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Accumulation of *trans*-piceid in sorghum seedlings infected with *Colletotrichum sublineolum*

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Abstract

Sorghum *SbSTS1*, a pathogen inducible gene, was previously demonstrated to encode an enzyme with stilbene synthase activity. In this study, we attempt to identify the stilbene derivatives that accumulate in infected sorghum seedlings after inoculation with the anthracnose pathogen *Colletotrichum sublineolum*. Scanning for precursor ions that produced the common stilbene aglycones as diagnostic ions was performed in a triple quadrupole mass spectrometer. It was found that infected sorghum seedlings accumulated *trans*-piceid as the major stilbene metabolite together with an unknown resveratrol derivative. Time-course accumulation of *trans*-piceid was examined in two sorghum cultivars, DK18 and DK77, which are resistant and susceptible to *C. sublineolum*, respectively. In both cultivars, *trans*-piceid was not detected until 48 h after inoculation, consistent with the late induction of *SbSTS1* reported previously in infected sorghum plants. The levels of *trans*-piceid detected in DK77 seedlings were approximately three times the levels detected in DK18 seedlings at 120 h after inoculation. In vitro assays demonstrated that *trans*-piceid did not exhibit significant toxicity on conidial germination and mycelial growth of *C. sublineolum*. Hence *trans*-piceid alone may not represent an important defense component against the anthracnose pathogen in sorghum seedlings.

Keywords: Anthracnose; *Colletotrichum sublineolum*; *Sorghum bicolor*; Poaceae; Precursor ion scan; Stilbene glycosides; Stilbene synthase; *trans*-Piceid

1. Introduction

Many plants use the products of secondary metabolism to protect themselves from pathogen attacks. In sorghum (*Sorghum bicolor*), this response is an active process that results in rapid accumulation of high levels of 3-deoxyanthocyanidins in infected tissues. These pigmented compounds were identified as luteolinidin, apigeninidin, and their methylated derivatives (Snyder and Nicholson, 1990; Lo et al., 1996; Wharton and Nicholson, 2000). We demonstrated previously that the 3-deoxyanthocyanidin phytoalexins represent a significant component of resistance against *Colletotrichum sublineolum* (Lo et al., 1999), the causal agent of anthracnose. The fungus causes severe foliar damage and results in substantial yield loss (Thomas et al., 1996).

Accumulation of the sorghum 3-deoxyanthocyanidin phytoalexins requires the activities of chalcone synthase (CHS) which catalyzes the first committed step in flavonoid biosynthesis (Lo and Nicholson, 1998). This enzyme is the prototype of the plant type III polyketide synthase family which also includes stilbene synthase (STS) enzymes. Both CHS and STS enzymes catalyze the condensation of same

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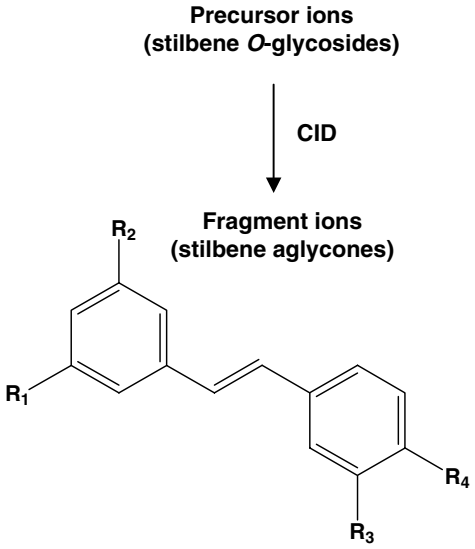
starter phenylpropanoid-CoA substrates with three molecules of malonyl-CoA, but follow different cyclization mechanisms leading to the formation of flavonoids and stilbenes, respectively (Austin and Noel, 2003). Recently we described the first example of a monocot STS gene, *SbSTS1*, isolated from sorghum (Yu et al., 2005). *SbSTS1* is a single-copy gene which is not constitutively expressed, but is inducible upon inoculation of different fungal pathogens (Yu et al., 2005). The gene is a late component expressed during defense response when compared to the induction of CHS genes which are required for 3-deoxyanthocyanidin accumulation. We provided evidences that *SbSTS1* encodes a functional STS enzyme. For example, recombinant SbSTS1 catalyzed the formation of *trans*-resveratrol (**1**) and *trans*-pinosylvin (**2**) using the starter substrates *p*-coumaroyl-CoA and cinnamoyl-CoA, respectively (Yu et al., 2005). In addition, transgenic Arabidopsis over-expressing *SbSTS1* accumulated *cis*-piceid as the major stilbene metabolite which was not present in wild-type plants (Yu et al., 2006).

