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Alternaria toxin-induced resistance in rose plants against rose aphid (*Macrosiphum rosivorum*): effect of tenuazonic acid*

Fa-zhong YANG[§1,2], Bin YANG[§2], Bei-bei LI[2], Chun XIAO[†‡1]

([1]*School of Plant Protection, Yunnan Agricultural University, Kunming 650201, China*)

([2]*Key Laboratory of Forest Disaster Warning and Control of Yunnan Province, Southwest Forestry University, Kunming 650224, China*)

[†]E-mail: x.chun@163.com

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Abstract: Many different types of toxins are produced by the fungus, *Alternaria alternata* (Fr.) Keissler. Little is known, however, regarding the influence of these toxins on insects. In this study, we investigated the toxin-induced inhibitory effects of the toxin produced by *A. alternata* on the rose aphid, *Macrosiphum rosivorum*, when the toxin was applied to leaves of the rose, *Rosa chinensis*. The results demonstrated that the purified crude toxin was non-harmful to rose plants and rose aphids, but had an intensive inhibitory effect on the multiplication of aphids. The inhibitory index against rose aphids reached 87.99% when rose plants were sprayed with the toxin solution at a low concentration. Further results from bioassays with aphids and high performance liquid chromatography (HPLC) analyses demonstrated that tenuazonic acid (TeA) was one of the most important resistance-related active components in the crude toxin. The content of TeA was 0.1199% in the crude toxin under the HPLC method. Similar to the crude toxin, the inhibitory index of pure TeA reached 83.60% 15 d after the rose plants were sprayed with pure TeA solution at the lower concentration of 0.060 μg/ml, while the contents of residual TeA on the surface and in the inner portion of the rose plants were only 0.04 and 0.00 ng/g fresh weight of TeA-treated rose twigs, respectively, 7 d after the treatment. Our results show that TeA, an active component in the *A. alternata* toxin, can induce the indirect plant-mediated responses in rose plants to intensively enhance the plant's resistances against rose aphids, and the results are very helpful to understand the plant-mediated interaction between fungi and insects on their shared host plants.

Key words: Toxin, Induced resistance, Fungus-plant-insect system, Plant-mediated interaction, Tenuazonic acid, *Alternaria alternata*, *Macrosiphum rosivorum*

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1 Introduction

Herbivorous insects and phytopathogenic fungi may encounter each other on the same individual host plant, and it is well-known that numerous plant-mediated interactions occur in fungus-plant-insect systems (Hatcher *et al*., 1995; Kruess, 2002; Rostás *et al*., 2003). The plant-mediated interactions may be direct interactions, indirect interactions, or both. Behaviors and performances of insects can be detrimentally or beneficially influenced by fungal infections through their shared host plants, and most of the reported studies demonstrated that preferences and performances of herbivorous insects were detrimentally affected by fungal infections of their host plants (Kruess, 2002; Rostás *et al*., 2003; Ma and Xiao, 2013). The metabolic changes of the plant induced by fungi are responsible for these plant-mediated indirect effects on insects, and then the systemic acquired resistance (SAR) and induced system resistance (ISR)

‡ Corresponding author
§ The two authors contributed equally to this work
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ORCID: Fa-zhong YANG, http://orcid.org/0000-0002-3962-6938; Chun XIAO, http://orcid.org/0000-0002-7245-4509

can be induced in the plant (Kruess, 2002). Rostás *et al*. (2003) reviewed the plant-mediated effects on a systemic scale (systemic effects) and the plant physiological changes induced by the fungal attack with effects on the herbivorous insects. The revelation of these indirect interactions is very important for understanding the decision of insects when they encounter their host plants infected by phytopathogenic fungi and, thus, for integrated pest management.

However, it is difficult to distinguish the plant-mediated indirect effects on insects from the direct effects in a fungus-plant-insect system. Possibly, some mycelia or the toxins produced by fungi are still remaining in the plants used in the bioassays. Therefore, the toxins produced by fungi, instead of the fungi themselves, should be used to test the effects of fungal infections on herbivorous insects.

