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Trichothecenes and aspinolides produced by *Trichoderma arundinaceum* regulate expression of *Botrytis cinerea* genes involved in virulence and growth

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Summary

Trichoderma arundinaceum **(Ta37) and** ***Botrytis cinerea*** **(B05.10) produce the sesquiterpenoids harzianum A (HA) and botrydial (BOT), respectively. TaΔTri5, an HA non-producer mutant, produces high levels of the polyketide compounds aspinolides (Asp) B and C. We analyzed the role of HA and Asp in the** ***B. cinerea*****-*****T. arundinaceum*** **interaction, including changes in BOT production as well as transcriptomic changes of** ***BcBOT*** **genes involved in BOT biosynthesis, and also of genes associated with virulence and ergosterol biosynthesis. We found that exogenously added HA up-regulated the expression of the** ***BcBOT*** **and all the virulence genes analyzed when** ***B. cinerea*** **was grown alone. However, a decrease in the amount of BOT and a down-regulation of** ***BcBOT*** **gene expression was observed in the interaction zone of B05.10-Ta37 dual cultures, compared to TaΔTri5. Thus, the confrontation with** ***T. arundinaceum*** **results in an up-regulation of most of the** ***B. cinerea*** **genes involved in virulence yet the presence of** ***T. arundinaceum*** **secondary metabolites, HA and AspC, act separately and together to down-regulate the** ***B. cinerea*** **genes analyzed. The present work emphasizes the existence of a chemical cross-regulation between** ***B. cinerea*** **and** ***T. arundinaceum*** **and contributes to understanding how a biocontrol fungus and its prey interact with each other.**

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Introduction

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is an airborne plant pathogen with a necrotrophic lifestyle affecting over 200 crop hosts worldwide causing the development of the "grey mould" proliferation. Infestation causes post-harvest rotting of vegetables, fruits, and flowers of apparently healthy crops and soft rotting of all aerial plant parts in some field- and greenhouse-grown horticultural crops prior to harvest (Williamson *et al.*, 2007). *B. cinerea* has a number of virulence genes which encode for infection structures, enzymes and toxins involved in the degradation of plant cutin and cell walls, host colonization, host cell killing, and/or the overcoming of oxidative bursts and host defenses (Nakajima and Akutsu 2014). One of these genes, *BMP1* [*Botrytis* mitogen-activated protein (MAP) kinase encoding gene], is necessary for penetrating and killing plant cells (Zheng *et al.*, 2000). A crucial point in the development of plant disease is the degradation of pectin, which allows penetration of the fungus into plant cells. At least five genes encoding endopolygalacturonases (endoPGs) have been described in *B. cinerea*. Mutants lacking *Bcpg1*, the gene that encodes a secreted endoPG, are less virulent against tomato leaves than the wild-type strain (Ten Have *et al.*, 1998). The ABC transporter (*BcatrB* gene) is involved in the sensitivity of

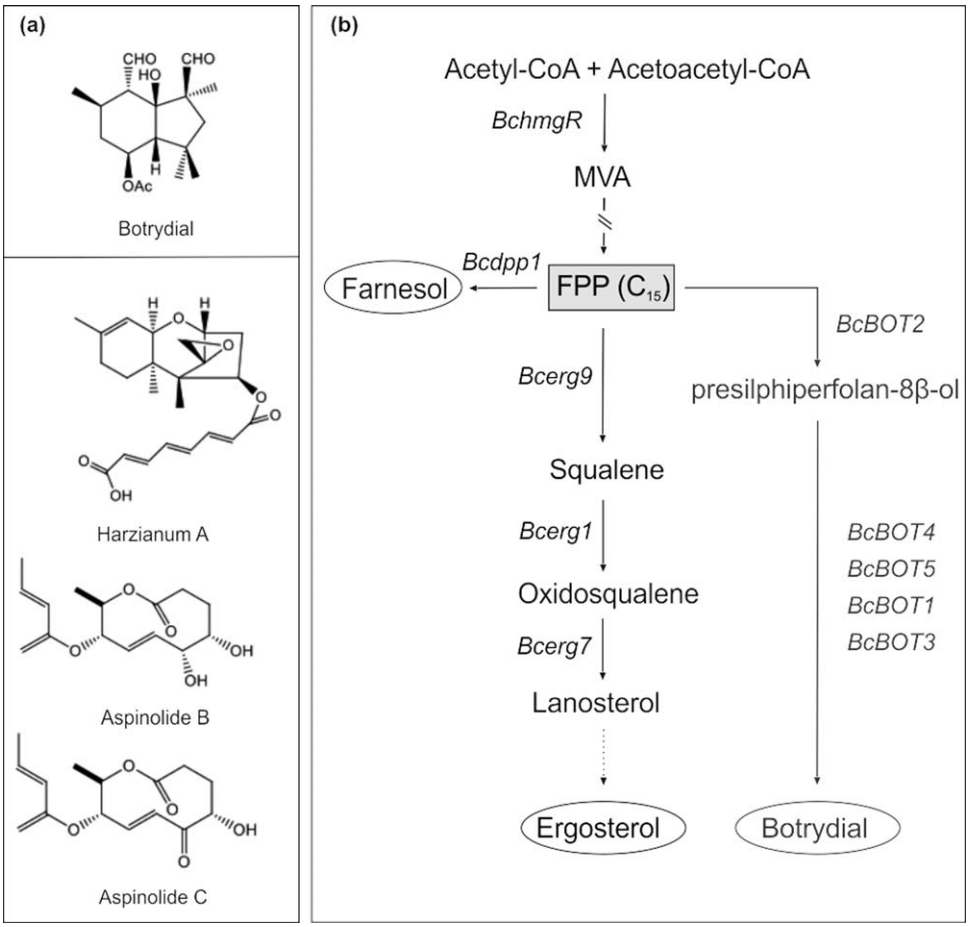

Fig. 1. (**a**) Chemical structures of SMs produced by *Botrytis cinerea* B05.10 (botrydial) or by *Trichoderma arundinaceum* (harzianum A, and aspinolides B and C) which have been analyzed or used in the present work.
(**b**) Schematic representation of the *Botrytis* terpene pathway including the branches for farnesol, ergosterol and botrydial biosynthesis from FPP. Genes are indicated in italics.

B. cinerea to the phytoalexin resveratrol and helps the pathogen escape plant defenses (Schoonbeek *et al.*, 2001). Another important virulence factor of *B. cinerea* is the superoxide dismutase (*bcsod1* gene) (Rolke *et al.*, 2004) that is involved in reactive oxygen species detoxification. Among the secondary metabolites (SMs) of *B. cinerea* that show phytotoxic activity are botrydial (BOT) (Fig. 1), a bicyclic sesquiterpene (Colmenares *et al.*, 2002) able to induce chlorosis and cell collapse during plant infection (Deighton *et al.*, 2001); and botcinic acid and its derivatives botcinins, which are polyketide compounds with a redundant virulence role with that of BOT (Dalmais *et al.*, 2011). BOT biosynthetic genes (*BcBOT* genes) are grouped in a cluster that includes *BcBOT1*, *BcBOT3*, and *BcBOT4* (all P450 monooxygenases), *BcBOT2* (sesquiterpene synthase), and *BcBOT5* (acetyl transferase) (Siewers *et al.*, 2005; Pinedo *et al.*, 2008). BOT is a sesquiterpene toxin that is synthesized from farnesyl diphosphate (FPP) produced by the mevalonate pathway. FPP is the branch point of several terpene pathways, among them ergosterol biosynthesis is especially important (Fig. 1), since it is a structural compound, that together with its intermediate squalene, strongly affect the rigidity or fluidity of the cell membrane (Spanova *et al.*, 2012), and are essential for fungal survival, as well as showing an important role in the modulation of plant defense responses and root colonization (Malmierca *et al.*, 2015c). Moreover, sterol biosynthesis is strongly regulated in the cell, and can have an effect on BOT production since both pathways have to compete for the FPP pool (Fig. 1).

