined, *sod1* and *bmp1*, was not significantly affected by the interaction with *Trichoderma* strains (Fig. 5B and C).

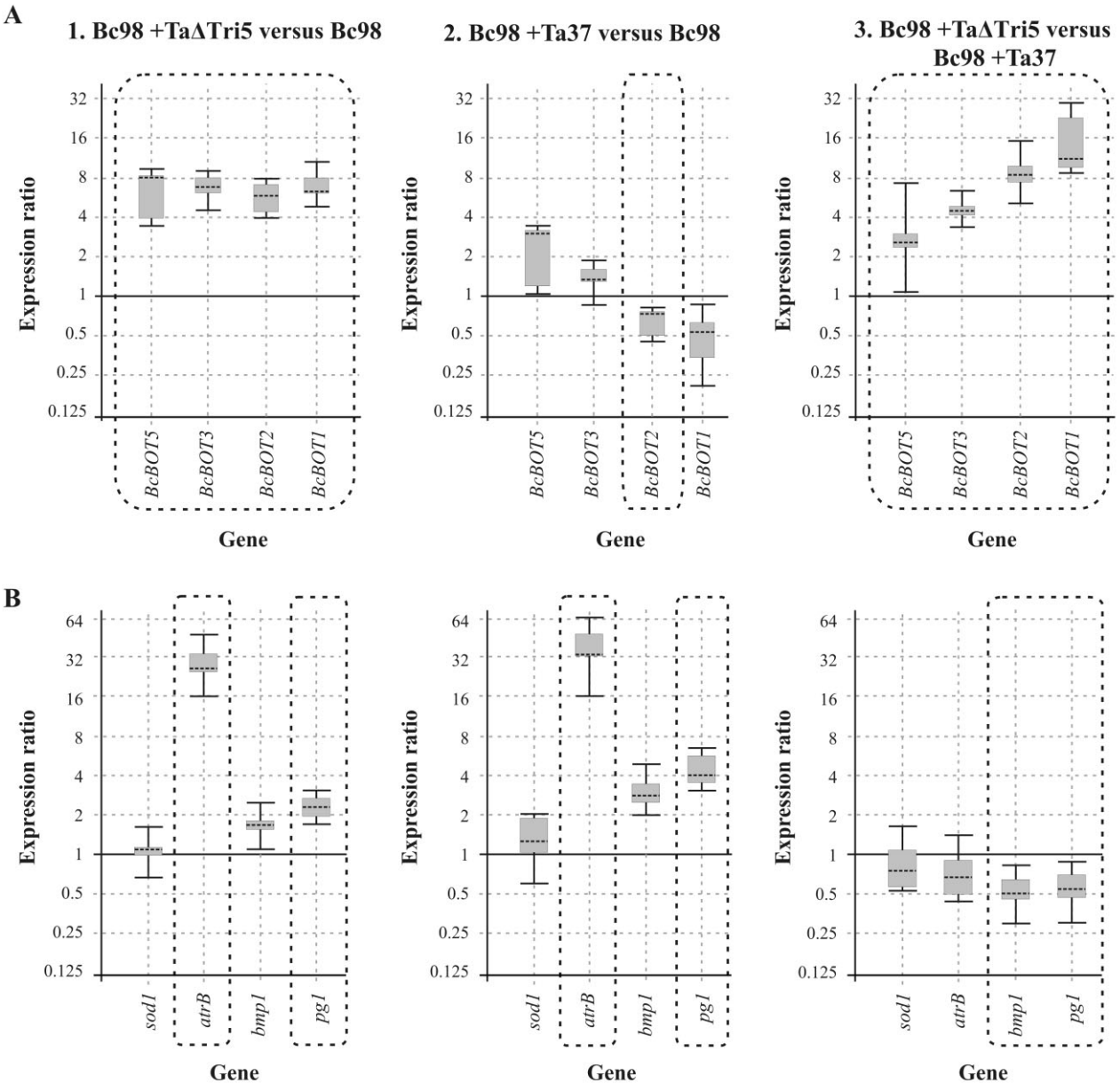
Bc98 regulates expression of tri *genes and HA production in the Ta37 strain*

The level of HA produced was measured in the same dual cultures of *T. arundinaceum* and Bc98 used above to study the level of expression of the *B. cinerea* virulence genes. The amount of HA produced by *T. arundinaceum* growing alone (44 ± 4.58 μg HA ml^{-1}) was much higher than the amount produced in the area of *interaction* with Bc98 (28.25 ± 0.92 μg HA ml^{-1}), suggesting that the interaction with Bc98 may have an inhibitory effect on HA biosynthesis. In addition, when the production of HA was quantified in Ta37-Bc98 dual cultures from samples taken from the *non-interaction* zone (see Fig. 2), an increase in HA production (56.78 ± 6.53 μg HA ml^{-1}) was observed, indicating that the inhibitory effect on HA production by Bc98 is restricted to the region where the interaction with *T. arundinaceum* takes place. However, it should be noted that the level of HA production in the *non-interaction* zone was higher, statistically significant, than when Ta37 was grown alone in the same conditions used for the dual cultures.

Ta37 cDNAs extracted from the *interaction* zone of confrontation experiments against Bc98 (see Fig. 2A) were analysed to determine the effect of this interaction on the level of expression of the Ta37 *tri* genes, under the same conditions used to study the expression of *Botrytis* virulence genes. All the *tri* genes, except *tri6*, were drastically repressed (Fig. 6) in Ta37 growing in the *interaction* zone, in comparison with the Ta37 strain growing alone, with expression ratios ranging between 0.007 ($P = 0.035$)-fold for *tri3* and 0.316 ($P = 0.026$)-fold for *tri5*. The expression of *tri6* was not significantly affected.

