

# Trichoderma-induced plant immunity likely involves both hormonal- and camalexin-dependent mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungus *Botrytis cinerea*

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**Key words:** Arabidopsis, Trichoderma, phytostimulation, defense responses, jasmonic acid, salicylic acid, camalexin

**Abbreviations:** WT, wild type; dai, days after inoculation; JA, jasmonic acid; SA, salicylic acid; ET, ethylene; ROS, reactive oxygen species; SAR, systemic acquired resistance; ISR, induced systemic resistance; PR, pathogenesis-related proteins; PAL, phenylalanine ammonia-lyase; ICAlD, indole-3-carboxaldehyde; Cys, cysteine; GUS,  $\beta$ -glucuronidase; GC-MS, gas chromatography-mass spectrometry

Filamentous fungi belonging to the genus *Trichoderma* have long been recognized as agents for the biocontrol of plant diseases. In this work, we investigated the mechanisms involved in the defense responses of *Arabidopsis thaliana* seedlings elicited by co-culture with *Trichoderma virens* and *Trichoderma atroviride*. Interaction of plant roots with fungal mycelium induced growth and defense responses, indicating that both processes are not inherently antagonist. Expression studies of the pathogenesis-related reporter markers *pPr1a:uidA* and *pLox2:uidA* in response to *T. virens* or *T. atroviride* provided evidence that the defense signaling pathway activated by these fungi involves salicylic acid (SA) and/or jasmonic acid (JA) depending on the amount of conidia inoculated. Moreover, we found that *Arabidopsis* seedlings colonized by *Trichoderma* accumulated hydrogen peroxide and camalexin in leaves. When grown under axenic conditions, *T. virens* produced indole-3-carboxaldehyde (ICAlD) a tryptophan-derived compound with activity in plant development. In *Arabidopsis* seedlings whose roots are in contact with *T. virens* or *T. atroviride*, and challenged with *Botrytis cinerea* in leaves, disease severity was significantly reduced compared with axenically grown seedlings. Our results indicate that the defense responses elicited by *Trichoderma* in *Arabidopsis* are complex and involve the canonical defense hormones SA and JA as well as camalexin, which may be important factors in boosting plant immunity.

## Introduction

Plants are in continuous interaction with a plethora of microorganisms, including pathogens and symbionts. In the rhizosphere, extensive communication occurs between plants and their associated microbes by exchange of different classes of microbial and plant compounds. This molecular dialog will determine the final outcome of the relationship, usually through highly coordinated cellular processes.<sup>1</sup>

Filamentous fungi of the genus *Trichoderma* are common rhizosphere inhabitants. They have been widely studied due to their capacity to produce antibiotics, parasitize other fungi or compete with deleterious plant microorganisms.<sup>2</sup> It has been known for

many years that *Trichoderma* species enhance plant growth and productivity in axenic systems and in soil.<sup>3-5</sup> In *Arabidopsis thaliana*, growth promotion by *T. virens* correlated with increased root biomass production and enhanced lateral root formation, which was attributed to the production of auxin signals by this fungus.<sup>6</sup> *Trichoderma* also induces plant defense responses. Co-cultivation of cucumber plants with *T. harzianum* increased production of peroxidase and chitinase, while *T. asperellum* T34 conferred protection against *Pseudomonas syringae* pv. *Lachrymans* through regulation of proteins involved in adaptation to stress, isoprenoid and ethylene biosynthesis, photorespiration and carbohydrate metabolism.<sup>7,8</sup> Nevertheless, the signaling mechanisms by which *Trichoderma* species activate plant immunity remain uncertain.

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Plants possess various inducible defense mechanisms for protection against pathogen attack. An example of this is systemic acquired resistance (SAR), which is activated by a wide range of pathogens, especially (but not only) those that cause tissue necrosis.<sup>9</sup> Similarly, colonization of plant roots by certain non-pathogenic rhizobacteria can induce systemic resistance (ISR) in the host plant.<sup>10</sup> Both pathogen-induced SAR and rhizobacteria-mediated ISR are effective against different types of pathogens, and are typically characterized by a restriction of pathogen growth and a suppression of disease development compared with primary infected, non-induced plants. However, the signaling pathways controlling pathogen-induced SAR and rhizobacteria-mediated ISR differ. Whereas SAR requires endogenous accumulation of salicylic acid (SA), the signaling pathway controlling ISR functions independently of SA and requires intact responsiveness to the plant hormones jasmonic acid (JA) and ethylene.<sup>10–12</sup> Additionally, it has been established that accumulation of phytoalexins and other low molecular weight antimicrobial metabolites is integral to plant protection. The chemical structures of phytoalexins vary among different plant families and include flavonoids, terpenoids and indoles.<sup>13</sup>

The antimicrobial properties of phytoalexins, which have been extensively investigated, suggest their potential function in the host defense machinery. However, only recently they have been found to make an important contribution to plant defense against particular pathogens.<sup>14</sup> Mutations that cause reduced phytoalexin levels can lead to increased susceptibility of plants to pathogens such as *Botrytis cinerea*.<sup>15–17</sup> The main phytoalexin detected in Arabidopsis is an indole derivative called camalexin (3-thiazol-2'-yl-indole).<sup>17–20</sup> Camalexin accumulates in tissue exposed to infection by either avirulent or virulent strains of the bacterium *Pseudomonas syringae* and after inoculation with the fungus *Cochliobolus carbonum* and is able to inhibit bacterial and fungal growth.<sup>19,21,22</sup>

The indole ring of camalexin is produced from Trp through at least three CYP (cytochrome P450) steps. Trp is converted to indole-3-acetonitrile (IAN) by CYP79B2/CYP79B3 and CYP71A13. Conversion of Cys(IAN) to dihydrocamalexin acid and subsequently to camalexin is catalyzed by CYP71B15.<sup>23,24</sup> Based on the *pad3* mutant phenotype CYP71B15 has been involved in camalexin biosynthesis by converting cysteine-indole-3-acetonitrile to camalexin.<sup>24–26</sup> Alternatively, camalexin biosynthesis may proceed through condensation of indole-3-carboxaldehyde (ICAlD) with cysteine (Cys) followed by cyclization and decarboxylation.<sup>27</sup>

*B. cinerea* is the causal agent of gray mold and causes rot symptoms in more than 200 different plant species, including Arabidopsis. An intact ethylene/JA signaling pathway is thought to be necessary for resistance to necrotrophic pathogens, such as *B. cinerea* and *E. carotovora*, whereas the SA signaling pathway is believed to mediate resistance to biotrophic pathogens. However, Arabidopsis defense responses against *B. cinerea* may also involve camalexin induction.<sup>16</sup> While SA or JA induce many defense responses, these signaling compounds are apparently not essential for camalexin production.<sup>17</sup> It is possible that several input signals are integrated to trigger camalexin biosynthesis. Based on this

information, it is tempting to speculate that biotic interactions capable of regulating multiple defense responses may increase the fitness of plants when challenged with pathogens.