Due to its high virulence versatility, *B. cinerea* is difficult to control. Considering the variety of modes of attack and diverse hosts as inoculum sources, the cost of bringing a new fungicide or biocontrol agent to market is so high that only major crops can attract sufficient economical interest (Williamson *et al.*, 2007). Biocontrol is a valid strategy against *B. cinerea* (Elad and Stewart 2007), with fungi of the genus *Trichoderma* having been reported as efficient antagonists (Harman *et al.*, 1996; Zimand *et al.*, 1996) that may be used to control this pathogen. Many *Trichoderma* strains produce SMs and enzymes with diverse industrial interest and biocontrol application (Lorito *et al.*, 2010). However, only species belonging to the clade Brevicompactum have the ability to produce the non-volatile sesquiterpenoid trichothecenes within the genus *Trichoderma* (Degenkolb *et al.*, 2008). Thus, *T. arundinaceum*

produces harzianum A (HA) (Fig. 1), which does not show *in vivo* phytotoxicity (Malmierca *et al*., 2012). In previous works we observed that the disruption of the trichodiene synthase encoding gene (*tri5*) (strain TaΔTri5) blocked the production of HA and reduced the biocontrol potential against *B. cinerea* (Malmierca *et al*., 2013), but also resulted in the production of high levels of aspinolides (Asp) B and C (Malmierca *et al*., 2015a) (Fig. 1). Among these polyketide compounds, AspC showed antifungal activity against *B. cinerea* (Malmierca *et al*., 2015a). Although the culture broth of TaΔTri5 failed to prevent tomato leaf lesion formation by *B. cinerea*, the size of the lesions was reduced due to the antifungal activity of AspC (Malmierca *et al*., 2013).

The aim of the present work was to analyze the role of HA, AspC, and AspB produced by *T. arundinaceum* in the confrontation with *B. cinerea*, in order to understand the impact of these sesquiterpene and polyketide metabolites on the behavior of its prey. To explore these mechanisms, wild-type strain Ta37 as well as the *T. arundinaceum* transformant blocked in HA biosynthesis (TaΔTri5) and *B. cinerea bcbot2*Δ mutant (in which *BcBOT2* is deleted) were used. The production of BOT, squalene and ergosterol by *B. cinerea* and the expression of (1) genes involved in BOT biosynthesis, (2) genes encoding for other virulence factors, and (3) genes belonging to the ergosterol biosynthetic pathway were measured in single cultures of *B. cinerea* and in *B. cinerea-T. arundinaceum* dual cultures with or without HA or Asps supplementation.

Results

BOT production by B05.10 and in the confrontation with Ta37 or TaΔTri5

B05.10 produced 6.2 μg/mL of BOT, when grown on MEA as single culture. In the dual culture confrontation plate experiments, BOT was extracted from both the interaction (IZ) and non-interaction (NIZ) zones (Fig. 2a). A greater amount of BOT (20.42 μg/mL) was detected in the NIZ of B05.10-Ta37 dual cultures than in the IZ (1.29 μg/mL), suggesting inhibition of BOT production by Ta37 (Table 1). B05.10-TaΔTri5 dual cultures showed BOT production was about the same in either the IZ or NIZ and about the same as in the NIZ of B05.10-Ta37 dual cultures (Table 1). This suggests that HA production is specifically inhibiting BOT production in the confrontation cultures, but only in the IZ.

Gene expression patterns in confrontation cultures

To determine the effect that Ta37, an HA producer strain, has on gene expression in *B. cinerea*, qPCR expression analyses were done on genes involved in BOT synthesis (*BcBOT*), on selected *B. cinerea* virulence genes, and on *B. cinerea* ergosterol biosynthetic genes during confrontation. Confrontation of *B. cinerea* B05.10 with Ta37 did not, or only slightly, affect *BcBOT* genes in the NIZ and slightly up-regulated most of *BcBOT* genes in the IZ (Fig. 2b). Moreover, *B. cinerea* virulence genes such as *BcatrB* (18 fold), *BMP1* (2.3 fold) and *Bcpg1* (2.5 fold) were also up-regulated in the IZ (Fig. 2b, Supporting Information Table S1a). Expression of genes involved in ergosterol biosynthesis in the IZ did not show a homogeneous pattern; *Bcerg1* was slightly down-regulated while expression of *BchmgR* and *Bcdpp1* was slightly increased relative to B05.10 growing alone (Fig. 2b, Supporting Information Table S1b).

Confrontation of B05.10 with TaΔTri5 showed little or no effect on *BcBOT* expression in the NIZ (Fig. 2c and d), but showed strong up-regulation on *BcBOT* genes (3 to 195 fold) in the IZ (Fig. 2d, Supporting Information Table S1a). The virulence genes, *BcatrB* (162 fold), *BMP1* (7 fold), and *Bcpg1* (7 fold) were also up-regulated in the IZ (Fig. 2d). Finally, the ergosterol pathway genes were up-regulated with *BchmgR* strongly up-regulated (1229 fold), *Bcerg7* highly up-regulated (128 fold), and the remaining ergosterol pathway genes moderately up-regulated (7.5-35 fold) in the IZ (Fig. 2d, Supporting Information Table S1b).

These data suggest a factor in *T. arundinaceum* that triggers BOT production in *B. cinerea*, as well as expression of *B. cinerea* virulence and ergosterol genes. However, endogenous HA production (by Ta37) represses *BcBOT* gene expression in the IZ, resulting in the lower accumulation of BOT seen in the IZ (Table 1). In the same way, endogenous HA also represses the B05.10 virulence and ergosterol biosynthetic genes in the IZ, compared with the confrontation with TaΔTri5.

Effect of exogenously added HA on the expression of B05.10 virulence genes

To analyze the direct effect of HA on the expression of selected B05.10 genes, this strain was grown as a single culture on MEA medium (control), or on MEA supplemented with purified exogenous HA (36 or 208 μg/mL). When HA was added at a concentration of 36 μg/mL, simulating the amount produced by *T. arundinaceum* in the IZ of B05.10-Ta37 dual cultures, a significant up-regulation of *BcBOT5*, *BcBOT3*, *BcBOT2*, and *BcBOT1* genes was detected compared to the expression levels detected in the control (Fig. 3a and b). Moreover, all the other virulence genes analyzed were also expressed at higher levels, with expression ratios ranging from 1.7 to 74.9 fold for *BMP1* and *Bcpg1*, respectively (Supporting Information Table S1a). In addition, all the ergosterol biosynthetic genes, except *Bcdpp1*, were also up-regulated by about 2 fold (Fig. 3b; Supporting Information Table S1b).

When exogenous HA (36 μg/mL) was added to dual cultures of B05.10 *vs* TaΔTri5, the relative level of expression