Interaction with plants: effect of TaΔTri5 mutant on the level of expression of tomato plant defence-related genes in comparison with the Ta37 wild-type strain

Comparative expression studies on plant marker genes linked to the SA and the jasmonic acid (JA) defence signalling pathways, under various treatments, allowed us to determine the individual effect of TaΔTri5 or Bc98 as well as their combined effects. To determine the individual effect of Bc98, gene expression in tomato plants infected with Bc98 (+B) was compared with the level of expression



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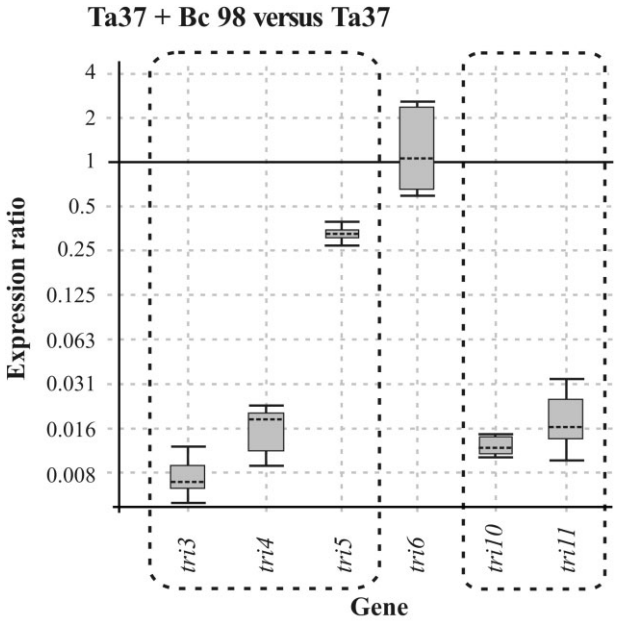
Gene	Expression ratio	p(H1)	Gene	Expression ratio	p(H1)	Gene	Expression ratio	p(H1)
BcBOT5	6.515*	0.037	***BcBOT5***	2.273	0.071	***BcBOT5***	2.731*	0.031
BcBOT3	6.611*	0.029	***BcBOT3***	1.394	0.139	***BcBOT3***	4.743*	0.030
BcBOT 2	5.762*	0.000	***BcBOT 2***	0.660*	0.000	***BcBOT 2***	8.511*	0.000
BcBOT1	6.910*	0.029	***BcBOT1***	0.485	0.100	***BcBOT1***	14.231*	0.000
sod1	1.078	0.498	***sod1***	1.347	0.299	***sod1***	0.800	0.290
atrB	27.179*	0.000	***atrB***	38.361*	0.000	***atrB***	0.709	0.199
bmp1	1.634	0.073	***bmp1***	2.961	0.073	***bmp1***	0.551*	0.000
pg1	2.267*	0.048	***pg1***	4.235*	0.000	***pg1***	0.533*	0.000

Fig. 5. qPCR analysis of the expression of (A) *BcBOT* genes involved in the biosynthesis of BOT and (B) other *Botrytis* virulence genes of Bc98 in confrontation experiments with strains TaΔTri5 and Ta37. (C) Relative expression data compared with the *B. cinerea* reference (housekeeping) gene *EF1b* are indicated in the bottom panel. The expression ratios as well as the statistical probability values were calculated using the REST 2009 software. Numeric data are illustrated at the bottom and are indicated with an asterisk and squared in the graphic representation for those which were statistically significant ($P < 0.05$).



Gene	Expression ratio	*p(H1)*
tri3	0.007*	0.035
tri4	0.015*	0.000
tri5	0.316*	0.026
tri6	1.184	0.644
tri10	0.011*	0.000
tri11	0.017*	0.036

Fig. 6. qPCR analysis of the expression of *tri* genes of Ta37 in confrontation experiments with Bc98 strains. The relative expression data compared with the Ta37 reference α-actin and *gpd* genes are indicated in the bottom panel. Comparative calculations and representations were carried out as indicated in the legend of Fig. 5.

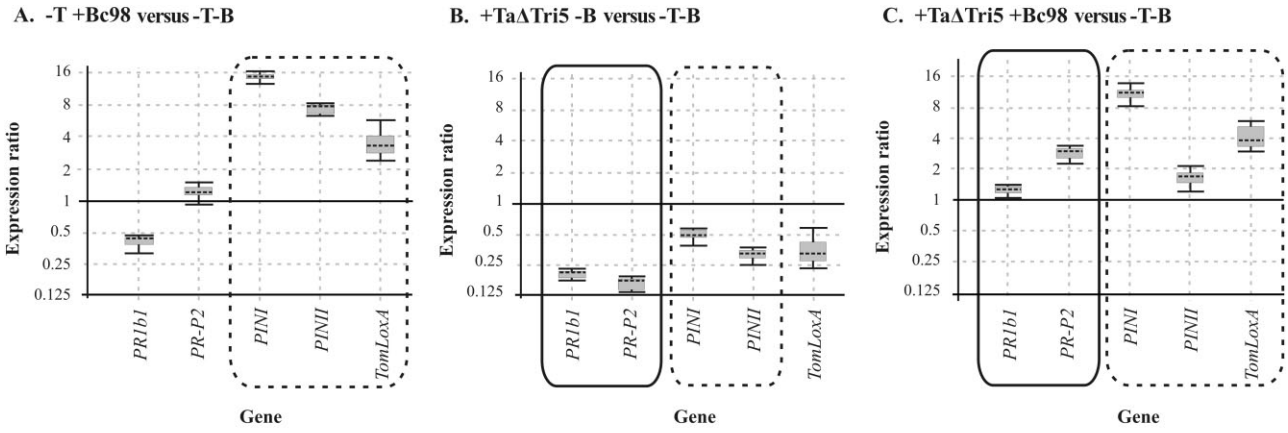
in plants not infected (–B). Only the genes in the JA pathway (*PINI*, *PINII*, *TomLoxA*) were significantly induced (Fig. 7A). Similarly, the effect of the lack of HA production, and the consequent production of aspinolides, on expression of tomato defence-related genes was determined by comparing the level of expression of those genes in tomato plants treated with TaΔTri5 (+TaΔTri5) in comparison with plants not treated with *Trichoderma* (–T). A repression of all the genes analysed was observed (Fig. 7B). Furthermore, the combined effects of pretreatment with *Trichoderma* and infection by Bc98 was determined in plants that were pretreated with TaΔTri5 and exposed to Bc98 (+TaΔTri5+B) and compared with plants with no pretreatment or exposure (–T–B). All the genes showed a significant increase in their expression ratios, showing values of induction between 1.295 ($P = 0.000$)-fold for SA-related *PR1b1* gene and 10.953 ($P = 0.000$)-fold for JA-related *PINI* gene (Fig. 7C).

Interaction with plants: in vitro *effect of aspinolide B and aspinolide C addition on plant growth and in the expression of plant defence-related genes*

A study using purified aspinolide B or aspinolide C to determine their effect on tomato growth and root architecture as well as on the expression of plant defence-related genes was carried out. The results showed that 50 μg ml^{-1} aspinolide B had a positive effect on the proliferation of lateral roots, without any negative effects on plant growth, in comparison with plants developed in the presence of ethanol, which was the solvent used to dissolve the aspinolide B. Aspinolide C had an almost opposite result. Plants grown in medium containing 50 μg ml^{-1} aspinolide C showed a significant reduction in the number of lateral roots and also in the size of the aerial parts of plants (average length 3.0 ± 0.41 cm) in comparison with control plants (average length 4.52 ± 0.22 cm). Furthermore, when a concentration of 250 μg ml^{-1} aspinolide C was used, a much more drastic effect was observed, with a very strong reduction in root and above ground development (average length 1.95 ± 0.39 cm) (Fig. 8A and B). The use of both aspinolides, B and C, mixed at a concentration of 50 μg ml^{-1} each, resulted in tomato plants with a similar phenotype to that observed when aspinolide C was used alone, with a reduction in the number of lateral roots and a reduction in the growth of the aerial parts when compared with tomato plants grown without aspinolides, in a proportion similar to that observed with aspinolide C (Fig. 8C).