An intriguing question remains regarding the identities of the stilbene-related metabolites that may accumulate in sorghum following fungal infections. In some plant species, such as grapevine and peanut, resveratrol (**1**) accumulates as phytoalexin in response to pathogen attack (Montero et al., 2003; Sobolev et al., 2007). Transgenic expression of resveratrol synthase gene from *Vitis vinifera* grapevine resulted in enhanced resistance in tobacco, tomato, and rice against the fungal pathogens *Botrytis cinerea*, *Phytophthora infestans*, and *Magnaporthe grisea*, respectively (Hain et al., 1993; Thomzik et al., 1997; Stark-Lorenzen et al., 1997). In members of the Poaceae, compound **1** has been isolated from endophyte-infected grasses such as fescue, ryegrass, barley, sleepygrass, and bluegrass (Powell et al., 1994). Piceatannol (**3**), which contains an additional hydroxyl group at the 5′-position, was detected in sugarcane stalks after inoculation with *Colletotrichum falcatum* (Brinker and Seigler, 1993). In this study, we employed LC-MS/MS analyses in precursor ion scan (PIS) mode to identify stilbene-related metabolites in sorghum using the mesocotyl inoculation system (Lo and Nicholson, 1998). Our results demonstrated the accumulation of *trans*-piceid (**6**) as the major stilbene derivatives in infected seedlings. Higher levels of pathogen-induced compound **6** were detected in a susceptible sorghum cultivar that does not accumulate the 3-deoxyanthocyanidin phytoalexins.

2. Results and discussion

2.1. Screening for stilbene glycosides in infected plant extracts

Since the discovery and characterization of the pathogen-inducible *SbSTS1* gene (Yu et al., 2005), we have initiated an intensive search for defense-related stilbene metabolites using our sorghum mesocotyl inoculation system. Polyphenolic compounds in plants often have different types and numbers of sugars conjugated to the aglycones through *O*-glycosidic linkages. During MS/MS experiments, glycosyl residues are frequently lost in the low energy collision-induced dissociation process.



1 Resveratrol (R_1, R_2, R_4 = OH; R_3 = H;), MW = 228
2 Pinosylvin (R_1, R_2 = OH; R_3, R_4 = H), MW = 212
3 Piceatannol (R_1, R_2, R_3, R_4 = OH), MW = 244
4 Pinostilbene (R_1 = OCH_3; R_2, R_4 = OH; R_3 = H), MW = 242
5 Pterostilbene (R_1, R_2 = OCH_3; R_3 = H, R_4 = OH), MW = 256

Fig. 1. Precursor ions (stilbene *O*-glycosides) which produced the common stilbene aglycones as diagnostic ions following collision induced dissociation (CID) were scanned. Structures and MWs of commonly found stilbene aglycones (**1**–**5**) are shown.

PIS experiments have recently emerged as a highly sensitive and specific MS technology for identification of glycosylated polyphenolic compounds in crude plant extracts (Parejo et al., 2004; Sánchez-Rabaneda et al., 2004; Tian et al., 2005). We used PIS under negative ion mode to survey glycosylated derivatives of different stilbenes in infected sorghum extracts prepared 72 h after inoculation (Fig. 1). The identities of stilbene glycosides could then be deduced based on the mass difference between the aglycones and the precursor ions.