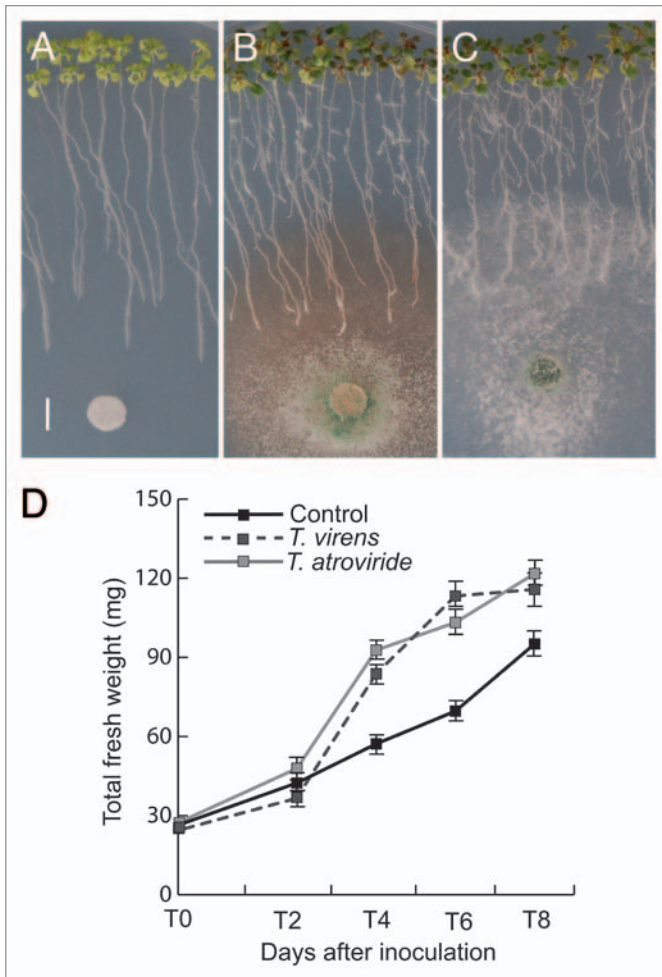
In this report, we used an in vitro Trichoderma-Arabidopsis interaction system,<sup>6</sup> to explore some of the signaling networks involved in defense responses triggered by *T. virens* and *T. atroviride* in plants. Our data show that co-cultivation of plant roots with these fungi induces hydrogen peroxide, SA and JA accumulation, which correlates with induction of pathogenesis-related reporter markers *pPr1a:uidA* and *pLox2:uidA*. We also found that both *T. virens* and *T. atroviride* increased camalexin accumulation in plants and conferred resistance to *B. cinerea*. Taken together, our results show the simultaneous involvement of hormonal signaling and camalexin biosynthesis in Trichoderma-induced plant immunity.

## Results

### Co-cultivation of Arabidopsis roots with Trichoderma stimulates lateral root development and plant biomass production.

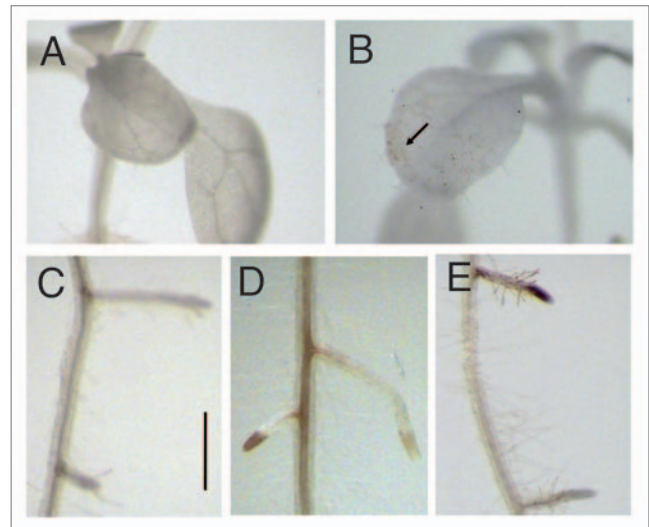
To evaluate the growth response of plants during physical contact with Trichoderma, we performed bioassays in which WT *Arabidopsis thaliana* ecotype Columbia (Col-0) seedlings were germinated and grown for 4 d on Petri plates, then, a drop of distilled water (control treatment) or a spore suspension from *T. virens* or *T. atroviride* was deposited at the opposite side of the plates. Six days after inoculation (dai), physical contact between the mycelium and the primary root tips could be observed, and two days later the fungi covered roughly 30% of the root system (Fig. 1A–C). In order to detect any potential deleterious effect of Trichoderma, total plant biomass in control or inoculated seedlings was determined every two days. During the first two days, no significant differences were observed in biomass production between control plants and plants co-cultivated with *T. virens* or *T. atroviride* (Fig. 1D). However, from 4 to 8 dai, a 40% increase in total fresh weight was evident in Trichoderma co-cultivated plants. Enhanced lateral root proliferation was a typical response of Arabidopsis roots colonized by the mycelia of *T. virens* or *T. atroviride*, and no deleterious symptoms such as chlorosis or necrosis could be observed in leaves (Fig. 1A–C). Interestingly, co-cultivation with Trichoderma increased anthocyanin production in leaves (Figs. 1A–C and S1), which might occur as a consequence/parallel effect of defense induction.

**Trichoderma induces hydrogen peroxide accumulation in Arabidopsis.** The production of reactive oxygen species (i.e., hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>) is an early response in plant-pathogen or elicitor recognition.<sup>31,49</sup> In Arabidopsis seedlings at 6 d of co-cultivation H<sub>2</sub>O<sub>2</sub> production was detected by DAB staining. The appearance of a brownish-red precipitate in plant tissues indicates hydrogen peroxide accumulation via polymerization with 3,3'-diaminobenzidine (Fig. 2A–E). *T. atroviride* induced accumulation of H<sub>2</sub>O<sub>2</sub> in leaves (Fig. 2B), in the primary root and mature lateral roots (Fig. 2D) and in meristems of young lateral roots (Fig. 2E) compared with the respective controls (Fig. 2A and C). H<sub>2</sub>O<sub>2</sub> induction was much weaker in leaves than in roots (Fig. 2A–E).



**Figure 1.** Effect of Trichoderma on Arabidopsis growth. Photographs of 10-d-old Arabidopsis (Col-0) seedlings grown on the surface of agar plates containing 0.2x MS medium. (A) Seedlings were treated with sterilized distilled water at day 4 and photographed 6 d later. Bar = 5 mm. (B) Representative photograph of Arabidopsis seedlings that were inoculated with *T. virens* at a distance of 5 cm from the root tip 4 d after germination and grown for a further 6 d period. (C) Photograph of Arabidopsis seedlings inoculated with *T. atroviride* at a distance of 5 cm from the root tip at 4 d after germination and grown for a further 6-d period. (D) Effects of Trichoderma on Arabidopsis biomass production. Mean  $\pm$  SD values were plotted at the indicated days in the kinetic experiment (n = 30). The experiment was repeated twice with similar results.