When expression of plant defence-related genes was analysed, aspinolide B (50 μg ml^{-1}) slightly repressed SA-related genes, while the effect on the expression of JA-related genes was not homogenous. Thus, *PINII* was induced by a factor of 3.974 ($P = 0.000$)-fold and *TomLoxA* was slightly repressed by a factor of 0.611 ($P = 0.000$)-fold, while *PINI* expression was not significantly affected. In contrast, aspinolide C (50 μg ml^{-1}) significantly induced the SA-related genes *PR1b1* and *PR-P2* by factors of 13.020 ($P = 0.000$)-fold and 2.957 ($P = 0.000$)-fold respectively. JA-related genes in plants grown in the presence of aspinolide C showed a similar pattern to that observed in aspinolide B-treated plants, with slight differences in expression (Fig. 8D–F).

Interestingly, a reduction in the level of expression of *PR1b1* was observed when aspinolide B and C were used together (50 μg ml^{-1} each), in comparison with the level when only aspinolide C was present. Expression of JA-related genes in tomato plants grown in the presence of aspinolide B and C was repressed in the case of *PINII*, whereas *TomLoxA* expression was induced and *PINI* was just slightly affected in comparison with the levels of expression in plants grown in the presence of either aspinolide C or aspinolide B.



Gene	Expression ratio	p(H1)
PR1b1	0.411	0.095
PR-P2	1.219	0.098
PINI	14.973*	0.000
PINII	7.633*	0.000
TomLoxA	3.603*	0.000

Gene	Expression ratio	p(H1)
PR1b1	0.206*	0.000
PR-P2	0.160*	0.000
PINI	0.518*	0.000
PINII	0.307*	0.000
TomLoxA	0.340	0.075

Gene	Expression ratio	p(H1)
PR1b1	1.295*	0.000
PR-P2	2.927*	0.000
PINI	10.953*	0.000
PINII	1.705*	0.000
TomLoxA	4.152*	0.000

Fig. 7. Upper panels. Comparison of expression levels of genes belonging to the salicylic acid (SA) (*PR1b1*, *PR-P2*) and jasmonic acid (JA) (*PINI, PINII, TomLoxA*) pathways in: (A) tomato plants infected with Bc 98 (+ Bc98) (B) tomato plants treated with TaΔTri5 mutant without Bc98 infection (+ TaΔTri5-B), and (C) tomato plants treated with TaΔTri5 mutant and infected with Bc 98. In all the cases, the comparison was performed with plants not treated with TaΔTri5 nor infected with Bc98 (-T-B). Comparative calculations, included in the lower panels, and graphic representations were carried out as indicated in the legend of Fig. 5.

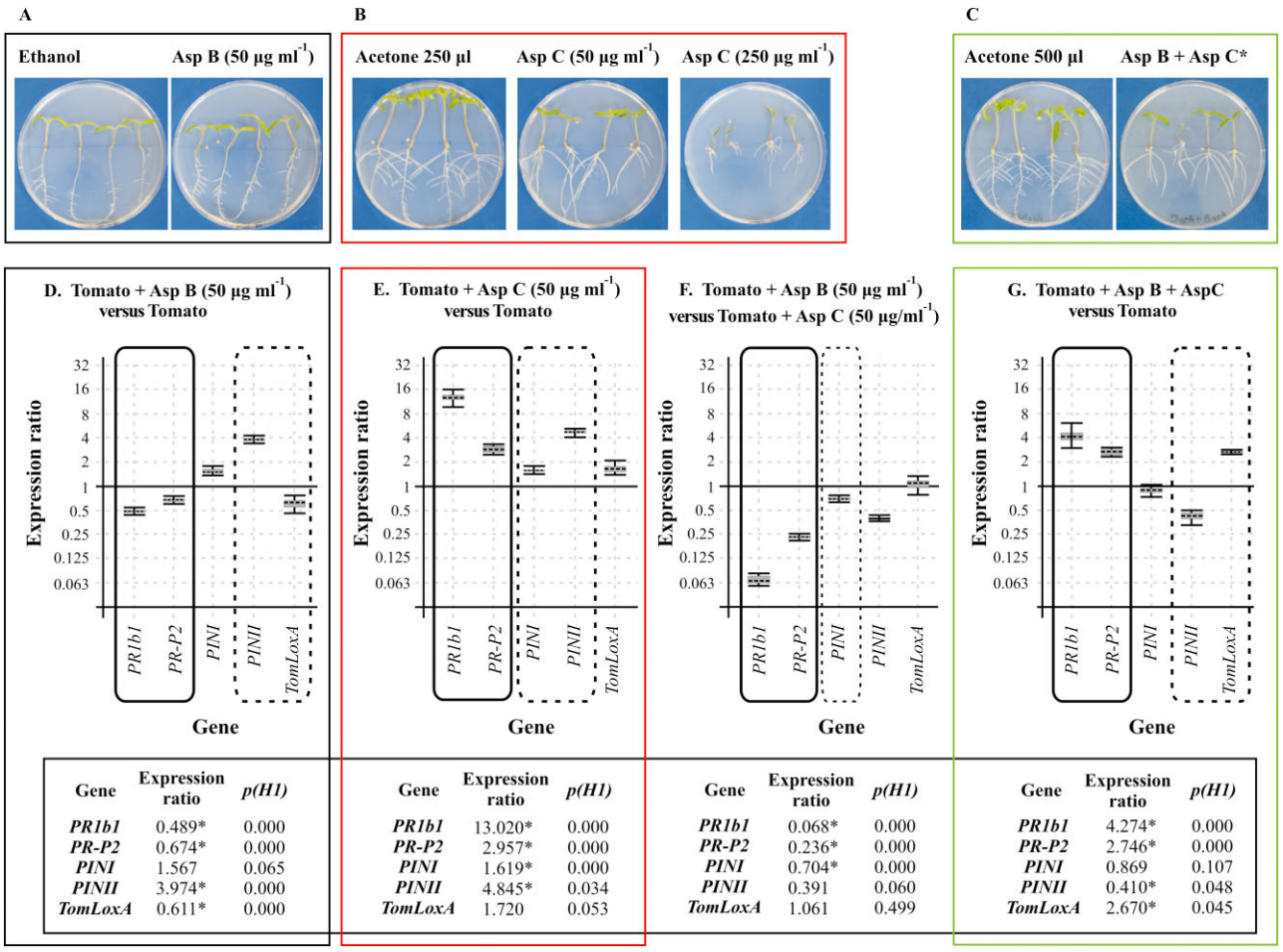
Discussion

The study of plant pathogen/host responses is a complex task, and involves DNA interactions (e.g. turning genes on and off), protein activity (e.g. hydrolytic enzymes) and toxin production (e.g. resulting in cell death) from both the aggressor and the host. Separating out the individual effects of any one of these responses is difficult. Adding a third level of biocontrol mechanisms from another micro-organism further complicates the study. Disrupting individual genes, and observing the effect, in any one of the three organisms (i.e. fungal pathogen, fungal antagonist and the host plant) allows one to define some of the complexity.