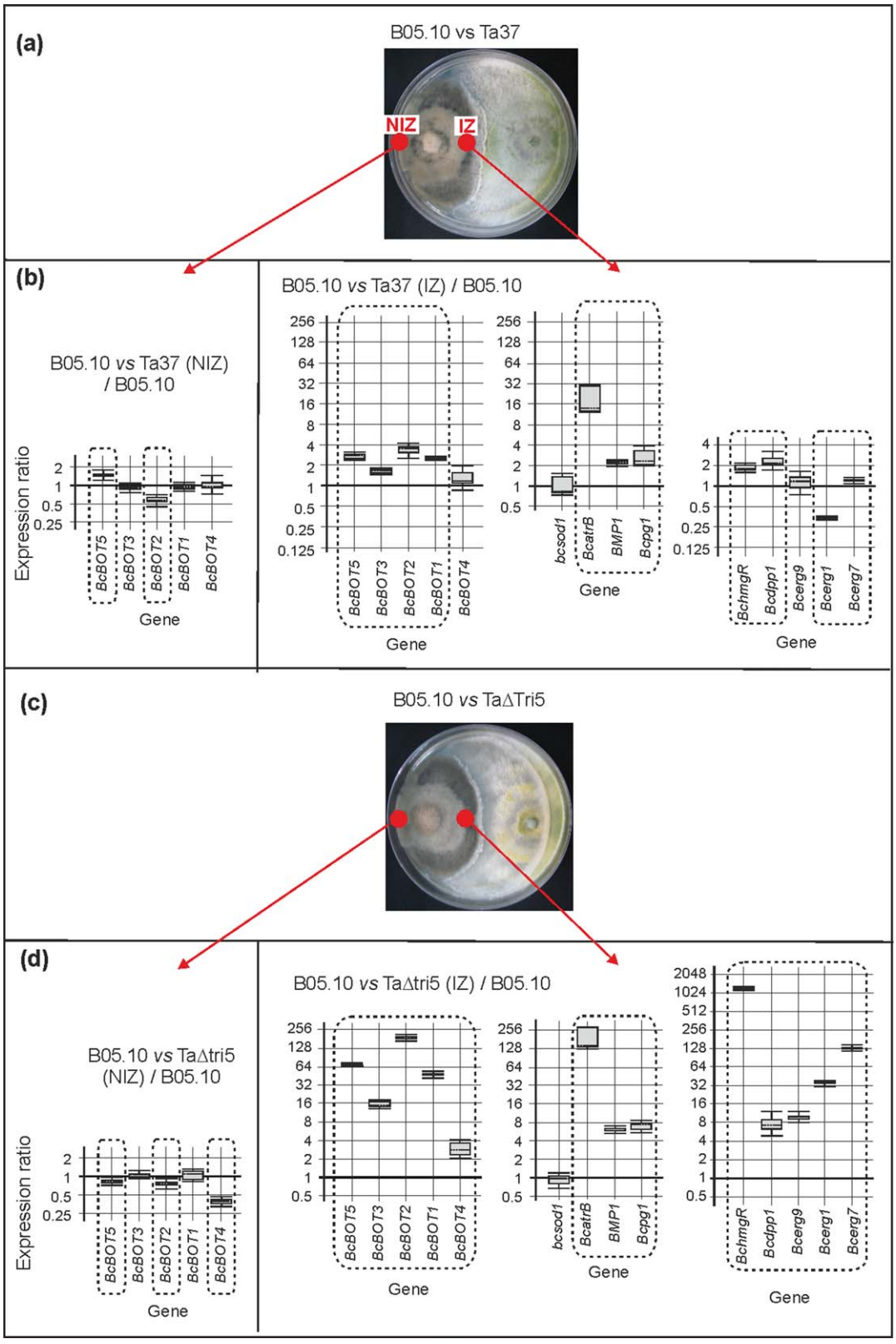
(a)
B05.10 vs Ta37
NIZ
IZ
(b)
B05.10 vs Ta37 (NIZ) / B05.10
Expression ratio
2
1
0.5
0.25
BcBOT5
BcBOT3
BcBOT2
BcBOT1
BcBOT4
Gene
B05.10 vs Ta37 (IZ) / B05.10
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
BcBOT5
BcBOT3
BcBOT2
BcBOT1
BcBOT4
Gene
bcsod1
BcatrB
BMP1
Bcpg1
Gene
4
2
1
0.5
0.25
0.125
BchmgR
Bcdpp1
Bcerg9
Bcerg1
Bcerg7
Gene
(c)
B05.10 vs TaΔTri5
(d)
B05.10 vs TaΔtri5 (NIZ) / B05.10
Expression ratio
2
1
0.5
0.25
BcBOT5
BcBOT3
BcBOT2
BcBOT1
BcBOT4
Gene
B05.10 vs TaΔtri5 (IZ) / B05.10
256
128
64
32
16
8
4
2
1
0.5
BcBOT5
BcBOT3
BcBOT2
BcBOT1
BcBOT4
Gene
bcsod1
BcatrB
BMP1
Bcpg1
Gene
2048
1024
512
256
128
64
32
16
8
4
2
1
0.5
BchmgR
Bcdpp1
Bcerg9
Bcerg1
Bcerg7
Gene

Fig. 2. Dual cultures of (**a**) B05.10 *versus* Ta37 and (**c**) B05.10 *versus* TaΔTri5. The interaction zone (IZ) and non-interaction zone (NIZ) from where the samples of mycelia were collected for further analysis are indicated in (**a**) and (**c**).
(**b, d**) Analysis of gene expression level of *B. cinerea* genes in mycelia isolated from the NIZ (left panel) and IZ (right panel) with (**b**) Ta37 or (**d**) TaΔTri5, *versus* gene expression level in B05.10 growing alone. Three sets of genes were analyzed: 1) BOT biosynthetic genes: (*BcBOT5, BcBOT3, BcBOT2, BcBOT1, BcBOT4*) analyzed in both zones; 2) other virulence genes *bcsod1*, *BcatrB*, *BMP1*, and *Bcpg1*; and 3) genes belonging to the ergosterol biosynthetic pathway (*BchmgR, Bcdpp1, Bcerg9, Bcerg1* and *Bcerg7*). The sets of genes 2) and 3) were only analyzed in the IZ. The expression ratios as well as the statistical probability values were calculated using the REST 2009 software (Pfaffl et al., 2002). Numeric values are included in Supporting Tables S1a and S1b. Those data statistically significant [$p(H1)<0.05$] are outlined in a square in the graphic representation.

of *BcBOT* in the IZ was much lower (Fig. 3c and d) than that seen in the dual culture of B05.10 *vs* TaΔTri5 (Fig. 2d), showing that the addition of HA down-regulated *BcBOT* expression in this confrontation. In fact, the relative expression of *BcBOT* was at a level very similar to the expression levels seen in B05.10 *vs* Ta37 (producing HA) (Fig. 2b).

The addition of exogenous HA (36 μg/mL) to B05.10 *vs* TaΔTri5 also relatively down-regulated the expression of the virulence genes and ergosterol biosynthetic genes (Fig. 3c and d; Supporting Information Table S1a, b), when compared with the same confrontation without adding HA.

When a higher amount of HA was added to the medium of B05.10 single cultures, at the non-biological concentration of 208 μg/mL, all the *BcBOT* genes were again up-regulated (Supporting Information Fig. S1), but not as highly as seen in cultures treated with 36 μg/mL HA. At this higher HA concentration, the ergosterol biosynthetic genes were mostly unaffected, except *BchmgR* and *Bcerg1* which were slightly up-regulated. Except for *BMP1*, most of the virulence genes were slightly up-regulated as well. The addition of 208 μg/mL HA to the dual culture of B05.10 *vs* TaΔTri5, resulted in a down-regulation of all *BcBOT*, except for *BcBOT4*, in the IZ (Supporting Information Fig. S2). The higher amount of HA up-regulated the virulence gene *BcatrB* but either down-regulated or had no effect on the other virulence genes in the IZ (Supporting Information Fig. S2). Similarly, the higher amount of HA had either a small effect or no effect on the expression of the ergosterol biosynthetic pathway genes (Supporting Information Fig. S2).

The results so far suggest that HA exogenously added to a single culture of B05.10 up-regulates gene expression of BOT, virulence, and ergosterol pathways. Yet the dual culture data suggest that the presence of HA in the IZ represses the expression of these genes.

Table 1. Production of BOT by B05.10 growing in dual cultures with Ta37 or TaΔTri5.

Conditions	NIZ μg BOT/mL	IZ μg BOT/mL
B05.10 *vs* Ta37	$20.42^{a} \pm 0.55$	$1.29^{b} \pm 0.16$
B05.10 *vs* TaΔTri5	$19.65^{a} \pm 1.76$	$17.87^{a} \pm 0.61$

*Production of B05.10 growing alone was calculated as $6.2^{c} \pm 0.61$ μg BOT/mL.
Krustal-Wallis test ($p < 0.05$; $n = 3$).
[a,b] For each region, values followed by different superscript letters are significantly different ($p < 0.05$).
NIZ= Non-Interaction Zone; IZ= Interaction Zone.

Production of squalene and ergosterol

The quantities of squalene and ergosterol were measured in single and dual cultures with or without exogenously added HA. Thus, the presence of endogenous HA in the dual culture of B05.10 *vs* Ta37 resulted in no significant change in squalene levels (precursor to ergosterol, Fig. 1) and a decrease of about 23% in ergosterol levels (Table 2). Gene expression levels indicated no change in *Bcerg9* (FPP→squalene), a slight decrease (0.3 fold) in *Bcerg1* expression, and a slight increase (1.2 fold) in *Bcerg7* (see Supporting Information Table S1b), the latter of which does not directly correlate with the drop in ergosterol.

Similarly, the increased relative gene expression levels in B05.10 *vs* TaΔTri5 (no HA present) did not correlate with the maintained levels of squalene and ergosterol (Table 2). Thus, as a consequence of the channeling of terpene precursors toward BOT biosynthesis, the ergosterol biosynthetic genes are overexpressed to compete with the BOT pathway for those precursors. HA (36 μg/mL) exogenously added to either B05.10 alone or to the dual culture of B05.10 *vs* TaΔTri5 decreased squalene accumulation ∼100% and ergosterol levels 40-57% (Table 2), which were much lower levels of production than those expected from the gene expression levels (Fig. 3).

These data suggest that HA affects the accumulation of squalene and ergosterol in a negative manner and that HA down-regulates relative gene expression of the ergosterol biosynthetic genes analyzed in the IZ, when compared with expression levels in the same cultures without HA.