Etiolated seedlings of the sorghum cultivars DK18 and DK77 with elongated mesocotyls were infected with *C. sublineolum*. To analyze plant metabolites, methanol extracts were prepared from mesocotyl tissues and separated on a C18 reverse phase column. Resveratrol (**1**), pinosylvin (**2**), piceatannol (**3**), pinostilbene (**4**), and pterostilbene (**5**) (Fig. 1) are the most commonly described stilbene aglycones in plants (Jeandet et al., 2002). Precursor ions of the [stilbene aglycone−H]$^-$ ions were scanned individually in a triple quadrupole mass spectrometer following HPLC separation. As shown in Fig. 2a, the total ion chromatogram of the PIS of *m*/*z* 227 [resveratrol (**1**)−H]$^-$ for infected plant extracts indicated a major peak (M1, *m*/*z* 389) at 5.2 min, which co-eluted with a *trans*-piceid (**6**) standard, and a minor peak (M2, *m*/*z* 475) at 6.3 min.

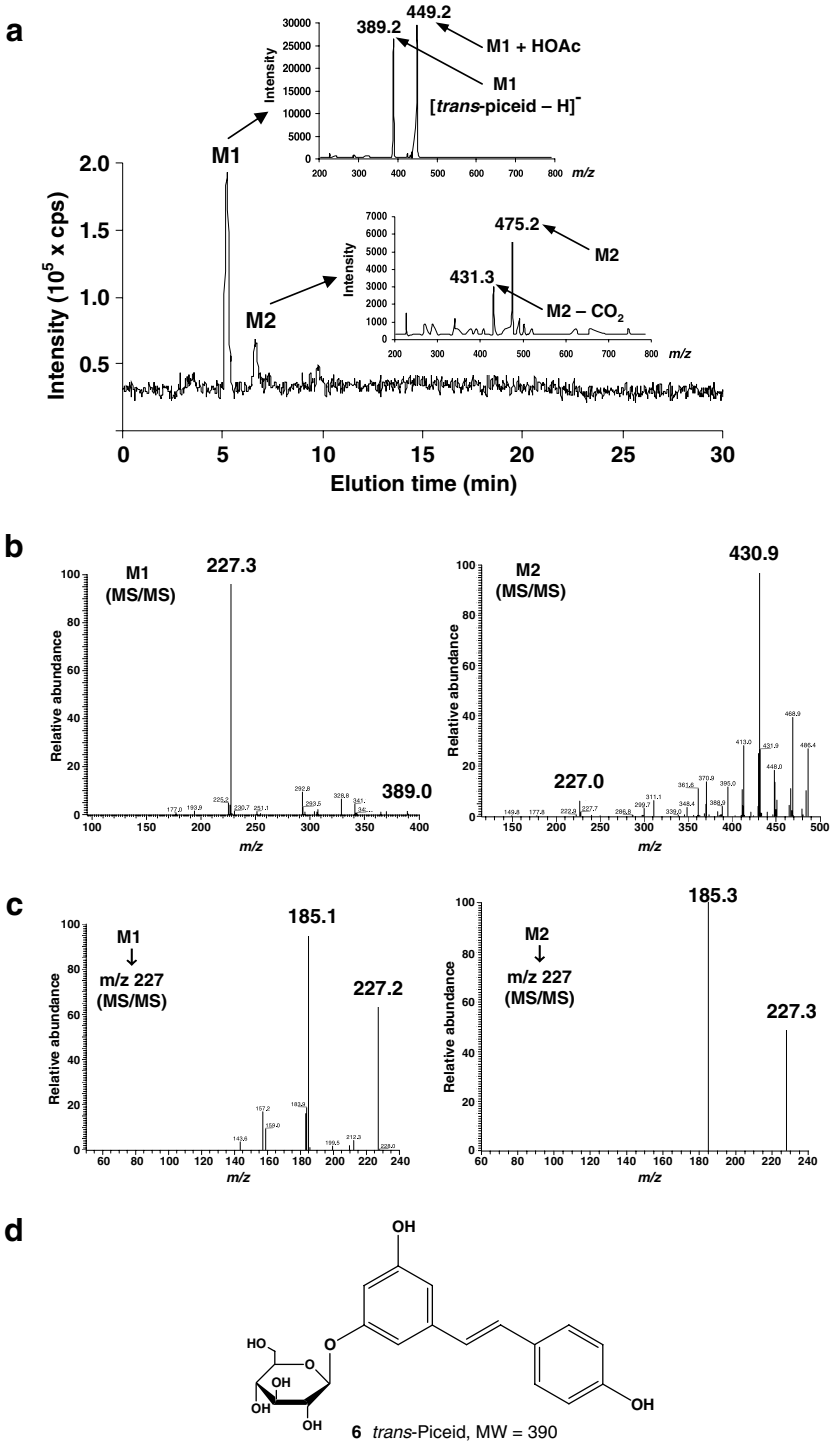


Fig. 2. Identification of stilbene glycosides in infected sorghum seedlings. (a) A representative LC-MS/MS-PIS chromatogram of infected sorghum extracts scanning for the precursor ions of *m/z* 227 [resveratrol (**1**)−H]. Precursor ions for the other stilbene aglycones were not detectable (data not shown). M1 is consistent with a [*trans*-piceid (**6**)−H]$^-$ ion which co-eluted with an authentic standard. The *m/z* 449 ion is an acetic acid adduct. M2 spectrum shows a major ion at *m/z* 475 and the *m/z* 431 ion represents the loss of one CO_2. (b) MS/MS spectra for M1 and M2 (c) MS^3 spectra for the *m/z* 227 daughter ions from M1 and M2. The characteristic *m/z* 185 ion [resveratrol (**1**)−H−CH_2CO]$^-$ was detected in both MS^3 spectra, demonstrating that M1 and M2 are derivatives of compound **1**. (d) Structure of *trans*-piceid (**6**, M1).