Trichoderma arundinaceum produces HA, an *in vivo* non-phytotoxic sesquiterpenoid compound with antifungal activity (Malmierca *et al*., 2012) that has a terpene-derived trichothecene ring structure and a polyketide-derived side-chain at the C-4 (Fig. 1). Disruption of *tri5* blocks the cyclization of FPP to form trichodiene and thus blocks production of HA (Malmierca *et al*., 2013). Interestingly, the *tri5* gene-disrupted transformant (TaΔTri5) produces the polyketides aspinolide B and aspinolide C, as well as smaller amounts of aspinolides D, E and G. Only small amounts of aspinolides D and F were found in the wild-type strain Ta37 (see Table 1).

Aspinolides have previously been reported only in *Aspergillus ochraceus* and a scheme for aspinolide B biosynthesis was proposed (Fuchser and Zeeck, 1997). As in the case of other decanolides (Mayer and Thiericke, 1993), the lactone skeleton of aspinolide B is formed by the polyketide pathway. The resulting pentaketide (decanolide) is further modified, first by oxidation at the C-4/C-5 double bond, and then by hydroxylation, followed by acylation with crotonic acid at C-8, giving rise to aspinolide B (Fuchser and Zeeck, 1997). Oxidation of aspinolide B at C-5 would give rise to aspinolide C (Fig. 3), which has increased antibiotic activity as well as other biological activities discussed later. Further modifications from these two aspinolides would give rise to aspinolides D, E, F and G. It is not clear if the increase in production of pentaketide aspinolides and the eight carbon HA side-chain are directly related; however, it is possible that polyketide biosynthesis is diverted to these compounds in the TaΔTri5 mutant, as a result of the *tri5* gene disruption.

Furthermore, of the two overproduced aspinolides, only aspinolide C displays antifungal activity against Bc98 (see Fig. 4), Bc05.10, and *F. sporotrichioides*, which suggests that it may be responsible in part for the remaining antagonistic effect shown by the TaΔTri5 mutant over *B. cinerea*.



Gene	Expression ratio	p(H1)
PR1b1	0.489*	0.000
PR-P2	0.674*	0.000
PINI	1.567	0.065
PINII	3.974*	0.000
TomLoxA	0.611*	0.000

Gene	Expression ratio	p(H1)
PR1b1	13.020*	0.000
PR-P2	2.957*	0.000
PINI	1.619*	0.000
PINII	4.845*	0.034
TomLoxA	1.720	0.053

Gene	Expression ratio	p(H1)
PR1b1	0.068*	0.000
PR-P2	0.236*	0.000
PINI	0.704*	0.000
PINII	0.391	0.060
TomLoxA	1.061	0.499

Gene	Expression ratio	p(H1)
PR1b1	4.274*	0.000
PR-P2	2.746*	0.000
PINI	0.869	0.107
PINII	0.410*	0.048
TomLoxA	2.670*	0.045

Fig. 8. Upper panels. Photographs illustrating the effect of (A) aspinolide B (Asp B),(B) aspinolide C (Asp C), and (C) aspinolides C and B (Asp B + Asp C) together, on the growth and root architecture of tomato plants. Lower panels. qPCR analysis of the relative level of expression of five tomato defence-related genes in the aerial parts of tomato plants grown under the effect of (D) 50 µg ml^{-1} aspinolide B, (E) 50 µg ml^{-1} aspinolide C, and (G) aspinolide B + aspinolide C (* = 50 µg ml^{-1} each). (F) represents the level of expression of those tomato defence-related genes in plants grown in the presence of aspinolide B versus the levels of expression in plants grown in the presence of aspinolide C. Comparative calculations, included in the bottom part of the figure, and graphic representations were carried out as indicated in the legend of Fig. 5.

Aspinolide C and HA may therefore have a redundant function in the environmental interactions between the fungi *T. arundinaceum* and the phytopathogen *B. cinerea*, whereby aspinolides are able to compensate by an unknown regulatory mechanism for the loss of HA. One hypothesis is that feedback control by HA on the signal transduction pathways regulates synthesis of both families of compounds, but such mechanisms have not been described so far in fungi. A similar effect has been described in *B. cinerea*, in which blocking BOT biosynthesis induces the synthesis of botcinic acid, a polyketide that may have a redundant role with that of BOT in the virulence of *B. cinerea* (Dalmais *et al.*, 2011). Expression analyses of the *T. arundinaceum* genes encoding polyketide synthases are in progress to identify aspinolide biosynthetic genes and to address these questions.

Many previous works have described changes in metabolite profiles in fungal mycelial interactions between different filamentous fungi (Hynes *et al.*, 2007; Peiris *et al.*, 2008; Rodriguez-Estrada *et al.*, 2011). However, to our knowledge, the present work is the first reference in which the effect of HA on *Trichoderma* as a natural antagonistic fungus is characterized at a molecular level.

Confrontation studies between Ta37 (producing HA) or TaΔTri5 (lacking HA) strains versus Bc98 resulted in an induction of the *B. cinerea* BOT biosynthetic genes only when HA was not produced. This result implies a modulation of the expression of the BOT biosynthetic genes by *T. arundinaceum* as a way of limiting virulence

of *B. cinerea*. A similar result was observed when this experiment was performed with a mutant in which the trichodiene oxygenase gene *tri4* was disrupted resulting in blocked HA biosynthesis (M. G. Malmierca, unpubl. data). The TaΔTri4 mutant produces 12,13 epoxytrichoene-2-ol derivatives, sesquiterpene metabolites that do not appear to affect the expression level of the BOT biosynthetic genes. This suggests that induction of BOT biosynthetic genes in confrontation studies with TaΔTri5 was due to the lack of HA rather than the increased production of aspinolides.