Effect of AspB and AspC on the relative expression of B05.10 genes

The data so far have shown that when HA was not present (B05.10 *vs* TaΔTri5), the expression of *BcBOT* was highly up-regulated in the IZ but not in the NIZ (Fig. 2, Supporting Information Table S1a). This suggests that a factor produced by TaΔTri5 is very important in affecting *BcBOT* expression. This factor is most likely to be at a higher concentration in the IZ of dual cultures than in the NIZ.

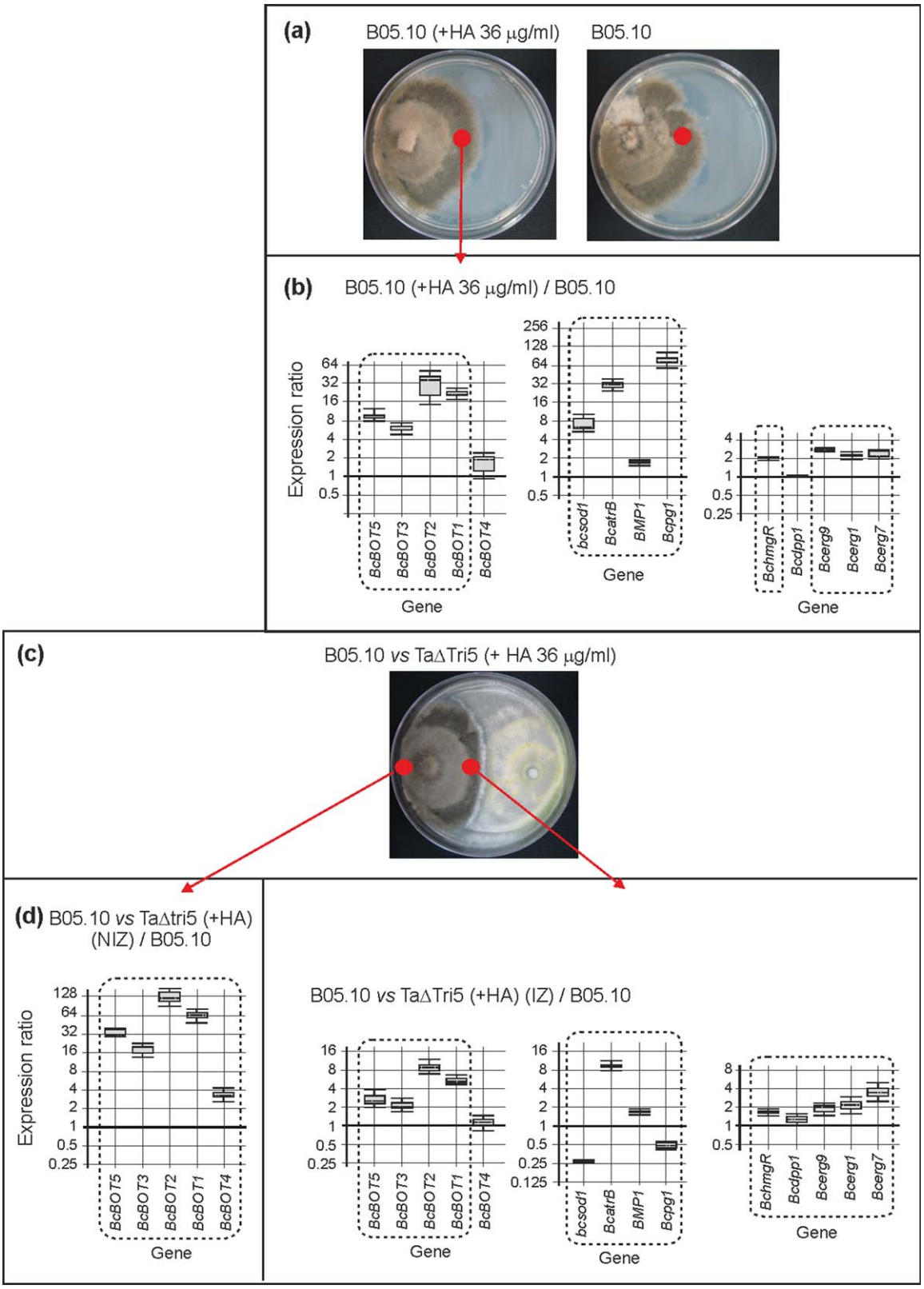
(a)
B05.10 (+HA 36 μg/ml)
B05.10
(b)
B05.10 (+HA 36 μg/ml) / B05.10
Expression ratio
64
32
16
8
4
2
1
0.5
BcBOT5
BcBOT3
BcBOT2
BcBOT1
BcBOT4
Gene
256
128
64
32
16
8
4
2
1
0.5
bcsod1
BcatrB
BMP1
Bcpg1
Gene
4
2
1
0.5
0.25
BchmgR
Bcdpp1
Bcerg9
Bcerg1
Bcerg7
Gene
(c)
B05.10 vs TaΔTri5 (+ HA 36 μg/ml)
(d) B05.10 vs TaΔtri5 (+HA) (NIZ) / B05.10
Expression ratio
128
64
32
16
8
4
2
1
0.5
0.25
BcBOT5
BcBOT3
BcBOT2
BcBOT1
BcBOT4
Gene
B05.10 vs TaΔTri5 (+HA) (IZ) / B05.10
16
8
4
2
1
0.5
0.25
BcBOT5
BcBOT3
BcBOT2
BcBOT1
BcBOT4
Gene
16
8
4
2
1
0.5
0.25
0.125
bcsod1
BcatrB
BMP1
Bcpg1
Gene
8
4
2
1
0.5
BchmgR
Bcdpp1
Bcerg9
Bcerg1
Bcerg7
Gene

Fig. 3. Photographs illustrating the growth of (**a**) B05.10 in the presence of 36 μg/mL HA (left panel) and without HA (right panel), and (**c**) dual culture of B05.10 *versus* TaΔTri5 in media supplemented with 36 μg/mL HA.
(**b, d**) Analysis of gene expression level of *B. cinerea* genes in (**b**) mycelia grown alone in the presence of 36 μg/mL HA, and (**d**) in *B. cinerea* mycelia isolated from the NIZ and IZ with TaΔTri5 in media supplemented with 36 μg/mL HA, in both cases *versus* gene expression level in mycelia of B05.10 growing alone. Comparative calculations, graphic representations were carried out as indicated in the legend of Fig. 2 and the numeric values are included in Supporting Tables S1a and S1b.

To test if secondary metabolites produced by TaΔTri5 have a role in B05.10 gene regulation, the effects of AspB and AspC, two polyketide compounds produced by TaΔTri5, were analyzed. AspB (50 μg/mL) caused an overall slight up-regulation of *BcBOT* expression on B05.10 alone (Supporting Information Fig. S3) but had only a minimal effect on the dual culture of B05.10 *vs* Ta37 in the IZ (Fig. 4). In the presence of AspC (50 μg/mL), all *BcBOT* genes were up-regulated in B05.10 alone (Supporting Information Fig. S3). However, in the dual culture of B05.10 *vs* Ta37, addition of exogenous AspC down-regulated the expression of *BcBOT* (Fig. 4). This down-regulation occurred in the presence of endogenous HA (Ta37) and such down-regulation was not observed in the absence of HA (B05.10 *vs* TaΔTri5 which is $HA^{-}AspC^{+}$) (Fig. 2d). This suggests that the combination of AspC and HA are down-regulating *BcBOT* genes in B05.10 confronted with *T. arundinaceum*. Neither AspB nor AspC had much effect on the virulence genes tested, except *BcatrB* which was strongly down-regulated by either one in the IZ of dual cultures of B05.10 *vs* Ta37 (Fig. 4; Supporting Information Fig. S3; Supporting Information Table S1a).

Summarizing these results, AspC showed a down-regulating effect on *BcBOT* and the virulence genes *BcatrB, BMP1*, and *Bcpg1* when in the presence of HA and/or other unidentified SMs produced by *T. arundinaceum*.