If the mechanism of the indirect effects on insects can be identified, a special means of insect control can be developed. The active metabolic chemicals, instead of the fungus itself, can be used on plants to control insects. Many studies have shown that *Alternaria* spp. can produce a variety of toxins, including either host-selective or non-host-selective toxins (Thomma, 2003). Generally, the spectrum of activities in a non-selective toxin is not limited to the phylogenetic specialisation of the producer pathogen, and the spectra of biological activities of toxins are concentration-dependent (Berestetskiy, 2008). Among the sorts of mycotoxins produced by fungi, the *Alternaria* toxins were widely applied. The toxins may provide the prospect for biocontrol of fungi and certain weeds (Strobel *et al*., 1991; Chelkowski and Visconti, 1992; Abbas *et al*., 1993; Chen *et al*., 2005).

Alternaria alternata (Fr.) Keissler produces a variety of secondary metabolites belonging to several classes of phytotoxic chemicals (Strange, 2003; Chen *et al*., 2005). For example, tenuazonic acid (TeA) is a non-host-selective toxin that can also be produced by many strains of *A. alternata*, other fungi of *Alternaria* spp., and even other mold fungi that do not belong to the genus *Alternaria* (Yekeler *et al*., 2001; Ostry, 2008).

Many studies have shown that pathogens or their toxins may induce numerous resistance-related plant responses (Wagner and Boyle, 1995; Heath and Skalamera, 1997; Stout *et al*., 1999; Paul *et al*., 2000; Bostock *et al*., 2001; Rostás *et al*., 2003; Simon and Hilker, 2005). The most important responses include syntheses of defensive components (Hammerschmidt, 1999) and occurrences of SAR and ISR (Cipollini *et al*., 2004). Many signaling pathways are involved during these plant defense responses, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). More importantly, numerous cross-talks between multiple signaling pathways have been demonstrated (Hunter, 2000; Zhao and Sakai, 2003)—for example, between the SA pathway, which is mainly related to the resistance to fungi, and the JA pathway, which is mainly related to the resistance to herbivorous insects. Thus, the ecological cross-effects between herbivores and toxins (or fungi themselves) may be plant-mediated, and the resistance of the host plants against insects can be obtained because of fungal infections in a fungus-plant-insect system.

While plant resistance to the original attacker can be enhanced via SAR and ISR, cross-effects on resistance to other pest organisms can be an important ecological consequence (Heil and Bostock, 2002; Cipollini *et al*., 2004) and may result from broad biological activity of induced defenses. For example, negative effects of *Alternaria brassicae*-infection on the leaf beetle, *Phaedon cochleariae*, were reported in an agricultural system by Rostás and Hilker (2002).

However, it remains unclear whether performances of herbivorous insects can be affected by either TeA or *A. alternata* fungus itself. If it can be verified that the insect's performance can be influenced by the *A. alternata* crude toxin, it still remains unclear whether the active compound(s) in the crude toxin is TeA and whether the plant's resistances against herbivorous insects were enhanced when host plants were treated with TeA, especially at a lower concentration.

With increasing the plantation area of the cut rose *Rosa chinensis* Jacq. cv. Movie Star, the infestation of the rose plants by the rose aphid, *Macrosiphum rosivorum* Zhang, has often been found throughout the year in greenhouses and is responsible for serious economic losses to the cut rose production industries. However, it has remained unclear up to now whether the rose aphid is detrimentally or beneficially affected by *Alternaria* toxins (including TeA). Even studies on the effect of the fungi of the genus *Alternaria* on insects and the literature related to the mechanisms of resistance induced by the *Alternaria* toxins on insects

are still significantly limited. So, in the present study, the toxicities of toxins produced by strain 0848 of *A. alternata* to rose leaves and aphids were determined (including TeA). We also aimed to test whether the resistance of rose plants against rose aphids can be induced by the *Alternaria* toxins and whether TeA is a resistance-related active component produced by *A. alternata*.