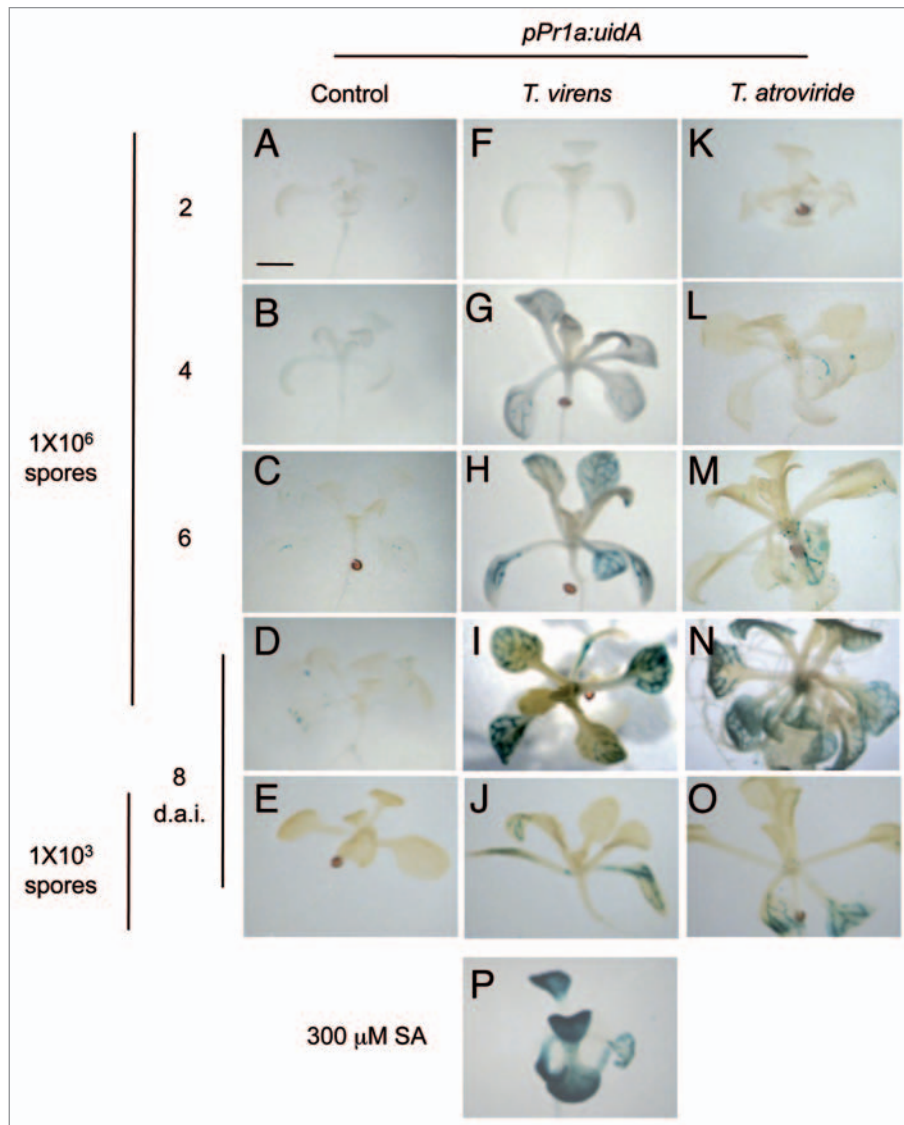
**Trichoderma induces hormone-dependent defense responses in Arabidopsis.** Infection of plants with pathogens or colonization of roots with certain beneficial microbes causes the induction of defense responses, which may depend on plant production of the alarm signals SA, JA and/or ET.<sup>12</sup> However, the defense signaling pathways that are activated in Arabidopsis upon exposure to Trichoderma remain unknown. To examine whether root colonization by Trichoderma involved altered SA or JA responses, we monitored expression of selected marker genes that are up-regulated by these hormones. We used transgenic Arabidopsis lines expressing  $\beta$ -glucuronidase (*uidA*, GUS) fusions to the *Pr1a* promoter, which is activated by SA,<sup>28</sup> and the *Lox2* promoter, activated by JA.<sup>29</sup> Arabidopsis transgenic seedlings expressing each



**Figure 2.** Effect of Trichoderma on H<sub>2</sub>O<sub>2</sub> accumulation in Arabidopsis. Representative photographs of leaves (A) and roots (C) from axenically-grown seedlings or from seedlings co-cultivated with *T. atroviride* for 6 d (B, D and E). Arabidopsis seedlings were treated with a solution of 3,3'-diaminobenzidine (DAB). In the presence of H<sub>2</sub>O<sub>2</sub>, DAB polymerizes, forming a dark red-brown coloration in plant tissues. Microscopy was performed using a Leica MZ6 Stereomicroscope. Bar = 1 mm. Arrow shows the spots of accumulated H<sub>2</sub>O<sub>2</sub>. Photographs show representative individuals of at least 15 seedlings stained with DAB. The experiment was repeated twice with similar results.

of these markers were co-cultivated with either *T. virens* or *T. atroviride* and the roots allowed to be colonized by the fungi. Axenically-grown seedlings did not show *pPr1a:uidA* expression in shoots at different time points in a time-course experiment (Fig. 3A–E). Interestingly, during pre-contact or physical contact between the roots and mycelium *pPr1a:uidA* expression was activated in shoots of plants co-cultivated with both *T. virens* and *T. atroviride* (Fig. 3F–O). The time required for the activation of the *Pr1* promoter depended on the fungal species and amount of conidia inoculated. *pPr1a:uidA* expression in plants co-cultivated with 10<sup>6</sup> conidia of *T. virens* significantly increased at 4-dai and continued to increase gradually to a maximum at 8 d. Changes in GUS activity by *T. atroviride* were evident at 6-dai and reached a maximum at 8 dai. In both cases, this response was apparently delayed when less conidia (10<sup>3</sup>) were inoculated and was detectable only at 8 dai in cotyledons and older leaves (Fig. 3J and O). SA clearly induced changes in *pPr1a:uidA* expression (Fig. 3P). In contrast, the JA-activated marker *pLox2:uidA* was better induced by low density of Trichoderma at 8-dai (Fig. 4A–P). These data suggest that SA and JA are likely involved in Arabidopsis responses to Trichoderma inoculation.