These two components (peaks) were not present in the control plant samples (data not shown). On the other hand, no precursor ions of other [stilbene aglycone−H]$^-$ ions were detected in the infected seedlings (data not shown).

Product ion scans were then performed for M1 and M2 in an ion-trap mass spectrometer with MS^3 experiments conducted concurrently for the *m/z* 227 product ions. The MS/MS spectrum of M1 (*m/z* 289) showed the

diagnostic *m/z* 227 ion, indicating the neutral loss of a hexosyl unit (162 Da) from the *m/z* 389 ion (Fig. 2b). MS^3 experiment of the *m/z* 227 ion produced the characteristic ions at *m/z* 185 [resveratrol (**1**)−H−CH_2CO]$^-$ (Fig. 2c). The methanol extracts were also treated with β-glucosidase and compound **1** was released as detected by HPLC-UV analysis (data not shown). Hence, together with its LC retention time and MS spectra, M1 was positively identified as *trans*-piceid (**6**) (Fig. 2d). Product ion spectrum for M2 showed the ions at *m/z* 431 (loss of 1 CO_2) and *m/z* 227 (Fig. 2b). MS^3 analysis of the *m/z* 227 ion also generated the characteristic ion at *m/z* 185 [resveratrol (**1**)−H−CH_2CO]$^-$ (Fig. 2c). However, the amount of M2 was too low for structural elucidation and we could only assign it as an unknown resveratrol derivative.

Conjugation of sugars generally increases the solubility and stability of plant polyphenols for storage in vacuoles and may serve to protect plant cells from their potential toxic effects. However, pathogen-induced phenolic compounds are often present in aglycone forms which are more toxic than the glycosides (Hipskind and Paiva, 2000). Hence it is quite uncommon that our infected sorghum plants accumulated compound **6** as the major pathogen-inducible stilbene metabolites. We also employed LC-MS in multiple reaction monitoring (MRM) mode to analyze the infected plant extracts prepared from different time points, but none of the common stilbene aglycones were detected (data not shown).