HA production was reduced by about 36% in *T. arundinaceum* in the interaction zone of confrontation experiments against Bc98, which correlates with the repression of *tri3*, *tri4*, *tri5*, *tri10* and *tri11* by this pathogen. Similar inhibitory effects on trichothecene synthesis during fungal interactions have been previously described. Thus, *Trichoderma* isolates producing 6-pentyl-α-pyrone (6-PP), a compound involved in the antifungal activity of *Trichoderma* (Rubio *et al*., 2009), reduced the production of the phytotoxic trichothecene deoxynivalenol by *Fusarium graminearum* from 66% to 81% (Cooney *et al*., 2001). In addition, production of 6-PP increased when *Trichoderma* was grown in the presence of specific pathogenic fungi (Cooney and Lauren, 1998).

The results obtained in the present work suggest that Bc98 may use a variety of virulence factors to defend itself from *T. arundinaceum*. The *atrB* gene encodes an ABC transporter that reduces the accumulation of toxic compounds (e.g. resveratrol) inside the cell, and therefore has an important role in protecting *B. cinerea* against damage caused by defensive metabolites secreted by plants or antagonistic fungi (Schoonbeek *et al*., 2001). Other ABC transporters have been shown to support both fungal growth and pathogenic action on the plant (Del Sorbo *et al*., 2008). In *T. atroviride*, upregulation of an ABC transporter encoding gene has been observed in the presence of pathogen-secreted mycotoxins and some fungicides (Ruocco *et al*., 2009). The strong induction of the *B. cinerea atrB* gene by the *T. arundinaceum* strains analysed in this work support the idea that this ABC transporter may also act as a resistance mechanism (Osbourn, 1999). However, it is interesting to note that this induction was produced by both the wild-type and mutant strains of *T. arundinaceum* used in this work. The induction of the ABC transporter in Bc98 appears to be a self-protection response to compounds or signals produced by *Trichoderma*, possibly HA in the case of the Ta37 strain, or aspinolide C in the case of TaΔTri5.

pg1 encodes an endoPG that participates in the degradation of polygalacturonic acid, located in plant cell walls. EndoPGs produced by *B. cinerea* release oligogalacturonides, which have been identified as elicitors of plant defence responses (de Lorenzo *et al*., 1990). *pg1* is overexpressed in Bc98 when the fungal antagonists TaΔTri5 or Ta37 are present, but especially with Ta37 due to the presence of HA. The level of expression of *pg1* in interaction with the *tri5* mutant is about 53% of the level observed when Bc98 is confronted with the wild-type strain. As Bc98 *pg1* is induced in the presence of *Trichoderma*, the resulting increased plant pectin degradation may result in induction of plant defence responses which may in turn slow the growth of Bc98. This hypothesis is supported by the observation that the TaΔTri5 mutant is not able to sensitize tomato plants to Bc98 invasion as efficiently as Ta37 (Malmierca *et al*., 2012).

A strong induction in the expression of all analysed defence-related genes was previously reported in plants treated with the Ta37 strain and infected with Bc98 (Malmierca *et al*., 2012). This increase in the expression of tomato defence-related genes in interaction with the Ta37 strain was understood as a result of the plant sensitization effect produced by *T. arundinaceum* in such a way that the genes involved in the tomato defence response were expressed faster and at a higher level after the infection with Bc98 when compared with plants not treated with *Trichoderma*. In this work, it has been shown that in the case of the TaΔTri5 strain, only the genes belonging to the SA pathway are positively sensitized, since their expression level was higher in plants treated with this mutant strain and infected with Bc98 than in plants infected with the phytopathogen alone or just treated with TaΔTri5. Meanwhile, *PINI* and *PINII*, involved in the JA pathway, appear to be downregulated, indicating that TaΔTri5 is not able to sensitize the plants against this pathogen through the JA pathway. Furthermore, the use of the TaΔTri5 mutant, lacking production of HA, led us to conclude that this metabolite is not the only compound responsible for the plant sensitization effect, since this mutant still has a significant sensitization effect on the SA pathway-related genes (Figs 7 and 9). This is an important difference in comparison to the TaΔTri4, a transformant that produces some early intermediates of the HA biosynthesis, in which the sensitization effect was not seen (Malmierca *et al*., 2012). Thus, the tomato plant sensitization effect observed with *T. arundinaceum* might not simply be due to HA or intermediates in HA biosynthesis, but probably is also due, at least in the case of the TaΔTri5 mutant, to the production of aspinolides (e.g. aspinolide C). The ability of aspinolide C to induce expression of SA-related genes observed in the present work serves to support this hypothesis. However, the induction of expression of SA-related genes observed in the *in vitro* Petri dish assays (Fig. 8) in which aspinolide C was added to the medium was determined in plantlets grown for just 6 days in the presence of this compound,