**Interaction between Arabidopsis roots and Trichoderma induces SA and JA accumulation in leaves.** To determine whether the changes in the expression of *pPr1a:uidA* and *pLox2:uidA* markers were associated with changes in endogenous SA and/or JA content in plants co-cultivated with Trichoderma, free SA and JA were quantified in WT Arabidopsis (Col-0) plants at 8 dai. A 3- to 4-fold increase in the accumulation of SA was observed in plants inoculated with Trichoderma (10<sup>6</sup> spores)



**Figure 3.** Effect of *T. virens* and *T. atroviride* on expression of the pathogen-related response gene marker *pPr1a:uidA*. In a time-course plant-fungus co-cultivation experiment GUS expression in seedlings was determined every two days after inoculation. SA was used as a positive control and dimethyl sulfoxide served as negative control. Bar = 1 mm. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results.

when compared with axenically-grown seedlings, with slightly higher levels upon treatment with *T. atroviride* (Fig. 5A). The levels of JA dramatically increased in plants co-cultivated with *T. virens* and to a lesser extent with *T. atroviride* (Fig. 5B). These data indicate that *T. virens* and *T. atroviride* can induce both SA and JA accumulation.

**Trichoderma induces camalexin accumulation in Arabidopsis and produces indole-3-carboxaldehyde.** The main phytoalexin that accumulates in Arabidopsis after infection by fungi or bacteria is 3-thiazol-2'-yl-indole (camalexin). To investigate whether Trichoderma inoculation could increase camalexin production, we determined camalexin levels in axenically-grown seedlings, seedlings treated with AgNO<sub>3</sub>, a well-known inducer of camalexin, or in seedlings at 8-dai with *T. virens* or *T. atroviride*. Gas chromatography-mass spectrometry

(GC-MS) analysis revealed that seedlings co-cultivated with *T. atroviride* as well as plants treated with AgNO<sub>3</sub> dramatically increased (15-fold) camalexin accumulation when compared with control seedlings. Co-cultivation with *T. virens* also resulted in strongly increased camalexin levels (Fig. 6). Previously, we determined the production of several indolic compounds by Trichoderma, including indole-3-acetic acid, indole-3-acetaldehyde and indole-3-ethanol.<sup>6</sup> Further analysis of the metabolites produced by Trichoderma by GC-MS revealed the presence of indole-3-carboxaldehyde (ICALd, retention time 10.79 min, *m/z* 144) (Fig. 7A–C), a compound previously reported by Zook and Hammerschmidt (1997), as a camalexin reactant.<sup>27</sup> When *A. thaliana* was grown in the presence of ICALd, the production of camalexin increased significantly (Fig. 7D), indicating that this compound is likely involved in camalexin biosynthesis. Supply of

L-Trp to *T. virens* culture medium increased ICAlD accumulation in culture supernatants (Fig. S2).

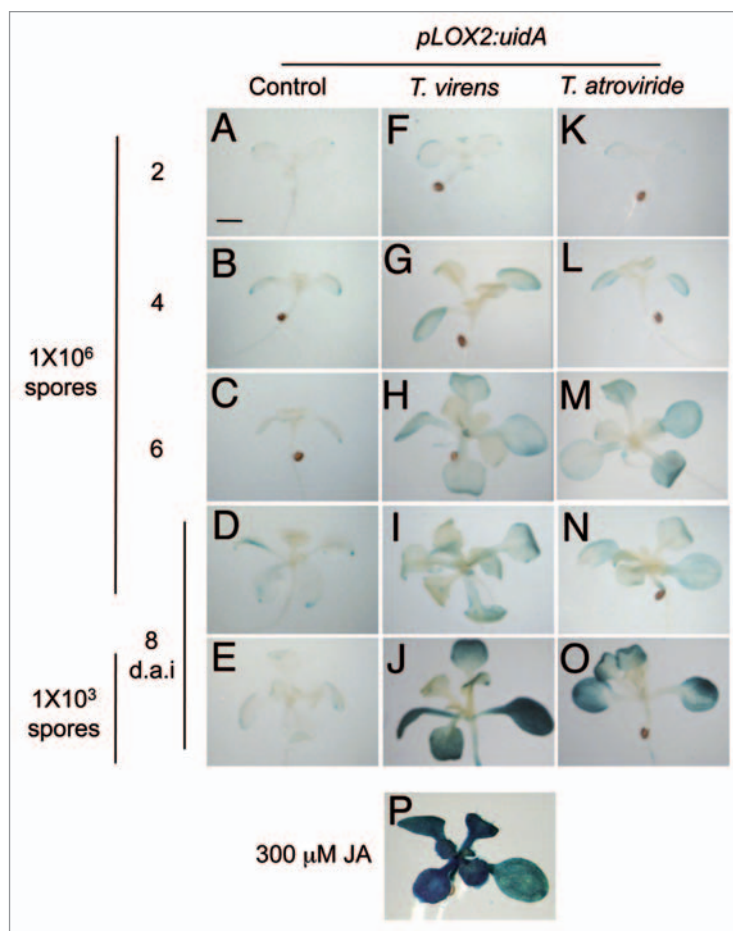
*Trichoderma confers resistance to Botrytis cinerea*. Camalexin is essential for resistance to *B. cinerea* in Arabidopsis.<sup>16,17</sup> The results from SA and JA accumulation, *pPr1a:uidA* and *pLox2:uidA* expression, and camalexin accumulation suggest that Trichoderma may act as potential defense-inducing fungus. To study whether Trichoderma could effectively activate defense mechanisms that lead to pathogen resistance, we tested the responses of 12-d-old Arabidopsis plants grown axenically or co-cultivated with *T. virens* or *T. atroviride* and further inoculated with the necrotrophic pathogen *Botrytis cinerea*, which causes spreading necrotic lesions on leaves. In these experiments, *B. cinerea* conidia were inoculated over the leaf surfaces and disease symptoms evaluated 3 d after inoculation with the pathogen. In control plants *B. cinerea* was found to induce necrotic lesions in over 82% of inoculated leaves (Fig. 8A). In contrast, in plants colonized by *T. virens* or *T. atroviride*, only 22% and 25% of leaves, respectively, presented necrotic lesions caused by *B. cinerea* infection (Fig. 8A). Five days after pathogen inoculation, it was found that *B. cinerea* caused death in about 70% of control plants; whereas only 10% of Trichoderma colonized plants were dead at this stage (Fig. 8B).

## Discussion

Fungi are an enormous resource of structurally diverse metabolites, which may affect interactions with plants and other organisms. Deciphering the signaling processes that mediate these interactions represents a continuous challenge. Although not directly economically important, the model plant *Arabidopsis thaliana* offers a number of experimental advantages over crop species, including its small size, short life cycle, and the suitability to be grown under axenic conditions. The adoption of an Arabidopsis-Trichoderma model has increased our knowledge about the molecular and physiological mechanisms by which Trichoderma modulate plant growth and development.<sup>6</sup>

Here we show that colonization of Arabidopsis roots by *T. virens* or *T. atroviride* sustained increased plant biomass production at 8 dai (Fig. 1). Root colonization by these fungi was associated to induction of lateral root development and anthocyanin accumulation in leaves (Figs. 1 and S1). Anthocyanin accumulation was highly dependent on the amount of conidia of Trichoderma applied. Importantly, we did not observe chlorosis or necrosis symptoms in leaves of seedlings co-cultivated with either *T. virens* or *T. atroviride* indicating that high fungal colonization in roots is not detrimental to plant growth and development.