2.2. Quantification of trans-piceid (6) accumulation in infected seedlings

Accumulation of compound **6**, the major pathogen-inducible stilbene metabolite identified, was examined in two cultivars, DK18 and DK77, at different time points following inoculation. Infected DK18 seedlings accumulated the 3-deoxyanthocyanidin phytoalexins which appear as restricted red lesions (Fig. 3). On the other hand, DK77 seedlings did not accumulate 3-deoxyanthocyanidins and were highly susceptible to *C. sublineolum*. Spreading necrotic lesions were observed in the mesocotyls 1 week after inoculation (Fig. 3). For quantification of compound **6** accumulation, we selected the key reaction *m/z* 389 → 227 for LC-MS MRM analysis (Yu et al., 2006). In both cultivars, compound **6** accumulation did not occur until 48 h after inoculation (Fig. 4), consistent with the late induction of the *SbSTS1*gene in infected seedlings (Yu et al., 2005). Interestingly, the susceptible cultivar DK77 accumulated approximately three times the levels of compound **6** detected in the resistant cultivar DK18. At 120 h post inoculation, the DK77 seedlings accumulated up to 35 μg g^{-1} FW of *trans*-piceid (**6**).

Biosynthesis of both stilbenes and flavonoids requires the activity of the general phenylpropanoid pathway (Yu et al., 2005). The CHS and STS enzymes compete for the same phenylpropanoid-CoA and malonyl-CoA substrates, leading to the formation of flavonoid and stilbene metabolites, respectively. Presumably the absence of 3-deoxyanthocyanidin (a flavonoid) accumulation in DK77 plants would allow more metabolic substrates for compound **6** biosynthesis after fungal inoculation. On the other hand, the active synthesis of 3-deoxyanthocyanidins, which are essential for defense against the anthracnose pathogen (Lo et al., 1999), would reduce the carbon flow in the stilbene pathway in DK18 plants. Previously we demonstrated the transcriptional repression of anthocyanin biosynthesis in infected sorghum DK46 plants (Lo and Nicholson, 1998). In response to light, seedlings of DK46 accumulate anthocyanin pigments in mesocotyls and suppression of this pathway was believed to play a compensatory role

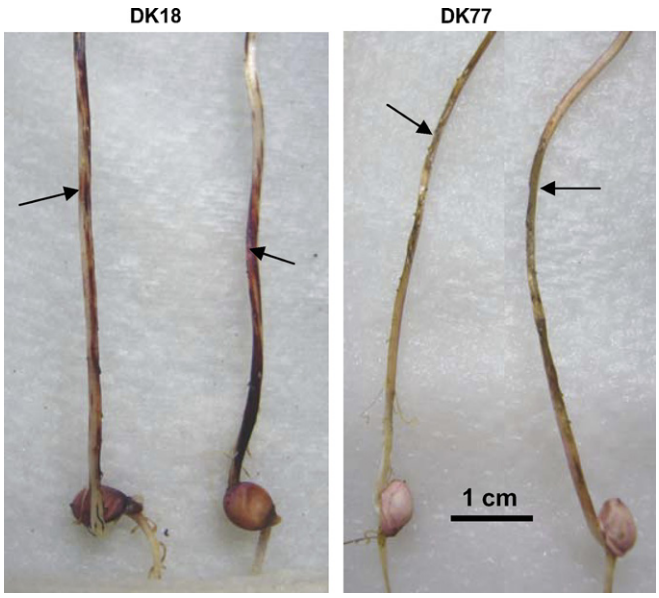


Fig. 3. Infection phenotypes of DK18 and DK77 mesocotyls 1 week after inoculation with *C. sublineolum*. DK18 plants showed resistant phenotypes with pigmented 3-deoxyanthocyanidin phytoalexins (arrows) which prevent fungal colonization. DK77 showed susceptible phenotypes with large and spreading necrotic lesions (arrows).