Correlation between the level of expression of Botrytis *virulence genes, production of BOT, and the overgrowth by* Trichoderma *strains*

The production of HA, Asps, and/or other SMs by *Trichoderma* affects gene regulation in *B. cinerea* during confrontation between the two strains. One would expect the up/down regulation of virulence genes produced by *B. cinerea* to have an effect on the growth pattern of *T. arundinaceum* over the respective *Botrytis* mycelia. Results of growth studies showed that the least amount of overlapping growth was seen in dual cultures of B05.10-TaΔTri5 and the most overlapping growth was seen in *bcbot2*Δ-Ta37 confrontation plates (Fig. 5). The expression levels of the virulence genes *BcatrB, BMP1* and *Bcpg1* in the IZ were higher when confronted with the TaΔTri5 strain (i.e., B05.10 *vs* TaΔTri5), which lacks HA production, and on the opposite end of the spectrum, they are expressed at the lowest level in the mutant lacking BOT production (i.e. *bcbot2*Δ against Ta37), which correlated with the overgrowth patterns. The expression of these genes followed a totally different pattern in the NIZ which would imply some kind of signal transduction through the *Botrytis* colony from the IZ towards the NIZ (Fig. 5; Supporting Information Table S1c).

Discussion

Mycoparasitism, the ability to antagonize, parasitize or even kill other fungi, is an ancestral property of certain *Trichoderma* species (Druzhinina *et al*., 2011). Recent studies identified the antifungal properties of the trichothecene HA and the polyketide AspC produced by *T. arundinaceum* against *B. cinerea* (Malmierca *et al*., 2013; 2015a). The sesquiterpene BOT is a virulence factor produced by *B. cinerea* (Deighton *et al*., 2001), and has the highest phytotoxic activity among the metabolites synthesized by this fungus (Colmenares *et al*., 2002).

Fungal co-cultures have been used to simulate physiological conditions potentially involved in SM production occurring in a given ecosystem (Brakhage, 2013). Mycelial

Table 2. Production of squalene and ergosterol by the control of B05.10 growing alone and in confrontation with Ta37 and TaΔTri5.

	DW (g)	Squalene (mgS/g DW)	% variation	Ergosterol (mgE/g DW)	% variation
B05.10	0.031	$1.350^{a} \pm 0.349$		$66.26^{a} \pm 0.28$	
B05.10-Ta37	0.021	$1.176^{a} \pm 0.179$		$50.81^{b} \pm 2.84$	−23.31
B05.10-TaΔTri5	0.018	$1.435^{a} \pm 0.323$		$67.97^{a} \pm 3.93$	
B05.10 (+HA)*	0.012	0^{b}	−100%	$28.72^{c} \pm 2.01$	−56.66
B05.10-TaΔTri5 (+HA)*	0.011	$0.013^{b} \pm 0.007$	−99.03	$39.64^{d} \pm 2.26$	−40.17

$n = 2$, ANOVA.
^{a,b,c,d} For each column, values followed by different superscript letters are significantly different ($p < 0.05$).
DW - Dry weight.
*In these conditions the cultures were supplemented with 36 μg/mL HA.

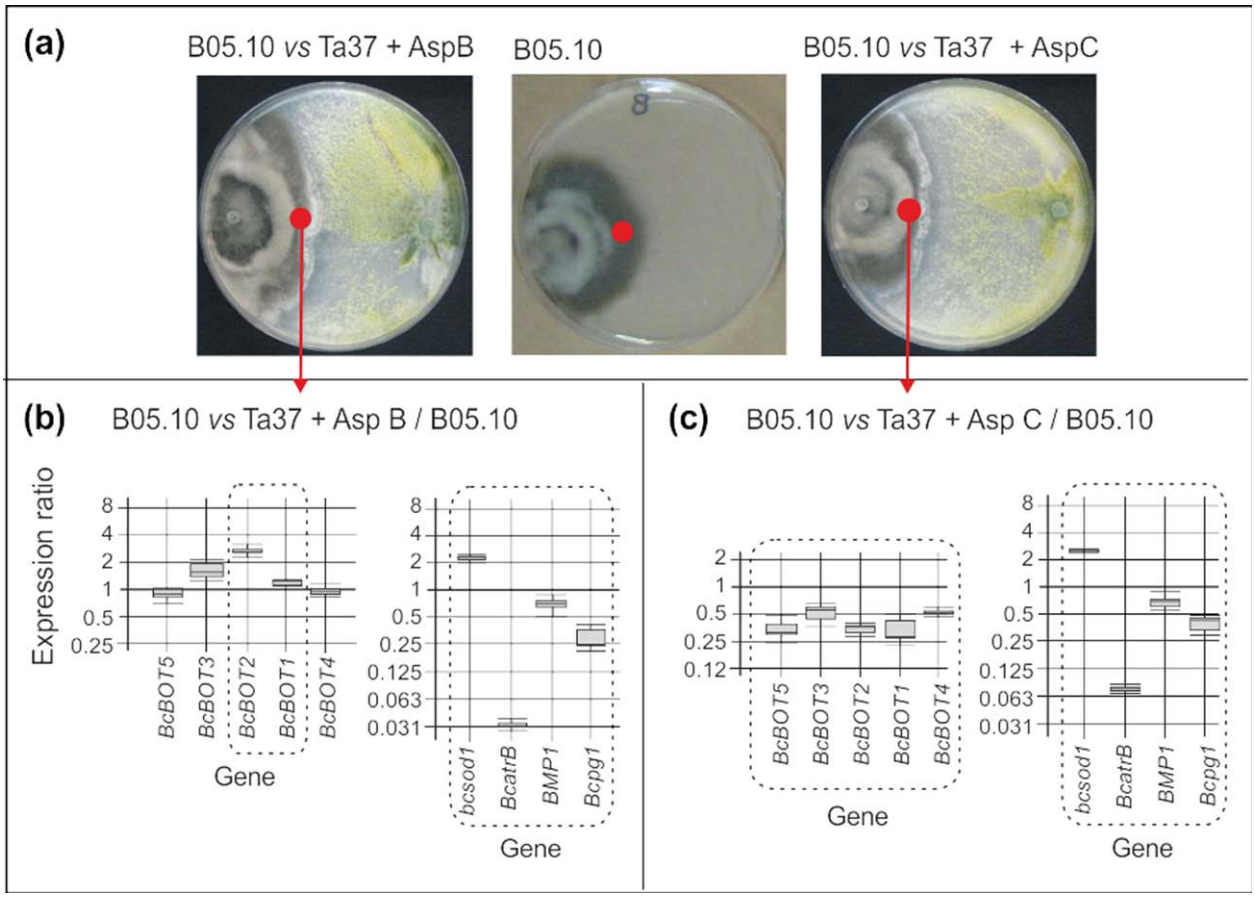

Fig. 4. (**a**) Photographs illustrating dual cultures of B05.10 *versus* Ta37 in the presence of 50 μg/mL AspB (left panel), B05.10 grown alone without Asps (central panel), and in the presence of 50 μg/mL AspC (right panel).
(**b, c**) Analysis of gene expression level in *B. cinerea* mycelia isolated from the interaction region with Ta37 in media supplemented with 50 μg/mL AspB (**b**) or AspC (**c**), *versus* gene expression level in mycelia of B05.10 grown alone in medium not supplemented with Asps. Comparative calculations and graphic representations were carried out as indicated in the legend of Fig. 2 and the numeric values are included in Supporting Tables S1a and S1b.

interaction experiments have allowed the detection of volatile compounds that are not produced in single cultures (Hynes *et al*., 2007) and of metabolite profiles that change depending on the prey fungus (Rodríguez-Estrada *et al*., 2011; Cooney *et al*., 2001; Cooney and Lauren, 1998). In a recent study we determined that BOT and botcinins produced by *Botrytis cinerea* regulate the expression of Ta37 genes involved in HA biosynthesis (Malmierca *et al*., in press). We have focused the present study on the interaction zones between *T. arundinaceum* and *B. cinerea* where significant changes in BOT production were detected (Table 1), in order to determine the effect of metabolites produced by *T. arundinaceum* on the regulation of B05.10 genes involved in (1) BOT biosynthesis, (2) *Botrytis* virulence, and (3) sterol biosynthesis.

To help define whether the trichothecene HA produced by *T. arundinaceum* is involved in BOT gene regulation, the TaΔTri5 mutant, which is unable to produce HA, was used. The confrontation of *B. cinerea* with TaΔTri5 triggered a strong up-regulation of *BcBOT* genes in the IZ, accompanied by a 13-fold increase in BOT concentration over that seen in B05.10 *vs* Ta37 (Table 1), which supports the theory that HA down-regulates BOT gene expression in confrontation cultures. Interestingly, the addition of 36 μg/mL or 208 μg/mL exogenous HA to the dual culture of B05.10-TaΔTri5 down-regulated *BcBOT* genes in a concentration-dependent manner. The results can be explained in two different ways, (i) HA itself down-regulates expression of *BcBOT* genes or (ii) expression of *BcBOT* genes in B05.10 is activated by metabolites produced by TaΔTri5, which are not produced, or are produced at a much lower level, by Ta37.