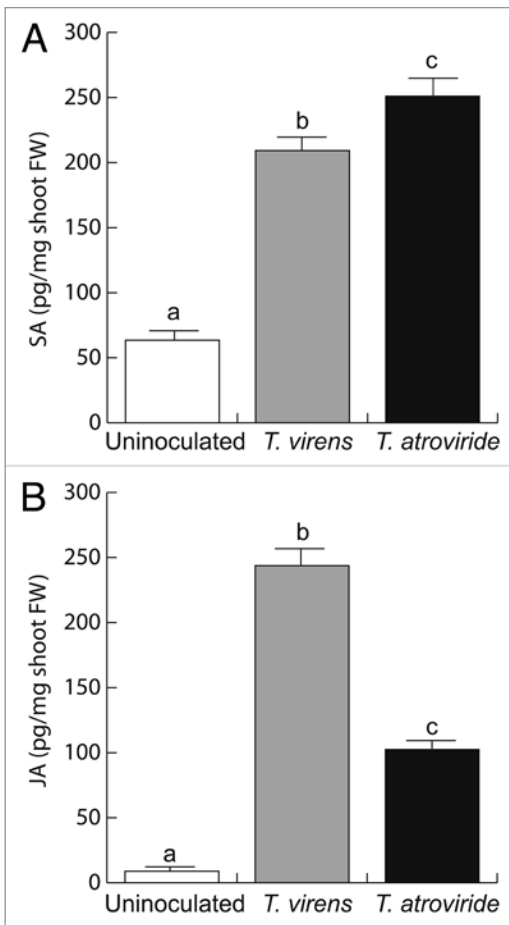
We found that Arabidopsis roots-colonized by Trichoderma accumulated H<sub>2</sub>O<sub>2</sub> (Fig. 2), indicating that Trichoderma triggers plant defense responses through reactive oxygen species (ROS). Interactions between plants and microbes involve recognition and signaling events that are distinct for different



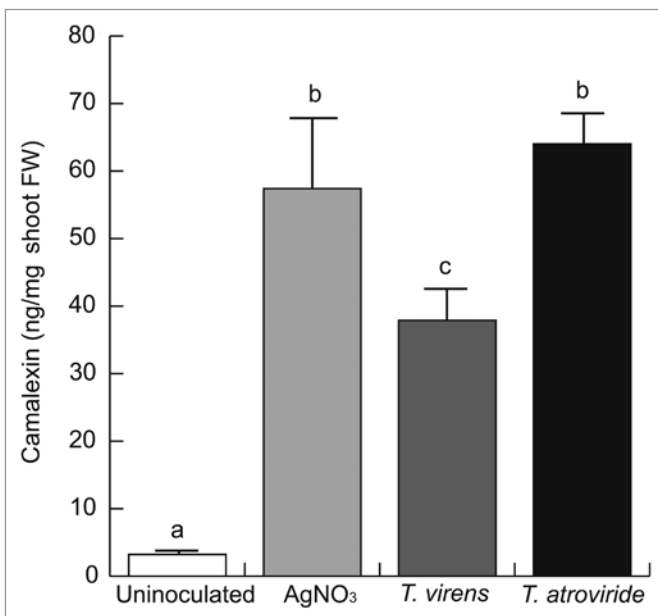
**Figure 4.** Effects of *T. virens* and *T. atroviride* on expression of the JA responsive gene marker *pLox2:uidA*. GUS expression in seedlings was determined every 2 d after inoculation in a time-course plant-fungus co-cultivation experiment. JA was used as a positive control and dimethyl sulfoxide served as negative control. Bar = 1 mm. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results.

microbial elicitors. These microbial elicitors may belong to pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs).<sup>30</sup> At the cellular level, the result of pathogen recognition often becomes apparent as a necrosis localized at the site of attack, called the hypersensitive response, which is accompanied by cellular changes such as an oxidative burst leading to the release of ROS, a rise in salicylic acid levels, and the induction of defense genes such as those coding for pathogenesis-related (PR) proteins.<sup>31-33</sup> The contribution of H<sub>2</sub>O<sub>2</sub> in plant protection by Trichoderma and its relationship with SA and JA pathways remains to be investigated.

Certain Trichoderma species have been previously found to elicit physical or chemical changes related to plant defense by activating ISR.<sup>34,35</sup> ISR elicited by Trichoderma may suppress plant diseases caused by a range of phytopathogens in both greenhouse and field conditions. In this study, we used transgenic Arabidopsis lines expressing  $\beta$ -glucuronidase (GUS) fusions to elucidate the signal transduction pathway by which *T. virens* and *T. atroviride* elicit plant defense responses. In our experiments, pre-contact or direct physical contact between the Arabidopsis