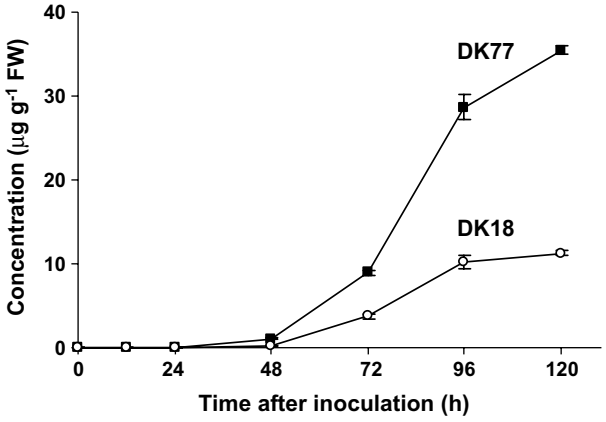


Fig. 4. Accumulation of *trans*-piceid (**6**) in mesocotyls of sorghum cultivars DK18 and DK77. Quantification was performed by LC-MS MRM analysis using the transition reaction *m/z* 389→227. Error bars represent standard deviations.

for the synthesis of 3-deoxyanthocyanidin phytoalexins during defense response. Phenomenon showing competition between stilbene and flavonoid pathways has been observed in grapevines. The level of resveratrol (**1**) accumulation in berry skin decreased while the level of anthocyanin pigmentation increased during fruit maturation (Bais et al., 2000).

2.3. Bioassay experiments

Following the identification of *trans*-piceid (**6**) as a pathogen-induced metabolite in sorghum, we addressed the question whether it served as a phytoalexin. We performed in vitro bioassays to examine the effects of *trans*-resveratrol (**1**) and compound **6** on spore germination of *C. sublineolum*. On hydrophobic surfaces, the fungal conidia germinate with short germ tubes and melanized appressoria formed within 12 h. As shown in Fig. 5, compound **6** is considerably less inhibitory than the aglycone compound **1**. Germination was affected by approximately 50% at 0.2 mM of compound **1** and was completely inhibited at 0.75 mM. In contrast, germination was only inhibited by less than 10% at 0.75 mM of compound **6**. Since compound **6** accumulated in infected plants long after spore germination (Fig. 4), which usually takes place by 12 h after inoculation (Lo et al., 1999), it would be important to determine whether the compound shows inhibitory effects on mycelial growth. However, both compounds **1** and **6** did not exhibit significant in vitro inhibitory effects on mycelial growth of *C. sublineolum* (Fig. 5), even at concentrations (up to 0.7 mM) which were known to be highly toxic on other fungal pathogens. Exposure of *B. cinerea* to compound **1** at concentrations ranging from 60 to 140 $\mu g\ ml^{-1}$ resulted in drastic reduction of mycelial growth and various degrees of cytological distortions (Adrian et al., 1997). On the other hand, compound **6** reduced the hyphal growth of *Phoma medicaginis* by 50% at 0.2 mM (Hipskind and Paiva, 2000) and completely inhibited penetration of *Venturia inequalis* into isolated apple cuticular membranes at 0.5 μM (Schulze et al., 2005).

The apparently low in vitro toxicity of *trans*-piceid (**6**) is consistent with the susceptibility of DK 77 seedlings to *C. sublineolum* although higher levels of compound **6** were detected. It is very likely that the proliferation of fungal biomass continued to induce the accumulation of compound **6** in the susceptible plants. However, in the absence of 3-deoxyanthocyanidins, compound **6** alone is unlikely to play an important role in defense against the anthracnose pathogen in the DK77 seedlings. Members of the Poaceae have been reported to accumulate different stilbenes in association with microorganisms, but the role for these metabolites as phytoalexins was never established. Sugarcane, a close relative of sorghum, produces piceatannol (**3**) aglycone in response to infection by *Colletotrichum falcum*. However, the levels of pathogen-induced compound **3** in a susceptible sugarcane cultivar were higher than those