For (ii), candidate metabolites were identified in TaΔTri5. The polyketide aspinolides AspB and AspC were produced at high levels in this mutant strain but were present in Ta37 at only trace amounts (Malmierca *et al*., 2015a). AspC had significant antifungal activity, although much less than HA (Malmierca *et al*., 2015a). Data from *BcBOT* gene

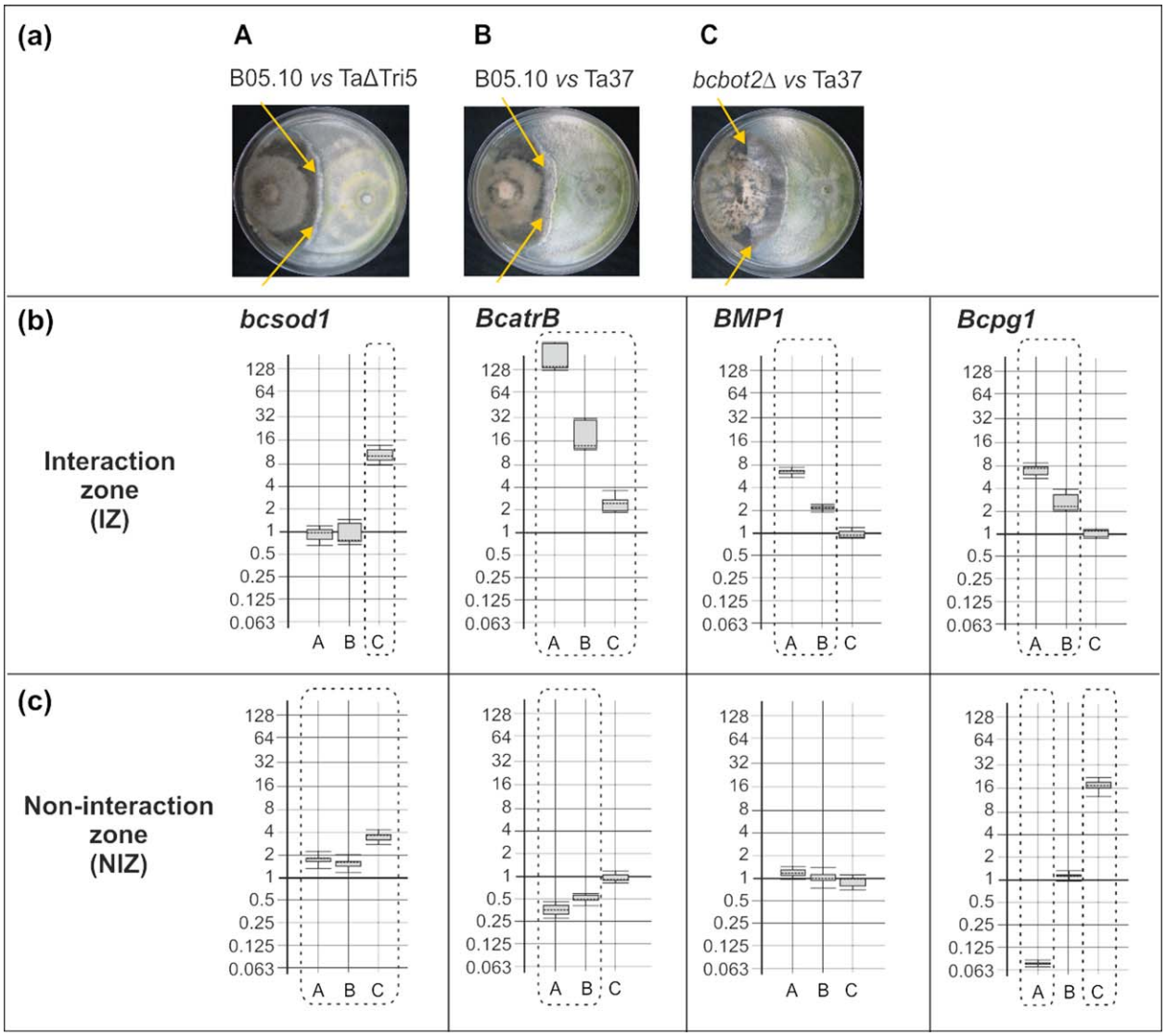

Fig. 5. (**a**) Photographs of **A**) B05.10-TaΔTri5, **B**) B05.10-Ta37, and **C**) *bcbot2*Δ-Ta37 dual cultures to observe the different ability of these *Trichoderma* strains to overgrow the *B. cinerea* mycelium.
Overgrow areas are indicated by yellow arrows. (**b, c**) Analysis of *bcsod1, BcatrB, BMP1*, and *Bcpg1* expression level in *B. cinerea* mycelia isolated from (**b**) IZ or (**c**) NIZ of the dual cultures *versus* expression level of these genes in mycelia of B05.10 growing alone. Comparative calculations and graphic representations were carried out as indicated in the legend of Fig. 2, and the numeric values are included in Supporting Table S1c.

expression in confrontation experiments with exogenously added AspB or AspC, led us to conclude that AspB has limited effect on the expression of *BcBOT* genes in B05.10 growing alone or in confrontation cultures with Ta37. However, AspC up-regulates the *BcBOT* genes in B05.10 single cultures but down-regulates these genes in confrontation cultures with Ta37, reinforcing the hypothesis that AspC and HA interact together to down-regulate these genes in the IZ of confrontation cultures.

In the presence of HA and *T. arundinaceum*, the *BcBOT* genes are down-regulated. The data from B05.10 *vs* TaΔTri5 + 36 μg/mL HA shows relative expression levels of the *BcBOT* genes (Fig. 3d) in the IZ that are similar to those seen in B5.10 *vs* Ta37 (Fig. 2b) confirming the hypothesis that HA in combination with AspC is responsible for the down-regulation of *BcBOT* genes.

During the mycoparasitic process, several *Trichoderma* mechanisms are activated (Seidl *et al.*, 2009; Suárez *et al.*, 2007), and a plethora of hydrolytic enzymes are produced, thus releasing oligomers from the pathogen and eventually from the *Trichoderma* cell walls that can act as elicitors for the production of SMs not only in *Trichoderma* (Druzhinina *et al.*, 2011) but also in the pathogen. Moreover, the data presented in this work are supported by a

previous study which showed the up-regulation of genes encoding hydrolytic enzymes in the interaction zone of B05.10-Ta37 dual cultures (Malmierca *et al.*, in press). It is noteworthy that in B05.10 cultures growing alone, the addition of HA, even at the highest concentration, induced all of the virulence genes assayed, indicating that this trichothecene itself is capable of stimulating *B. cinerea* virulence. However, in the confrontation experiments without HA, TaΔTri5 did not induce *bcsod1* but did induce all other B05.10 virulence genes, indicating that the confrontation itself induces *B. cinerea* virulence and that HA attenuates *BcatrB* expression. *bcsod1* encodes a superoxide dismutase, which detoxifies reactive oxygen species (ROS) (Rolke *et al.*, 2004), and was up-regulated when *B. cinerea* was grown alone in the presence of HA. This gene may be involved in *B. cinerea* pathogenicity by inducing generation of ROS in plants, but may also have a role in the ROS detoxification by *B. cinerea* induced by trichothecenes. The generation of reactive oxygen species and oxidative stress induced by *Fusarium* trichothecenes in interaction with plants and mammalian cells has been previously described (Kosawang *et al.*, 2014; Zhang *et al.*, 2009).

The data showed that HA, either endogenous or exogenous, up-regulated *BcatrB* (encoding an ABC transporter) expression (Figs. 2 and 3; Supporting Information Table S1a) and that AspC also up-regulates expression (Supporting Information Fig. S3). This indicates that these metabolites are being perceived as a hazard by B05.10. When HA is in the presence of AspC, there is a down-regulation of *BcatrB* (Fig. 4). When examining the data from the confrontation cultures with exogenously added Asps, it must be taken into account that the confrontation assays with these compounds were carried out against the strain Ta37, which produces HA. Thus, this would further support that Asps and HA have a combinational, perhaps even synergistic, effect on the *BcatrB* down-regulation. Moreover, the up-regulation of *BcatrB* observed in B05.10 grown alone with the addition of HA or AspB or AspC is in agreement with that described for an ABC transporter encoding gene of *T. atroviride* reacting to mycotoxins secreted by its fungal prey (Ruocco *et al.*, 2009).