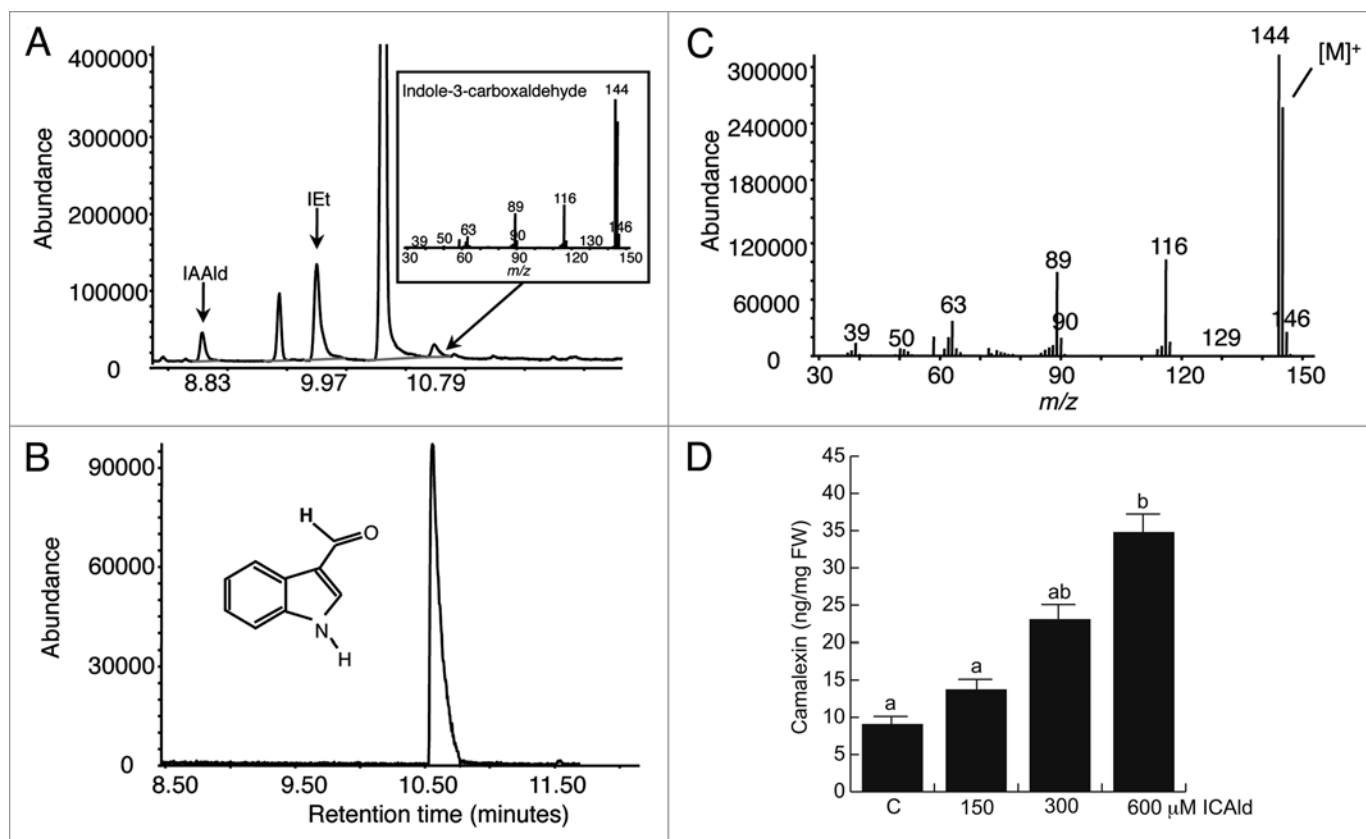
**Figure 5.** Effect of Trichoderma co-cultivation on SA and JA accumulation in Arabidopsis leaves. (A) Free SA or (B) JA at 8 d of co-cultivation with either *T. virens* or *T. atroviride*. Error bars represent the SE. Different letters are used to indicate means that differ significantly ( $p < 0.05$ ). The experiment was repeated three times with similar results.



root system and Trichoderma stimulated *pPr1a:uidA* expression and SA accumulation in plants (Figs. 3 and 5A). The concerted action of PR proteins, some of which display antimicrobial activity may act restricting pathogen growth. Low fungal concentration was also found to induce *pLox2:uidA* and JA accumulation (Figs. 4 and 5B). Both SA and JA changes have been previously described in cucumber plants inoculated with *Trichoderma asperellum* strain T34,<sup>36</sup> indicating that hormonal defense protection of plants is widespread among Trichoderma species. These results are in agreement with previous reports that *T. virens* induces the expression of PAL in maize through a JA-dependent response.<sup>34,35</sup> Moreover, our data suggest that SA and JA are involved in the signal transduction events mediating Trichoderma induced defense responses and are even consistent with the SA/JA antagonism. Similar defense activation patterns have been previously identified in Arabidopsis seedlings inoculated with plant growth promoting rhizobacteria.<sup>37-39</sup>

Antimicrobial compounds can be produced during normal plant growth and are usually stored in specialized organs or tissues such as trichomes, oil glands or epidermal cell layers.<sup>40</sup> Arabidopsis is a member of the Brassicaceae family, which is known for the production of various indolic constituents, including several indolic glucosinolates and closely related indolyl thiazoles, including camalexin and 6-methoxycamalexin.<sup>40-42</sup> Camalexin production can be elicited by bacterial and fungal phytopathogens and possess antimicrobial activity. In this work, we showed that Arabidopsis seedlings colonized with *T. virens* or *T. atroviride* accumulate greater levels of camalexin than axenically-grown plants (Fig. 6). Interestingly, we found that *T. virens* also produces ICALd, a compound related to indole-3-acetic acid metabolism likely involved in camalexin biosynthesis.<sup>43</sup> ICALd concentration in *T. virens* liquid extracts increased when Trp was supplied in the culture medium (Fig. S2), suggesting that Trp might be a precursor for ICALd. A number of aldehydes possess the ability to react with Cys to form the corresponding thiazolidinecarboxylic acid.<sup>27</sup> It has been reported that the synthesis of camalexin may proceed through the condensation of indole-3-carboxaldehyde with cysteine followed by a two-step oxidation and decarboxylation.<sup>44</sup> We found that application of ICALd increased camalexin accumulation in Arabidopsis seedlings (Fig. 7). These results indicate that Trichoderma species can induce both SA- and JA-mediated defense responses and camalexin accumulation. Hormone-dependent signaling and camalexin accumulation are considered independent or complementary mechanisms involved in plant immunity. The results that *T. virens* produces ICALd suggest that plants might use this compound for camalexin production by either direct or indirect means. The activity of ICALd on plant signaling was evidenced

**Figure 6.** Effect of Trichoderma co-cultivation on camalexin accumulation in Arabidopsis. Camalexin levels were determined in leaves of axenically-grown WT Arabidopsis seedlings, in seedlings treated with 5 mM AgNO<sub>3</sub> or co-cultivated 8 d with *T. virens* or *T. atroviride*. Bars show the mean  $\pm$  SD of five independent biological replicates. Each replicate included 20 seedlings. Different letters are used to indicate means that treatments differ significantly ( $p < 0.05$ ). The experiment was repeated twice with similar results.