2 Materials and methods

2.1 Plants and insects

Plants were sampled from the susceptible rose cultivar Movie Star (about two years old) in greenhouses for rose cut flower production in Yunnan Province, which is in the southwest of China, and were grown in a fungus-free greenhouse compartment of our laboratory at about 24 °C and 80% relative humidity (RH) with a 16 h/8 h (light/dark) photoperiod regime. Rose plants were used for bioassays when they had developed 6–10 expanded leaves after about four weeks.

Rose aphids were collected from a naturally occurring rose aphid colony at the same site as the rose plants and were continuously reared on seedlings of roses grown inside another greenhouse compartment, as described above.

2.2 Toxin purification

Samples (stems and leaves) were collected from wild and cultivated rose plants (*Rosa rugosa* Thunb.). For isolation of the *Alternaria* fungi from the samples, the procedure following Kaul *et al*. (2008) was adopted, with slight modifications. Out of a total of ten fungus strains of *A. alternata*, strain 0848 was used for the production of toxins in the present study.

Alternaria toxin was obtained from the culture of strain 0848 of *A. alternata* by first transferring conidia into a potato dextrose agar (PDA) medium and thereafter transferring mycelial culture into a 200-ml potato dextrose broth in a 500-ml conical flask at 25 °C for 10 d. Culture filtrate (5 L) was extracted with ethyl acetate, and the extract was passed through a column of macroporous resin D101 (26–60 mesh, Tianjin Haiguang Chemistry Company, China), the column being eluted with alcohol. The alcohol-diluted extract was concentrated by use of a rotary evaporator at 50 °C (pressure 0.075 MPa) until partially purified crude solid extract was obtained.

2.3 Toxicity of crude toxin extract on rose leaves and aphids

2.3.1 Leaf-puncture bioassay

The toxicity of the toxin of strain 0848 of *A. alternata* was assayed by leaf-puncture bioassay on rose leaves of *R. chinensis* (method A) (Abbas *et al*., 1993). The fully expanded leaves from the greenhouse-grown plants were washed for 10 min in running tap water, sterilized in 1% (0.01 g/ml) sodium hypochlorite for about 1 min, and aseptically rinsed thoroughly with sterile distilled water. Finally the leaves were placed on moistened filter paper and punctured by a sterile needle on the lower surface. The toxin was dissolved in sterile deionized water at concentrations of 5.0, 10.0, 20.0, 40.0, 80.0, and 100.0 μg/ml. Droplets (10 μl) of the test solutions were applied to the wounded leaves and the leaves were then incubated in transparent plastic boxes at 24 °C under a 16-h photoperiod, with the lower surface up. The diameters of the necrotic lesions (mm) were measured with a venire calliper (Shanghai exactitude apparatus factory, China) once every 8 h for 48 h. Other rose leaves with droplets of sterile water served as the control. In addition, the total increased percentages of the lesions diameter (%Δd) against the control were calculated 48 h after treatment, i.e. $\%\Delta d=(d_t-d_c)/d_c\times 100\%$, where d_t is the diameter of lesion 48 h after treatment and d_c is the diameter of control lesion. Ten replicates were conducted for each concentration and for the control.

2.3.2 Bioassay on live plants

The same series of crude toxin solutions and the control (water) were directly applied to the leaves of live rose plants in greenhouses (10 ml each time for each concentration), i.e. the leaves of the healthy plants were evenly sprayed with the solutions once every 3 h (three times each day) over 3 d (method B). On the second day after the treatments ended, the leaf areas of the necrotic lesions produced by the toxin were measured using a transparent scale-paper, and the total leaf areas of the lesions were then calculated as mm^2 per twig. Four replicates were performed at each concentration.