It is interesting to note that *Bcpg1*, a gene encoding an endoPG involved in plant virulence, followed a similar pattern to that observed for *BcBOT* genes. *T. arundinaceum* and *B. cinerea* can compete for aboveground plant parts and for debris in soil, and an increase of polygalacturonase activity would facilitate the survival of *B. cinerea* on these substrates. The down-regulation of *Bcpg1* by HA or other SMs (e.g. Asps) is in agreement with previous reports showing a reduction in the level of *Botrytis* endoPG activity when a *Trichoderma* biocontrol agent was applied to bean leaves infected with the pathogen (Zimand *et al.*, 1996), and could indicate that these metabolites cannot only regulate biosynthesis of related SMs in the pathogen but, in addition, can control its pathogenicity by regulating the expression of genes encoding proteins directly involved in plant attack, as is the case of the endoPG1.

The role of the virulence genes analyzed in the interaction with other antagonistic fungi has not been described. Based on the results presented in this work, we propose that these genes, mainly *Bcpg1*, *BMP1* and *BcatrB* are also involved in the response of *Botrytis* against competitor fungi like *T. arundinaceum*, and their regulation is mediated by the ability of these fungi to produce sesquiterpene compounds (see Fig. 5). This agrees with earlier work which showed a metabolic dialogue between *T. arundinaceum* and *B. cinerea* that is mediated by sesquiterpenes (BOT) and polyketides (Botcinins) (Malmierca *et al.*, in press).

The remainder of the genes analyzed in this study can be separated into 2 groups: (i) *BchmgR* and *Bcdpp1*, which are active early in the biosynthetic pathway of terpenes and BOT, and (ii) *Bcerg9, Bcerg1*, and *Bcerg7* which are specific to ergosterol biosynthesis (Fig. 1). In fact, *BchmgR* can be considered as a "gateway" gene, since it is the first step in the common pathway, before splitting at FPP, for the two separate terpenoid pathways being investigated. If there are high gene expression ratios for *BchmgR*, one would expect high levels of farnesol, ergosterol, and BOT in the media. The data presented here confirm that high expression of *BchmgR* is only seen when high levels of BOT are present and HA is missing (in dual cultures of *B. cinerea* and TaΔTri5). The presence of HA in dual cultures down-regulates this high expression of *BchmgR*, which can be understood as a way to reduce the BOT production by reducing the flux of precursors, e.g. acetyl CoA, toward terpene biosynthesis. These conclusions are in agreement with previous reports showing that there is a channeling of precursors toward the ergosterol biosynthetic pathway in competition with sesquiterpenoid pathways (Asadollahi *et al.*, 2010; Malmierca *et al.*, 2015b, c; Cardoza *et al.*, 2015).

Fungi as well as other microorganisms use SMs to control diverse cellular processes and/or intra- and interspecies communication (Brakhage, 2013). In *Trichoderma*, in addition to HA and Asps, other SMs have been reported to contribute to its biocontrol potential, for example an enhanced level of 6-pentyl-2H-pyran-2-one (6PP) was found in response to the presence of a target fungus (Cooney and Lauren 1998). The deletion of a transcription factor gene in *T. harzianum* was linked to a loss of two SMs derived from 6PP, resulting in a reduced antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (Rubio *et al.*, 2009).

The present work shows, as summarized in Fig. 6, a cross-gene regulation between *B. cinerea* and *T. arundinaceum*, in which sesquiterpenes produced by both partners have a role and contribute to the understanding of the significance of BOT for *B. cinerea* physiology. In addition, the

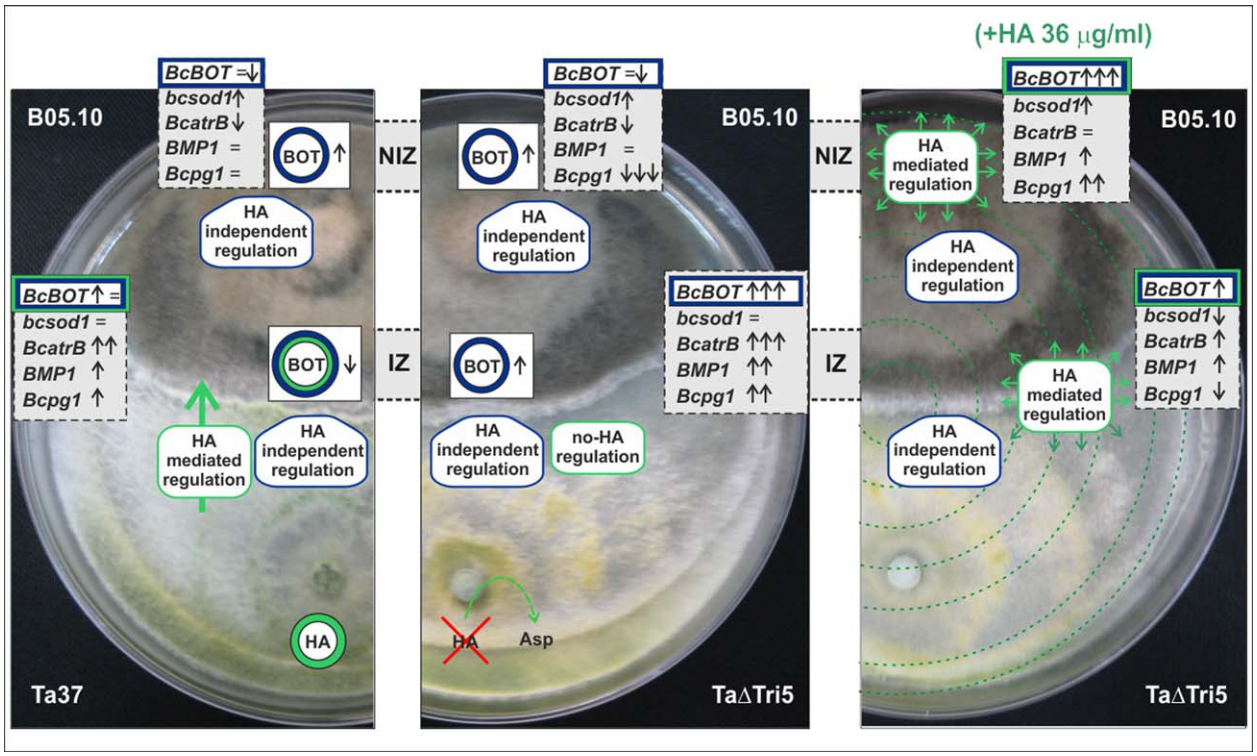

Fig. 6. Graphical summary of the key phenomena observed in the interaction- (IZ) and non-interaction (NIZ) zones of B05.10-Ta37 (left panel), B05.10-TaΔTri5 (central panel), and B05.10-TaΔTri5 (+HA 36 μg/mL) (right panel) dual cultures, compared with B05.10 grown alone. Black up/down arrows represent gene up- or down-regulation. Arrows, boxes or circles in green indicate responses that can be attributed to HA (=HA mediated regulation), and the boxes or circles in blue represent the HA-independent regulatory effects caused by signals generated by the interaction zone between both fungi (=HA-independent regulation). Boxes with both green and blue represent responses which are the result of a combination of both regulations (i.e. HA-independent and HA-dependent regulation) (e.g. the reduction in the level of BOT production in the IZ of B05.10 *vs* Ta37 dual cultures can be attributed to the effect of HA produced by Ta37 and also to the signals generated in the IZ). The dashed, green, concentric circles in the right panel indicate the uniform presence of the exogenously added HA to the medium (both in the IZ and NIZ).

regulation of BOT biosynthesis by HA and AspC, has implications for other biosynthetic pathways (e.g. polyketide) that should be further explored. The results of our study will lead to a better understanding of the counter-attacks of two fungi as a part of the molecular cross-talk of a biocontrol agent and its prey.

Experimental procedures

Strains and culture media

B. cinerea B05.10 (B05.10) is derived from a vineyard field isolate (Quidde *et al.*, 1999). *B. cinerea* B05.10 *bcbot2*Δ strain is a BOT mutant (now referred to as *bcbot2*Δ) in which *BcBOT2* is deleted (Pinedo *et al.*, 2008). This gene encodes presilphiperfolan-8β-ol synthase that catalyzes the multistep cyclization of FPP to presilphiperfolan-8β-ol, the precursor of BOT.