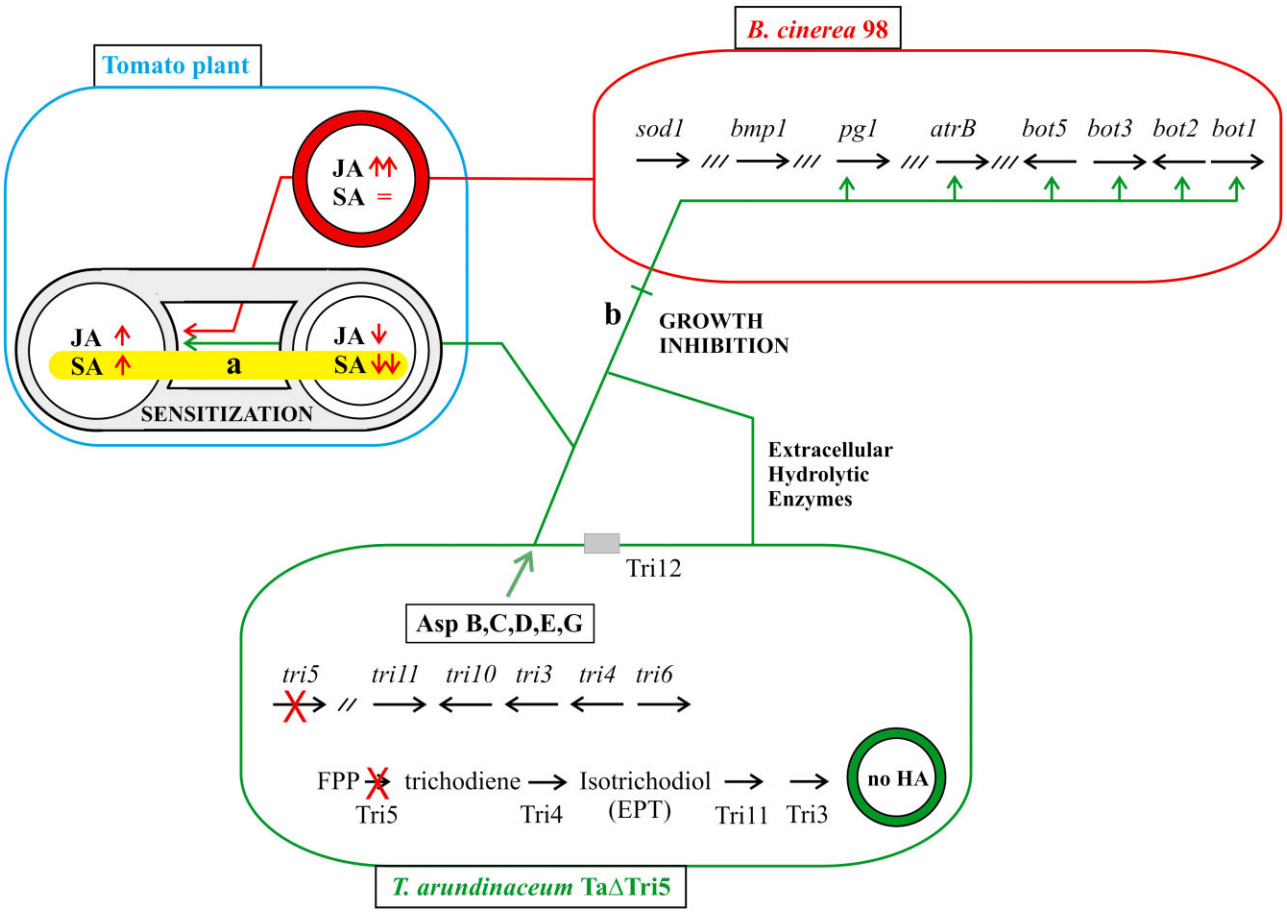


Fig. 9. Schematic representation of the interactions established among tomato plants, Bc98, and TaΔTri5, as deduced from the present work. Arrows indicate stimulatory response or gene up-regulation, and blunt-ended lines indicate gene repression or growth inhibition. In the tomato plant box, arrows pointing up and down indicate upregulation and downregulation, respectively, of the JA- or SA-related genes. Red and green lines indicate interactions mediated by Bc98 or T*richoderma* respectively.
a. sensitizing effect observed in TaΔTri5-pretreated plants caused by a compound, probably aspinolides (Asp C) produced by this fungal strain.
b. action of extracellular hydrolytic enzymes and aspinolides produced by TaΔTri5 to inhibit Bc98 growth.
Production of aspinolides B, C, D, E, G by the TaΔTri5 strain is also indicated.

while the *in vivo* assays (Fig. 7) were done on plants grown for 4 weeks from seeds inoculated with TaΔTri5. These differences in time of growth and also in the substrate in which the plants were grown may explain the differences observed in plants just treated with TaΔTri5 strain in the *in vivo* experiment.

Another possibility is that this sensitization effect is due to compounds synthesized as a result of channeling of the FPP from the trichothecene pathway towards other terpene-related compounds. In fact, it was shown that the *tri5*-disrupted mutant has about 42% more ergosterol at 96 h of growth, indicating that at least a fraction of the FPP that is not processed through the trichothecene biosynthetic pathway is channeled to ergosterol biosynthesis (Malmierca *et al.*, 2013). Ergosterol is synthesized from FPP and its primary function is as a major structural constituent of the fungal cell membrane. Ergosterol is also considered a general elicitor recognized by plants as a pathogen-associated molecular pattern (Nürnberger *et al.*, 2004) that induces several changes in various plant models, including modifications of H^+ fluxes (Kasparovsky *et al.*, 2003), changes in membrane potential, and increases in the level of reactive oxygen species (Rossard *et al.*, 2006). As demonstrated in sugar beet, JA and SA signalling are involved in ergosterol-induced elicitation (Rossard *et al.*, 2010). Thus, the increased level of expression of the tomato SA-related genes may also be due to the increase in the level of ergosterol observed in TaΔTri5, but further investigations are required to confirm this hypothesis.

Taking together these results, we can conclude that the loss of HA production in the *T. arundinaceum tri5*

deletion mutant resulted in an alteration in the expression of genes encoding for virulence factors in Bc98 during confrontation experiments. In addition, *tri5* gene disruption resulted in the production and accumulation of the polyketides aspinolides B and C. Aspinolide C showed a significant antifungal activity and also induced expression of tomato SA-related genes, which indicates that it may play a role in the sensitization effect of tomato defence-related genes (Fig. 9 for synopsis). In summary, *Trichoderma* trichothecenes can be considered as key molecules in the fungal metabolomics network and they play a role in the environmental interactions established by these fungi with other organisms present in the rhizosphere.

Experimental procedures

Fungal strains, plants, culture media and culture conditions

Trichoderma arundinaceum IBT 40837 (= Ta37) (IBT Culture Collection of Fungi at the Department of Biotechnology, Technical University of Denmark) is an HA producer strain. TaΔTri5 mutant is a strain previously obtained (Malmierca *et al.*, 2013) by genetic disruption of *tri5* gene which is blocked in the HA biosynthesis. For trichothecene analysis, *T. arundinaceum* strains were grown using a two-step procedure, first in CM [0.5% malt extract (Becton Dickinson, Heidelberg, Germany = BD), 0.5% yeast extract (BD), 0.5% glucose (Panreac Applichem, Barcelona, Spain = PA)] followed by growth in potato dextrose broth (PDB) medium (BD) as described previously (Cardoza *et al.*, 2011). For production and purification of aspinolides B and C, TaΔTri5 was grown on 5-GYEP [5% dextrose (PA), 1% yeast extract (BD), 1% peptone (BD)]. PDA medium [PDB medium + 2.5% Agar (BD)] was used to compare the profile of metabolites produced by the *Trichoderma* strains analysed in the present work with that observed by growth in MEA [2% glucose (PA), 2% malt extract (PA), 1% peptone (BD), 2% agar (BD), pH 5.6] medium. Finally, for sporulation, *T. arundinaceum* strains were grown on potatoes glucose (PPG) [2% mashed potatoes (Nestlé, Barcelona, Spain), 2% glucose (PA), 2% agar (BD)] medium at 28°C for 5 days.

The fungal phytopathogen Bc98 was isolated from a diseased strawberry plant. It was maintained on MEA medium and grown at 21°C for 5–7 days with a photoperiod of 16 h light/8 h dark. *Botrytis cinerea* Bc05.10 was maintained in MEA medium and grown in a similar way to that described for Bc98, and *F. sporotrichioides* was grown in PDA medium at 28°C for 5–7 days.

Solanum lycopersicum var. Marmande (Semillas Battle S.A., Barcelona, Spain) was used for fungal–plant and metabolite–plant interaction studies.