2.3.3 Bioassay with aphids

To determine whether the aphids were directly affected by the *Alternaria* toxin of strain 0848, we tested its toxicity to the aphids under laboratory conditions using the slide-dipping method (commonly used for the determination of insecticidal activity) as described by Stribley *et al*. (1983). With a stereomicroscope and a wetted fine brush, 20 adults were affixed to double-face scotch tape stuck tightly to a side on the dorsal part, and the slides were then dipped into the toxin solution for 5 s. The excess was blotted off with filter paper. Aphids were maintained at 22 °C and 70% RH. The toxicity was tested at six different concentrations of 5.0, 10.0, 20.0, 40.0, 80.0, and 100.0 μg/ml, and six replicates were carried out for each concentration. The same method was used with the control treatment using water. Mean mortality percentages were recorded 24 h after treatment, and all insects that responded to touching with a fine brush were considered to be alive (Sugimoto and Osakabe, 2014).

2.4 Resistance bioassay

To test the *Alternaria* toxin-induced resistance against aphids, the 10 ml toxin solutions at 5.0, 20.0, 35.0, and 50.0 μg/ml were evenly and separately sprayed to the surfaces of each rose plant (leaves and stems) with a sprayer similar to a perfume container twice per day for two days for each concentration. Five days after the treatment ended, each potted rose was infested with 10 wingless adult aphids by means of a wetted fine brush and a stereomicroscope, and the whole plant was covered with a cage of fine-mesh gauze to avoid aphids moving to neighboring plants. Another water-spraying rose infested by 10 aphids served as the control. Six replicates were performed for treated and control rose plants. All rose plants were left in the greenhouse under the conditions previously described. The number of aphids in each cage was separately recorded on each of three days until the fifteenth day after infestation to calculate the inhibitory index (II) of rose aphid multiplication using the following formula: $II=(N_c-N_t)/N_c\times100\%$, where N_c is the mean number of aphids on control plants and N_t is the mean number of aphids on toxin-treated plants.

2.5 HPLC analyses of crude extract of *Alternaria* toxin

To test whether TeA existed in the crude toxin extract produced by *A. alternata* and was an active component that can induce the resistance of rose plants against aphids, the partially purified crude extract of *Alternaria* toxin and pure TeA standard (purity>97%; Sigma-Aldrich, St. Louis, MO, USA) were separately dissolved in acetonitrile-water mixtures (90:10, v/v) to obtain the solutions of 100.00 and 0.50 μg/ml, respectively, and were analyzed using a high performance liquid chromatograph (HPLC) system (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler, thermostated column compartment, and Agilent technologies ChemStation software for LC (B.02.01). Analyses of TeA in the crude extract and pure TeA were performed using a 250 mm×4.6 mm i.d. Zorbax SB-C18 reversed phase column, with a PDA detector set at 226 nm as the integration wavelength. A mixture of acetonitrile and 2% formic acid in water (90:10, v/v) was used as the mobile phase at 0.7 ml/min. In the meantime, pure TeA (0.10, 0.30, 0.50, 0.70, 0.90, and 1.00 μg/ml, in acetonitrile-water mixtures (90:10, v/v)) was used as an external standard to calculate the concentration of TeA in the crude extract. TeA peaks were identified by comparison of HPLC retention time and ultra violet (UV) spectra with TeA standard kindly provided by Prof. Yu-hui CHEN (Experimental Center of Chemistry, Southwest Forestry University, Kunming, China). Furthermore, pure TeA was added into the solution of the crude toxin and its concentration in the mixture reached 0.1 μg/ml. The mixture was analyzed as previously described to more accurately ascertain whether TeA existed in the crude *Alternaria*-toxin and to test the accuracy of the method. Four replicates were performed for each solution at each concentration.

2.6 Toxicity of TeA to rose plants and aphids and TeA-induced resistance against aphids

The experiments were conducted to test whether the resistance of rose plants against aphids can be induced by TeA. Pure TeA was dissolved in water at concentrations of 0.006, 0.024, 0.042, and 0.060 μg/ml, and its toxicity was tested using the method previously

described for the bioassays for the toxicity of the crude toxin (including the leaf-puncture bioassay and the bioassay that TeA was applied to the live rose plants). Similarly, the toxicity of TeA to rose aphids was tested using the method described previously (the slide-dipping method). Water served as controls for all of the bioassays.