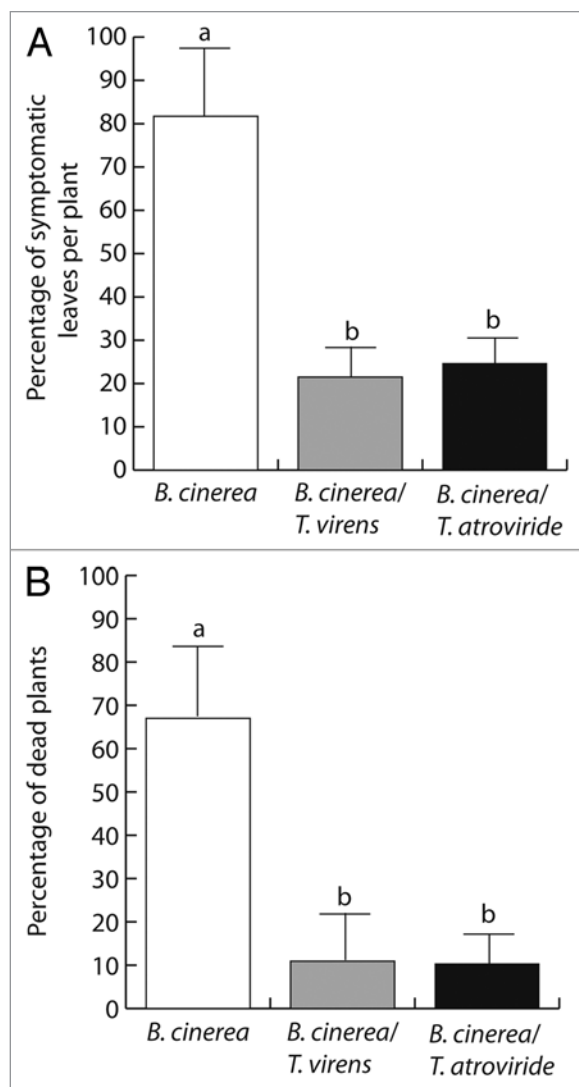
**Figure 7.** Identification of indole-3-carboxaldehyde from underivatized samples from *T. vires* growth medium by GC-MS. (A) chromatogram from neutral ethyl acetate extract obtained from 1 l of culture medium of *T. vires*, arrows indicate the presence of different indolic compounds, indole-3-acetaldehyde (IAAld), indole-3-ethanol (IEt) and indole-3-carboxaldehyde (ICAld). (B) ICAld standard. (C) the 70-eV electron-impact full-scan mass spectra from  $m/z$  50 to 500 of ICAld standard. (D) Indole-3-carboxaldehyde increases camalexin biosynthesis. Induction of camalexin in shoots of *Arabidopsis* after treatment with 300  $\mu$ M ICAld for 72 h. Bars shown in (D) represent the mean  $\pm$  SD of three independent replicates. Each replicate included 20 seedlings. Different letters are used to indicate means that differ significantly ( $p < 0.05$ ). The experiment was repeated twice with similar results.

monitoring primary root growth and adventitious root formation, which were clearly altered by ICAld supply to the medium (Fig. S3). The combination of such complex defense and/or plant growth regulating mechanisms may account for better performance of plants inoculated with *Trichoderma*.

In accordance with its involvement in activating defense-signaling pathways, *T. vires* and *T. atroviride* were found to be effective against the fungal necrotizing pathogen *B. cinerea*, a gray mold that causes rot symptoms in more than 200 different plant species. Our results of disease susceptibility showed that colonization of *Arabidopsis* roots by *T. vires* or *T. atroviride* reduced disease symptoms and plant death caused by this pathogen in leaves (Fig. 8). The correlation found between defense gene expression,  $H_2O_2$  induction, SA and JA accumulation, camalexin production and reduced disease symptoms in *Arabidopsis* colonized by *Trichoderma*, suggests that the combined activation of these defense pathways might be important to confer plant immunity against a fungal necrotizing pathogen. The characterization of two ISR elicitors secreted by *T. vires* was recently described. Peptides with antimicrobial activity

termed peptaibols, which are produced by *T. vires* were shown to have ISR elicitor effects, and they systemically induced defense in cucumber leaves.<sup>45</sup> The second ISR elicitor of *T. vires* is an extracellular small protein (Sm1), which is overproduced in the presence of cotton plants.<sup>34,46</sup> In maize, the metabolic pathways that lead to the establishment of Sm1-mediated ISR involves the signaling networks associated with salicylic acid, green leaf volatiles and jasmonic acid metabolism.<sup>34,35,46</sup> Our results are in agreement with these findings by showing that *Trichoderma* species can confer protection in *Arabidopsis* seedlings against *B. cinerea*, possibly by producing diffusible signals prior or during direct contact with roots.

We conclude that *Trichoderma* species are capable of regulating multiple defense responses. In addition to the defense gene induction and hormone biosynthesis, we now provide chemical evidence supporting a role for *Trichoderma* in phytoalexin induction, an important defense response less characterized. The combined effect of these responses may ultimately determine the role played by particular *Trichoderma* isolates in conferring disease resistance to crops.



**Figure 8.** Trichoderma confers protection against *Botrytis cinerea* in Arabidopsis seedlings. Twelve-day-old axenically-grown *A. thaliana* seedlings or seedlings co-cultivated 3-d with *T. virens* or *T. atroviride* in vitro were placed in Petri dishes containing 0.2x MS medium and mock (water) treated or inoculated with 5  $\mu$ l of  $1 \times 10^6$  *Botrytis cinerea* spores per ml solution/per leaf, depositing the inoculum on leaf surfaces. The number of symptomatic leaves per plant (A) and dead plants (B) are shown. Bars show the mean  $\pm$  SD of 30 Arabidopsis seedlings. Different letters are used to indicate means that treatments differ significantly ( $p < 0.05$ ). The experiment was repeated three times with similar results.

## Materials and Methods

**Plant material and growth conditions.** All mutant and transgenic lines were derived from parental Arabidopsis ecotype Columbia-0 (Col-0). Arabidopsis transgenic lines used in this work expressed a JA-inducible *lipoxygenase2* (At3g45140) or the *pathogenesis-related1* (At2g14610) gene promoters fused to the *uidA* reporter gene, which are referred as *pLox2:uidA*<sup>29</sup> and *pPr1a:uidA*,<sup>28</sup> respectively. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) household bleach (6% NaOCl) for 7 min. After five washes in distilled water, seeds were germinated and grown on

agar plates containing 0.2x MS medium (Murashige and Skoog basal salts mixture, Cat M5524; Sigma, St. Louis). Plates were placed vertically at a 65 degrees angle to allow root growth along the agar surface and unimpeded aerial growth of the hypocotyls. Plants were grown at 24°C in a growth chamber with a 16 h light (200  $\mu$ molm<sup>-2</sup>s<sup>-1</sup>)/8 h darkness photoperiod.

**Fungal growth and plant co-cultivation experiments.** The following fungal strains were used in this work: *Trichoderma virens* Gv.29-8 and *Trichoderma atroviride* (Formerly *Trichoderma harzianum*) IMI 206040. *T. virens* and *T. atroviride* were evaluated in vitro for their ability to elicit defense responses in Arabidopsis. Fungal spore densities of  $\sim 1 \times 10^6$  or  $1 \times 10^3$  spores were inoculated by placing the spores at 5 cm in the opposite ends of agar plates containing 4-d-old germinated Arabidopsis seedlings (10 seedlings per plate). Plates were arranged in a completely randomized design. The seedlings were cultured for different time periods in a Percival AR95L growth chamber. The percentages of primary roots colonized by Trichoderma were determined with a ruler by measuring the primary root length and the surface covered by fungal hyphae.

**Histochemical analysis.** For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated 12 to 14 h at 37°C in a GUS reaction buffer (0.5 mg/ml of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997).<sup>47</sup> For each marker line and for each treatment, at least 15 transgenic plants were analyzed. A representative plant was chosen and photographed, using a Leica MZ6 stereomicroscope.