For analysis of metabolite production, the Ta37 and TaΔTri5 strains were grown on 12 Petri dishes containing 100 ml of MEA medium. Each Petri dish was inoculated with two 1 cm diameter agar plugs and grown for 12 days at 25°C under white light.

Analysis of the TaΔTri5 metabolite profile: general procedure

Purification by semipreparative and analytical HPLC was performed with a Hitachi/Merck L-7100 apparatus equipped with a differential refractometer detector (RI-7490). A LiChrospher® Si 60 (5 μm) LiChroCart® (250 mm × 4 mm) and a LiChrospher® Si 60 (10 μm) LiChroCart® (250 mm × 10 mm) columns were used in isolation experiments. Silica gel (Merck, Whitehouse Station, NJ, USA) was used for column chromatography. TLC was performed on a Merck Kieselgel 60 F_{254}, 0.25 mm thick. Optical rotation was determined with a digital polarimeter. Infrared spectra were recorded on a Fourier transform infrared spectroscopy (FT-IR) spectrophotometer and reported as wave number (cm^{-1}). ^{1}H-NMR and ^{13}C-NMR measurements were recorded on Agilent 400 MHz and Agilent 500 MHz spectrometers with $SiMe_4$ as the internal reference at 25°C. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.25, δ_C 77.0). NMR assignments were made using a combination of one-dimensional and two-dimensional techniques. Multiplicities are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. HRMS was recorded with a double-focusing magnetic sector mass spectrometer in positive ion mode or with a quadrupole time of flight (QTOF) mass spectrometer in positive ion electrospray mode at 20 V cone voltage. High resolution electrospray ionisation mass/mass spectrometry (HRESIMS/MS) experiments were performed with a QTOF mass spectrometer at 20 V cone voltage and 10 eV collision energy.

Analysis of the TaΔTri5 metabolite profile: metabolite analysis

The MEA medium from *Trichoderma* cultures was extracted with ethyl acetate (3 × 50 ml) using an ultrasonic bath for 30 min. The organic extracts were dried over Na_2SO_4 and concentrated to dryness. Evaporation of the solvent under reduced pressure afforded dense oil from Ta37 and TaΔTri5 cultures (756.6 mg and 918.8 mg, respectively) that were separated by column chromatography on silica gel eluted with mixtures containing increasing percentages of ethyl acetate/hexane (10–100%) and methanol as solvent. Final purification of selected fractions was carried out by HPLC.

The isolated metabolites were characterized by extensive spectrometric and spectroscopic analysis by HRMS, ^{1}H-NMR and ^{13}C-NMR (Table 2).

Analysis of the TaΔTri5 metabolite profile: production and purification of aspinolides C and B

For production of aspinolides C and B, TaΔTri5 strain was grown in 1 l liquid 5-GYEP [5% glucose (Fisher Scientific, Fair Lawn NJ, USA), 1% yeast extract (BD), 1% peptone (BD)] cultures for 6 days. Culture broths were filtered with Miracloth (EMD Millipore, Darmstadt, Germany) and the filtrate was then extracted with equal volumes of ethyl acetate. The extract was concentrated to a syrup with a rotary evaporator and then separated on a silica gel column eluted with n-hexane-ethyl acetate-methanol (48:92:10). Column frac-

tions were monitored for aspinolides with gas chromatography low-resolution mass spectrometry using a Hewlett Packard 6890 gas chromatograph fitted with an HP-5MS column (30 mm by 0.25 mm film thickness) and a 5973 mass detector. The identity of the isolated aspinolides was confirmed with ^{1}H-NMR and ^{13}C-NMR. Column fractions containing purified aspinolides were concentrated with a rotary evaporator and finally dried under a stream of nitrogen. Aspinolides C and B were resuspended in acetone and ethanol, respectively, and stored at −30°C until use.

Antifungal assays

In order to determine the antifungal activity of aspinolides C and B against Bc98 and Bc05.10, these strains were grown on MEA medium at 21°C during 7 days with a photoperiod of 16 h light/8 h dark. In the case of *F. sporotrichioides*, this strain was grown on PDA medium at 28°C in the dark during 5–7 days. Spores from these plates were recovered and added at a final concentration of 1×10^5 spores ml^{-1} to a bottle containing 100 ml of melted MEA medium, containing 1% agar, previously cooled to 50°C. Then, 20 ml of the medium, containing the fungal spores, were spread on Petri dishes of 9 cm diameter. Once the medium was solidified, five plugs of 7 mm diameter were removed from each plate, and finally 60 μl of aspinolide C or B dilution were added to each hole. The center hole in each plate was filled with acetone or ethanol as control for aspinolides C or B respectively. Plates were then kept at 4°C during 5 h, to allow metabolite diffusion, and finally incubated at 21°C for 24–48 h to detect and measure the diameter of the inhibition areas. The MIC of aspinolide C against the assayed fungi was calculated as the concentration that corresponded to an inhibition area of 7 mm diameter (the size of the holes).

Confrontation assays between T. arundinaceum *and* B. cinerea *strains*

In order to obtain RNA samples from the interaction zone between both fungi, *in vitro* confrontation assays were performed on MEA plates as described previously in Malmierca and colleagues (2012). After 7 days of incubation at 28°C, mycelia of *T. arundinaceum* and Bc98 (see Fig. 2A) were collected using a 5 mm-wide sterile spatula and immediately frozen in liquid nitrogen for RNA extraction.

Extraction and chemical analysis of HA

For analysis of HA produced in solid media, eight 1 cm diameter MEA plugs were removed from the *interaction* and *non-interaction* zones between the fungi (see Fig. 2B) and placed in a 10 ml tube. Four millilitres of ethyl acetate were added, vortexed for 1 min and removed. Another 4 ml of ethyl acetate were added, shaken for 3 h and recovered. The combined extracts were evaporated to dryness in a rotary evaporator at room temperature, resuspended in 150 μl of acetonitrile and stored at −20°C until further use. The extracts were analysed by HPLC for HA quantification as previously described (Corley *et al.*, 1994; Cardoza *et al.*, 2011). The data of HA production are expressed in micrograms of HA per millilitre of medium (μg HA ml^{-1}).

Tomato plant assays

Surface sterilized tomato seeds were inoculated with the TaΔTri5 mutant or the Ta37 wild-type strain, as previously described (Malmierca *et al.*, 2012). They were then sowed in commercial loamy field soil [Kekkilä 50/50 (Projar S.A., Valencia, Spain), refer to Malmierca and colleagues (2012) for composition]. Pots were incubated in a greenhouse at 21 ± 2°C with a photoperiod of 16 h light/8 h dark, and watered as needed. After 4 weeks, the tomato leaves were inoculated with 15 μl of Bc98 conidial suspensions [5×10^5 conidia ml^{-1} in germination buffer (20 mM glucose; 20 mM KH_2PO_4)]. After 4 days of incubation, leaves with or without pathogen inoculation were collected to extract RNA, in order to analyse the expression of tomato marker genes involved in defence responses as previously described (Malmierca *et al.*, 2012).