The resistance of TeA against rose aphids was also tested under the method previously described for the resistance of the crude toxin, i.e. the rose plants were sprayed with the same series of pure TeA solutions, and the aphid numbers were recorded to calculate the inhibitory index 15 d after infestation. Similarly, water served as the control.

2.7 HPLC analyses for TeA in rose plants

In total, 144 rose plants were grown for analyses of TeA on the surface of rose plants and in the inner portion of the plants. The plants were sprayed with 0.060 μg/ml TeA once every 3 h (12 times in total, over 3 d), as previously described for the treatment with the crude toxin solution. Forty minutes after the treatment (Day 0) and the first, third, fifth, and seventh days after the treatment (i.e. Days 1, 3, 5, and 7), TeA was first extracted from the surfaces of the intact leaves and stems. Each sample consisted of ten twigs of about 80-cm length that were cut from the base of the twigs, and three replicates were conducted for each sample. Directly after cutting, the twig was dipped for 15 s in 450 ml of methanol. After the extraction of TeA from the surfaces, the leaves and stems were immediately frozen at −80 °C, freeze-dried, weighed, and ground to a fine powder. For each sample, the TeA in the inner portion of the plants was extracted from the fine powder using methanol. The methanol in the two types of solutions was evaporated from the crude methanol dip-volumes with a rotary evaporator (R-3000, Büchi, Switzerland). The two extracts were separately re-dissolved in 0.8 ml of acetonitrile-water mixture, and then analyzed under the same HPLC method. The water-sprayed plants served as the control. The TeA contents were calculated in ng/g fresh weight of plant material.

2.8 Statistical analysis

Statistical analysis was performed using SPSS Version 13.0 (SPSS Inc., Chicago, IL, USA). A binomial test was used to analyze the mortality percentage of aphids from the slide-dipping bioassay. One-way analysis of variance (ANOVA) was used to compare the means of the diameters of the necrotic lesions, the leaf area of the necrotic lesions produced by the toxin or TeA, the inhibitory index that was calculated from the aphid number, and the data from the HPLC analysis. Post hoc tests were performed using Fisher's least significant difference (LSD) method.

3 Results

3.1 Toxicity of crude toxin on rose plants and aphids and resistance of crude toxin against rose aphids

The toxicity of partially purified *A. alternata* toxin on rose plants is presented in Fig. 1 (with the leaf-puncture bioassay, i.e. method A) and in Fig. 2 (the crude toxin was directly applied to the surface of the rose plants, i.e. method B). Under method A, the diameters of the lesions produced by the toxin increased only slightly with the increasing of the test time when compared with the control. The differences in the lesion diameters among different toxin concentrations were not significant (F[6, 63]=0.828, 0.816, 0.775, 0.982, 0.627, 0.835, and 0.819; P=0.210, 0.235, 0.171, 0.453, 0.106, 0.199, and 0.236, for seven periods of time, respectively; comparisons were performed within the same periods of time). The same was true for the total increased percentages of lesion diameter (F[6, 63]= 0.782, P=0.587; Fig. 1). The percentages did not increase in the range of toxin concentrations from 0.0 to 80.0 μg/ml, with slight increases at concentration of 100.0 μg/ml. Under method B, the leaf area of the lesions per twig of rose also did not increase with the increasing of the toxin concentration (F[6, 21]=0.085, P=0.967; Fig. 2).

Similarly, no significant differences in the mortality percentages of the rose aphids at different concentrations of the crude toxin were found in the slide-dipping bioassay (Binomial test, P=0.407, 0.529, 0.411, 0.327, 0.259, and 0.428, for concentrations of 5.0, 10.0, 20.0, 40.0, 80.0, and 100.0 μg/ml, respectively, compared with the control; Fig. 3).

However, the inhibitory index increased significantly with the increase in time (Fig. 4) for all concentrations of crude toxin produced by *A. alternata*.