**Determination of H<sub>2</sub>O<sub>2</sub> production.** The production of H<sub>2</sub>O<sub>2</sub> in Arabidopsis seedlings co-cultivated with Trichoderma was determined at 6 dai. The seedlings were included in 1 mg/ml solution of 3,3'-diaminobenzidine (DAB; Sigma), incubated 2 h, fixed and cleared in alcoholic solution. In presence of H<sub>2</sub>O<sub>2</sub>, DAB polymerizes, forming a dark red-brown precipitate staining. After these procedures the seedlings were examined for the production of H<sub>2</sub>O<sub>2</sub> by microscopy. For each treatment at least 15 plants were analyzed. A representative plant was chosen and photographed, using a Leica MZ6 stereomicroscope.

**Anthocyanin determination.** Anthocyanin content was determined in WT Arabidopsis (Col-0) seedlings 6 d after Trichoderma inoculation. 100 mg leaf samples were placed in an Eppendorf tube containing 1 ml of 0.1% HCl in methanol for 48 h at 4°C. After this period, the methanol extracts were analyzed in a spectrophotometer at 530 nm. The amount of anthocyanin in the extracts was reported as described by Pirie and Mullins (1976).<sup>48</sup>

**SA and JA extraction and measurements.** SA and JA extraction and determinations were performed in *Arabidopsis thaliana* (ecotype Col-0) shoots at 8 d after Trichoderma inoculation. For sample preparation, plants were sectioned at the root/shoot interface. Plant tissues were frozen and ground in liquid N<sub>2</sub>. Three hundred milligrams ground tissue was placed in an eppendorf tube, homogenized with 500  $\mu$ l isopropanol/H<sub>2</sub>O/concentrated HCl (2:1:0.002, v/v), and 200 ng orto-anisic acid (OA; Sigma) added to serve as internal standard for SA and shaken for



30 sec. Samples were centrifuged at 11,500 rpm for 3 min, supernatants were collected and subjected to SA extraction with 200  $\mu$ l of dichloromethane. SA and JA were derivatized with acetyl chloride in methanol (1 ml/250  $\mu$ l), sonicated for 15 min and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and resuspended in 25  $\mu$ l of ethyl acetate for GC-MS analysis. Gas chromatography-selected ion monitoring mass spectrometry (GC-SIM-MS) and retention time were established for SA-ME (2.3 min,  $m/z$  152), OA-ME (3.2 min,  $m/z$  166) and (7.5 min,  $m/z$  224) respectively. JA was quantified by comparison with a standard curve obtained by using purified Me-JA (Sigma).

**Camalexin determination.** Camalexin was extracted from leaves of WT Arabidopsis seedlings 8 d after *T. virens* or *T. atroviride* inoculation. As a positive control for camalexin induction, 12-d-old plants were treated with 5 mM AgNO<sub>3</sub> for 12 h, or 300  $\mu$ M indole-3-carboxaldehyde (Sigma) for 72 h. Camalexin levels were determined as described by Glazebrook and Ausubel (1994),<sup>21</sup> and GC-MS analysis performed. For GC-MS analysis 100 mg per sample of shoot material were submerged in 800  $\mu$ l of methanol and kept at 80°C for 20 min. The supernatant was transferred to a vial, evaporated under a stream of nitrogen and redissolved in 10  $\mu$ l of methanol, and injected 2  $\mu$ l for GC-SIM-MS analysis. The ions with  $m/z$  58, 142 and 200 were monitored. Camalexin (Rt 18.0 min) was quantified by comparison with a standard curve obtained by using purified camalexin kindly provided by Prof. J. Glazebrook (University of Minnesota) and dissolved in methanol for chemical analysis.

**Identification and quantification of indole-3-carboxaldehyde.** For ICAld determination, an inoculum of  $1 \times 10^6$  conidia of *T. virens* was added to 1 l potato dextrose broth (Sigma), and grown for 3 d at 28°C with shaking at 200 rpm. To evaluate the effect of Trp supply on ICAld accumulation, the medium was supplemented with L-Trp (Merck) at a concentration of 100 mg/l. For ICAld determinations, the fungal culture was filtered and the pH of the supernatant adjusted to 7 using 2 N NaOH. Indolic compounds in supernatant filtrate were extracted three times with 1 l of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of nitrogen, taken up and diluted 1:10 (v/v) without L-Trp in the medium and 1:100 (v/v) with L-Trp before GC-MS analysis. ICAld was identified by comparison with mass spectra from the library (NIST/EPA/NIH, "Chem Station" Agilent Technologies Rev. D.04.00 2002). The identity of ICAld was further confirmed by comparison of retention time in the fungal extract with samples of the pure ICAld (Sigma). The molecular ion was monitored after electron impact ionization (70 eV). ICAld,  $m/z$  144. To estimate the amount of ICAld produced by *T. virens*, we constructed a standard curve.

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**Mass spectrometry analysis.** Identification and determination of all compounds was performed using a gas chromatography-mass spectrometry system. Samples were injected in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and 30-mm x 0.2 mm x 0.25 mm, 5% phenyl methyl silicone capillary column (HP-5 MS). The operating conditions used were: 1 ml min<sup>-1</sup> helium as carrier gas, 300°C detector temperature and 250°C injector temperature. The column was held for 5 min at 150°C and programmed at 5°C min<sup>-1</sup> to a 278°C final temperature for 5 min.

**Bioassays for Trichoderma-induced resistance against *B. cinerea*.** To test plant protection conferred by *T. virens* and *T. atroviride* against *B. cinerea*, Arabidopsis seedlings were inoculated with a fungal density of  $\sim 1 \times 10^6$  spores by placing the spores at 1 cm at the opposite ends of agar plates containing 9-d-old germinated Arabidopsis seedlings (10 seedlings per plate). *Trichoderma* was allowed to grow for 3-d to elicit defense responses by the physical contact of the mycelium with the root system. *B. cinerea* was grown on PDA medium for 12 d, at this time, the conidia were collected and resuspended in sterilized distilled water. Arabidopsis shoots were then inoculated with  $\sim 1 \times 10^6$  conidia of *B. cinerea* and leaves exhibiting soft rot symptoms were determined by visual inspection 3 d later. Numbers of symptomatic leaves per seedling were counted as a measure of disease severity. The percentage of dead plants was determined 5 d after pathogen inoculation for a total of 30 plants. Plants were grown at 24°C in a chamber with a 16 h light (200  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>)/8 h darkness photoperiod.

**Data analysis.** Experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyzes with a Tukey's post hoc test were used for testing differences in the biochemical analysis for SA, JA, ICAld, camalexin and anthocyanin measurements, number of lesions and percentage of dead plants in WT Arabidopsis. Different letters are used to indicate means that differ significantly ( $p \leq 0.05$ ).

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## Note

Supplemental materials can be found at: [www.landesbioscience.com/journals/psb/article/17443](http://www.landesbioscience.com/journals/psb/article/17443)

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