In vitro *analysis of the effect of aspinolide B and aspinolide C on tomato growth and on the expression of plant defence-related genes*

Tomato seeds (five per plate) sterilized as described previously (Malmierca *et al.*, 2012) were placed on 150 mm round Petri dishes containing 60 ml of Murashige and Skoog (MS) medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated in a greenhouse at 21 ± 2°C with a photoperiod of 16 h light/8 h dark for 4 days to allow germination. Four germinated tomato seeds were placed on another 150 mm round Petri dish with 50 ml of MS medium containing: 50 μg ml^{-1} aspinolide B, diluted in 250 μl ethanol or 250 μl of ethanol as a control; 50 μg ml^{-1} and 250 μl ml^{-1} of aspinolide C, diluted in 250 μl acetone or 250 μl of acetone as a control; 50 μg ml^{-1} aspinolide B + 50 μg ml^{-1} aspinolide C, diluted in 500 μl acetone or 500 μl acetone as a control. Each round Petri dish was sealed with adhesive tape and parafilm, placed in a slanted position and incubated in a greenhouse at 21 ± 2°C for 6 days. Plants were then photographed to observe differences in the main and secondary root length and in the above ground growth. Then, RNAs were extracted from the aerial parts of the plants and cDNAs were analysed by qPCR to determine the effect of aspinolides C or B treatment on expression of tomato genes involved in defence response as described above.

Nucleic acids extraction and manipulation

To isolate total RNA, mycelia from the 5 mm interaction zone in confrontation assays were frozen with liquid nitrogen and ground in a mortar. RNA was extracted with the phenol-SDS method (Ausubel *et al.*, 1987) and treated with DNase and RNase protector (Fermentas, Vilnius, Lithuania). The same procedure was also used to extract RNA from tomato leaves and from the aerial part of tomato plants in the plate assays.

The cDNAs were synthesized using 1 μg of total RNA and a reverse transcription system based on the use of an Oligo(dT)$_{15}$ primer (Promega, Madison, WI, USA).

Table 3. Oligonucleotides used for qPCR studies of *B. cinerea* genes.

Gene	Forward/reverse primer	Sequence 5′-3′
bmp1	BcMAPK-F	CCGCAAATGTTCTACACAGAG
	BcMAPK-R	CAGAGTTATCTTCTTGGGATGC
pg1	BcPG1-F2	TGTCAAGAACTCTGCCAACG
	BcPG1-R2	AGTGATGGGGACACCTGAAG
atrB	BcABC-F	CCGAAGTCACCGATTCATT
	BcABC-R	GGCGATTTGAGCGATACA
sod1	BcSOD-F	ACATTTCAACCCACACGG
	BcSOD-R	CAGAGCCAGTAGCATTTCCT

Note: oligonucleotides of *BcBOT* genes and the housekeeping *EF1b* gene were those described in Pinedo and colleagues (2008).

qPCR experiments

In order to perform comparative studies, previously described oligonucleotides of the *T. arundinaceum tri* genes, *B. cinerea BcBOT* genes and tomato plant defence-related genes were used (Pinedo *et al*., 2008; Cardoza *et al*., 2011; Tucci *et al*., 2011). The oligonucleotides used to analyse the expression of *B. cinerea bmp1*, *pg1*, *atrB* and *sod1* virulence genes were designed in the present work (Table 3). For *T. arundinaceum* gene expression studies, according to GeNorm software (Vandesompele *et al*., 2002) results, α-actin and *gpd* were used as reference genes. For tomato defence-related gene expression assays and for *B. cinerea* virulence gene analysis in confrontation assays, we used α-actin and EF1b, respectively, as the housekeeping genes. The qPCR reactions were carried out using the system Step One Plus (Applied Biosystems, Foster City, CA, USA). The reactions were performed in a total volume of 20 μl adding the following components/reaction: 10 μl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.4 μl Forward Primer 10 μM, 0.4 μl Reverse Primer 10 μM, 5 μl cDNA, and H_2O to 20 μl. The REST 2009© software (Pfaffl *et al*., 2002) was used to calculate the relative expression values and the significance of the differences between the gene expression levels. For each primer pair used in this work, we performed a standard curve with 320, 160, 80, 40, 20 and 10 ng cDNA for *Trichoderma* and *Botrytis* genes or 160, 80, 40, 20, 10 and 5 ng cDNA for tomato defence-related genes to determine the polymerase chain reaction amplification efficiency (E value) (Supporting Information Table S3). Each measurement was made in triplicate.

Statistical analysis

ANOVA and Kruskal–Wallis tests were performed with IBM SSPS Statistics 19 Software.

Acknowledgements

This research was supported by grants from MICINN (AGL2009-13431-C02) and MINECO (AGL2012-40041-C02 and AGL2012-39798-C02-01) and from Junta de Castilla y León (LE125A12-2). M. G. Malmierca was granted a FPU fellowship by the Spanish Ministry of Science and Innovation (AP2007-02835). J. Barua is grateful to ITAIPU Binacional (Paraguay) for his research fellowship. We thank Ulf Thrane from the Technical University of Denmark for kindly providing the *T. arundinaceum* IBT 40837 strain. Use of NMR and mass spectrometry (QTOF) facilities at the Servicio Centralizado de Ciencia y Tecnología (SCCYT) of the University of Cádiz is acknowledged.

We thank K. Vermillion (USDA/ARS, Peoria) for NMR analysis and J. Álvarez from the University of León, and J. Teresi and K. MacDonald from the Bacterial Foodborne Pathogens and Mycology Unit USDA/ARS, for their excellent technical assistance.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. 1a. ^{1}H-NMR Spectrum of Asp D (**3**).
1b. ^{13}C-NMR Spectrum of Asp D (**3**).
2a. ^{1}H-NMR Spectrum of Asp E (**4**).
2b. ^{13}C-NMR Spectrum of Asp E (**4**).
3a. ^{1}H-NMR Spectrum of Asp F (**5**).
3b. ^{13}C-NMR Spectrum of Asp F (**5**).
4a. ^{1}H-NMR Spectrum of Asp G (**6**).
4b. ^{13}C-NMR Spectrum of Asp G (**6**).
Table S1. Observed correlations in gHMBC experiments for aspinolides D–G.
Table S2. NOEs Observed for aspinolides D–G (**3–6**).
Table S3. Efficiency of the qPCR oligonucleotide pairs used in this work.