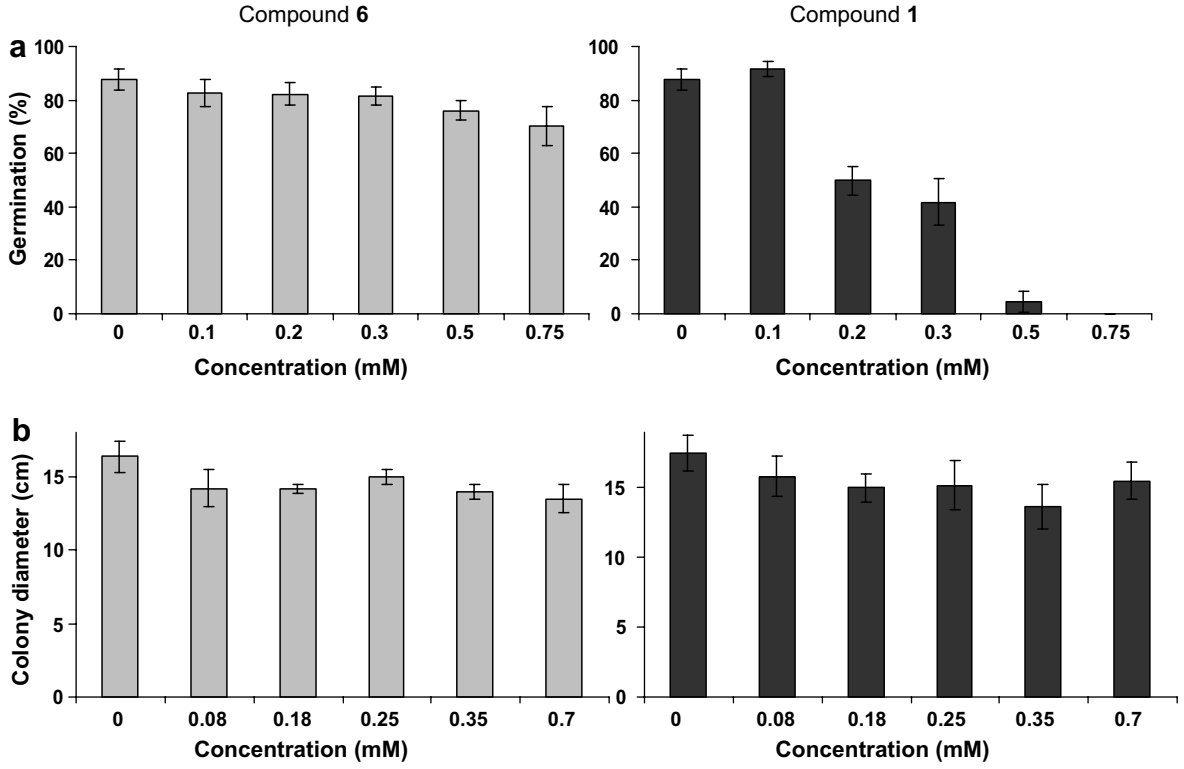


Fig. 5. Effects of *trans*-resveratrol (**1**) and *trans*-piceid (**6**) on spore germination (a) and mycelial growth (b) of *C. sublineolum*. Data represent average values obtained from at least three independent experiments and error bars represent standard deviations.

in a resistant cultivar (Brinker and Seigler, 1993). Thus, the role of stilbenes in defense against the *Colletotrichum* pathogens appears to be secondary in both sugarcane and sorghum.

While *trans*-piceid (**6**) may not play an important role in defense response, our studies demonstrated the capability of sorghum to produce stilbene metabolites. In addition to their use as animal feeds, sorghum grains have been used for food, beverage, and liquor production around the world. Resveratrol (**1**) and other stilbenes are well recognized for their impressive list of health benefits, such as anti-oxidation, anticancer, blood thinning, and blood pressure lowering activities. Sorghum is known to be a rich source of natural products including a wide range of polyphenolic compounds (Awika and Rooney, 2004). Currently there are no reports of stilbene accumulation in sorghum grains, but their agricultural and nutritional values of sorghum would be significantly improved if we can manipulate and optimize the production of these beneficial compounds during seed development.

3. Concluding remarks

This is the first report of stilbene accumulation in sorghum, which is well known to be a rich source of phytochemicals. Following our previous characterization of a pathogen-inducible STS gene, we identified *trans*-piceid (**6**) as the major stilbene metabolite in seedlings infected with *C. sublineolum* by LC-MS/MS analysis. However, compound **6** alone may not constitute a significant defense mechanism against the anthracnose pathogen because (1) higher levels of compound **6** were detected in a susceptible cultivar that does not accumulate the 3-deoxyanthocyanidin phytoalexins; and (2) compound **6** showed very low toxicity on spore germination and mycelial growth. Nonetheless, the ability of sorghum to accumulate stilbenes may be exploited for nutritional improvement.