T. arundinaceum IBT 40837 (=Ta37) (IBT Culture Collection of Fungi at the Department of Biotechnology, Technical University of Denmark), was kindly provided by Ulf Thrane. TaΔTri5, is a transformant derived from Ta37 that is disrupted in the *tri5* gene (encoding trichodiene synthase, the enzyme responsible for the production of trichodiene from FPP), and blocked in the biosynthesis of HA (Malmierca *et al.*, 2013).

B. cinerea and *T. arundinaceum* strains were maintained on MEA (2% glucose, 2% malt extract, 1% peptone, 2% agar, pH 5.6) and PPG (2% mashed potatoes, 2% glucose, 2% agar) respectively.

Confrontation assays

Confrontation assays between *T. arundinaceum* and *B. cinerea* strains were carried out as described previously (Malmierca *et al.*, in press). Mycelia were collected for RNA extraction, in three biological replicates from 1-cm bands of the IZ and NIZ (see Fig. 2a) between both microorganisms. Single cultures, incubated in the same conditions as the dual cultures, were also performed for comparative purposes.

Chemical analysis

General procedures. Unless otherwise noted, materials and reagents were obtained from commercial suppliers and were used without further purification. Purification by semipreparative and analytical HPLC was performed with a Hitachi/Merck

L-6270 apparatus equipped with a differential refractometer detector (RI-7490). A LiChrospher® Si 60 (5μm) LiChroCart® (250 mm × 4 mm) column and a LiChrospher® Si 60 (10μm) LiChroCart® (250 mm × 10 mm) were used in isolation experiments. Silica gel (Merck) was used for column chromatography. TLC was performed on a Merck Kiesegel 60 F_{254}, 0.25 mm thick. Infrared spectra were recorded on a FT-IR spectrophotometer and reported as wave number (cm^{-1}). ^1H and ^{13}C NMR measurements were recorded on an Agilent 400 MHz and Agilent 500 MHz spectrometers with $SiMe_4$ as the internal reference at room temperature. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.25, δ_C 77.0). NMR assignments were made using a combination of 1D and 2D techniques. Mass spectroscopy (MS) was recorded with a GC-MS Thermoquest Voyager spectrometer (Thermo Finnigan) in positive ion mode. High-resolution mass spectroscopy (HRMS) were recorded with a QTOF mass spectrometer (Water Synapt G2) in positive ion electrospray mode at 20 V cone voltage and 10 eV collision energy.

Extraction and chemical analysis of BOT, HA, and Asps. Samples were collected from the different *T. arundinaceum-B. cinerea* cultures. Eight 1-cm-diameter MEA plugs were taken after cellophane sheet removal (which removed the mycelia) from the IZ and NIZ between both fungi or from single cultures of B05.10, and separately placed in 10-ml tubes. The solid MEA plugs, or from B05.10 single cultures, were extracted 3 fold with 50 ml ethyl acetate using an ultrasonic bath for 15 minutes. The solvent was filtered, dried over Na_2SO_4 and further concentrated to dryness. Evaporation of the solvent under reduced pressure afforded a dense oil that was separated by column chromatography on silica gel eluted with mixtures containing increasing percentages of ethyl acetate/hexane (10-100%) and methanol as solvent. Extensive spectroscopic analyses by ^1H-NMR and ^{13}C-NMR were used to detect the presence of BOT in each fraction. Candidate fractions were further purified by HPLC with a mixture of solvents, hexane:ethyl acetate (80:20), F = 3 mL/min. The toxin structures were confirmed by ^1H NMR and ^{13}C NMR spectroscopic methods and directly compared to authentic samples previously isolated from strains of *B. cinerea*. BOT quantities were determined by HPLC as described previously (Schumacher *et al*., 2012).

HA was purified from liquid PDB cultures from Ta37 strain as described by Cardoza *et al*., 2011. Extraction of Aspinolides from TaΔTri5 strain was carried out from solid MEA cultures as described by Malmierca *et al*., 2015a.

RNA extraction and cDNA synthesis. Mycelia were collected from the IZ and NIZ of the *B. cinerea-T. arundinaceum* dual cultures (see Fig. 2a) or from B05.10 grown in single cultures, and RNA was extracted and cDNA synthesized as described previously (Malmierca *et al*., in press).

qPCR experiments. In the present work, the expression of three sets of genes has been analyzed: 1) *B. cinerea* structural genes involved in the BOT biosynthesis (*BcBOT* genes); 2) genes encoding for *B. cinerea* virulence factors (*bcsod1, BcatrB, BMP1*, and *Bcpg1*), and 3) genes involved in the biosynthesis of ergosterol and/or farnesol (*BchmgR, Bcdpp1, Bcerg9, Bcerg1, Bcerg7*). In order to perform comparative studies, previously described oligonucleotides of the *B. cinerea BcBOT*, *BMP1, Bcpg1, BcatrB*, and *bcsod1* were used (Pinedo *et al*., 2008; Malmierca *et al*., 2015b). To design oligonucleotides for qPCR analysis of B05.10 genes involved in the ergosterol biosynthetic pathway (Supporting Information Table S2), the sequences of those genes were obtained from the B05.10 genomic data base, through the Broad Institute web page: http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/GenomeDescriptions.html#BC1. According to GeNorm software (Vandesompele *et al*., 2002) results, the *BcEF1b* gene was used for the *B. cinerea* endogenous control. The qPCR reactions were carried out using the system Step One Plus™ (Applied Biosystems, Foster City, CA) and following the manufacturer's instructions. The reactions were performed in a total volume of 20 μl by 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 160, 80, 40, 20, 10 and 5 ng cDNA to determine the PCR amplification efficiency (E value) (Supporting Information Table S3). Each measurement was made in triplicate using cDNA pooled from three biological replications.

Quantification of ergosterol and squalene. Squalene and ergosterol contents were calculated as reported previously (Cardoza *et al*., 2007; Ghimire *et al*., 2009) from mycelia of *Botrytis* grown alone and also from mycelia of this fungus collected from the IZ of the primary dual cultures analyzed in the present work.

Statistical analysis. Kruskal-Wallis tests were performed with IBM SSPS Statistics 19 Software (search: IBM + Spss + Statistics + 19).

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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. (a) Photographs illustrating the grown of B05.10 in the presence of 208 μg/mL HA (left) or without HA (right). **(b, c)** Analysis of gene expression levels of *Botrytis* genes in mycelia grown in the presence of 208 μg/mL HA *versus* gene expression levels in mycelia of B05.10 grown alone without HA. Three sets of genes were analyzed: 1) (**left panel**) BOT biosynthetic genes: (*BcBOT5, BcBOT3, BcBOT2, BcBOT1, BcBOT4*), 2) (**central panel**) other virulence genes *bcsod1*, *BcatrB*, *BMP1*, and *Bcpg1*, and 3) (**right panel**) genes belonging to the ergosterol biosynthetic pathway *BchmgR, Bcdpp1, Bcerg9, Bcerg1* and *Bcerg7*. The expression ratios as well as the statistical probability values were calculated using the REST 2009 software (Pfaffl *et al*., 2002). Those data statistically significant [$p(H1) < 0.05$] are indicated with an asterisk and outlined in a square in the graphic representation. Pfaffl, M.W., Horgan, W., and Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.

Fig. S2. (**a**) Dual culture of B05.10 *versus* TaΔTri5 in media supplemented with 208 μg/mL HA. (**b, c**) Analysis of gene expression level in *B. cinerea* mycelia isolated from the interaction region with TaΔTri5 in media supplemented with 208 μg/mL HA *versus* gene expression level in mycelia of B05.10 growing alone. Comparative calculations and graphic representations were carried out as indicated in the legend of Fig. S1.

Fig. S3. **(a)** Photographs illustrating the growth of B05.10 in the presence of 50 μg/mL AspB (left panel) and 50 μg/mL AspC. (**b, c**) Analysis of gene expression level of *Botrytis* genes in mycelia growth in the presence of AspB (left panels) or AspC (right panels) *versus* gene expression levels in mycelia of B05.10 grown alone without Asps. Comparative calculations and graphic representations were carried out as indicated in the legend of Fig. S1.

Table S1. Numeric values of the expression ratios observed for the genes analyzed in the present work (***#,##***).

Table S2. Oligonucleotides used for qPCR analysis of the *Botrytis cinerea* genes involved in the ergosterol biosynthesis

Table S3. Efficiency of the qPCR reactions analyzed in this work.