4. Experimental

4.1. Plant materials and fungal inoculation

Sorghum (*Sorghum bicolor*) hybrid seeds and fungal strains used were kindly provided by R Nicholson (Purdue University, West Lafayette, IN, USA). Seeds of the sorghum cultivars DK18 and DK77 were planted on rolls of germination paper and kept in the dark for 4 days at 28 °C. For inoculation, etiolated seedlings with elongated mesocotyls were sprayed with conidial suspensions of *C. sublineolum* isolate TX430BB (2.0×10^6 spores ml^{-1}) containing Tween 20 (1 μl ml^{-1}), incubated at 100% relative humidity for 24 h under constant light. Inoculated mesocotyls were excised and placed in methanol, and metabolites were allowed to leach from the tissues at 4 °C overnight.

4.2. Triple quadrupole and ion trap MS experiments

LC-MS/MS operated in the PIS mode was performed to screen for stilbene *O*-glycosides in the infected seedlings. LC separation on a Zorbax Eclipse XDB-C18 column (5 μm, 150 × 2.1 mm; Agilent Technologies) was performed using a Perkin–Elmer HPLC system (Series 100 pump). A mobile phase of 0.1% AcOH in H_2O (v/v) (A) and CH_3CN (B) with a linear gradient of 20–60% B over 25 min was used. Flow rate was maintained at 0.2 ml min^{-1} and LC elution was analyzed by PIS scan experiments conducted on the heated TurboIonSpray™ interface of an API-2000 triple quadrupole mass spectrometer (Applied Biosystems). For quantification of *trans*-piceid (**6**) in the plant extracts, elution from LC was analyzed by monitoring the transition reaction of *m/z* 389→227. A calibration curve over the 1–100 μg ml^{-1} range was constructed using a commercial standard. Product ion scans and MS^3 experiments for the precursor ions were performed in an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). MS conditions were optimized for maximum sensitivities for both instruments and the parameters were as follows: API2000, ion spray voltage −4.5 kV, source temperature 500 °C, nebulizer gas (N_2) 35 (arbitrary units), curtain gas (N_2) 35 (arbitrary units), collision gas 4 (arbitrary units), entrance potential −12 V, declustering potential −5 V, focusing potential −300 V and collision energy −20 V in negative ion mode; LCQ, capillary temperature 300 °C, sheath gas (N_2) 70 (arbitrary units), auxillary gas (N_2) 20 (arbitrary units), ESI spray voltage 5.5 kV, capillary voltage −18 V, a multipole 1 offset voltage of 5.75, a multipole 2 offset voltage of 10 V and an inter-multipole voltage of 20 V in the negative ion mode. Data acquisition, peak integration, and calculation were interfaced to a computer workstation running the Analyst 1.3.1 software (Applied Biosystems).

4.3. In vitro fungitoxicity assays

For germination assays, conidia were dislodged with water from the surface of *C. sublineolum* cultures growing on oatmeal agar, and filtered to remove hyphae and debris. Spore suspensions (20 μl; 5.0×10^4 ml^{-1}) containing different concentrations of either compound **1** or **6** (Alexis Chemicals) dissolved in EtOH (4% final concentration) were inoculated on the surface of plastic petri plates. Controls were prepared by adding EtOH (4%) without stilbenes in the suspensions. After overnight incubation at room temperature, germination and appressorium formation was observed under a dissecting microscope. For agar-plate assays, ethanol solution of either compound **1** or **6** in EtOH were added to molten potato dextrose agar to achieve the desired concentrations. A small agar plug of *C. sublineolum* was placed in the centre of each agar plate and the plates were incubated at room temperature. The extent of mycelial growth was measured after 5 days.

Acknowledgements

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