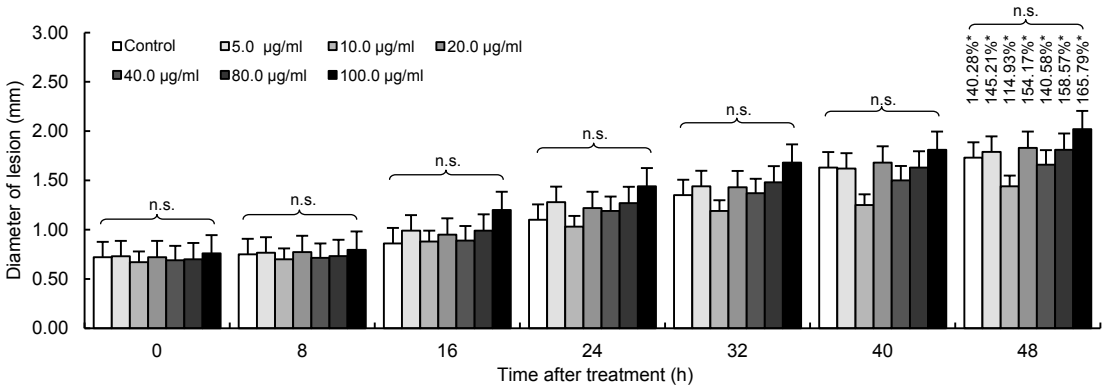

Fig. 1 Toxicity of purified crude toxin produced by *A. alternata* to rose leaves with leaf-puncture bioassay

Six concentrations of the crude toxin were tested against the control (0.0 μg/ml, only water used). The values are expressed as mean (SD). Ten replicates were conducted for each concentration for each time. The data with an asterisk (*) represents the total increased percentage of the lesion diameter compared to the control (i.e. $\%\Delta d=(d_t-d_c)/d_c\times100\%$, where d_t is diameter of lesion 48 h after treatment and d_c is diameter of lesion of control). No significances (n.s.) were found among the different concentrations within the same period of time

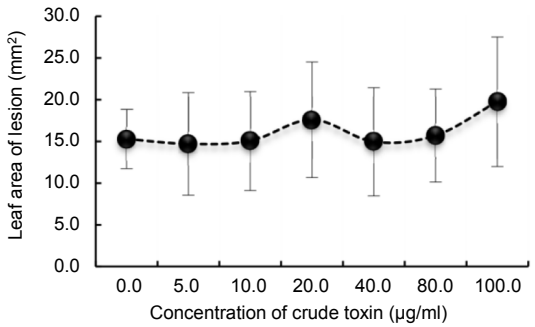

Fig. 2 Leaf area of lesions per twig

Crude *A. alternata* toxin was applied directly to the surfaces of the leaves of live potted roses for 3 d. Six concentrations of the toxin were tested against the control (0.0 μg/ml, only water used). The values are expressed as mean±SD. Four replicates were conducted for each concentration. No significances were found among the different concentrations

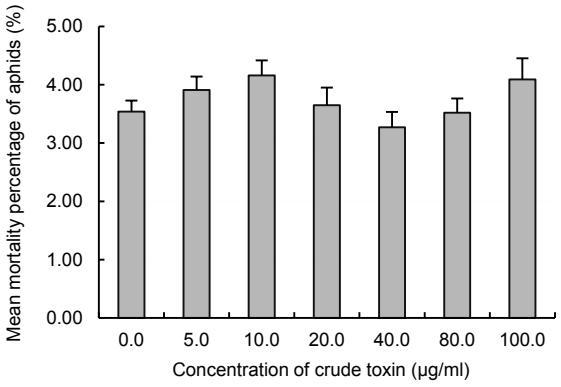

Fig. 3 Toxicity of *A. alternata* toxin to rose aphids

The aphids (n=20 for each treatment) were dipped into the toxin solution for 5 s using the slide-dipping method. Mortality percentages (mean (SD)) of the aphids were calculated against the control (0.0 μg/ml, only water was used). Six replicates were conducted for each concentration. No significances were found among the different concentrations

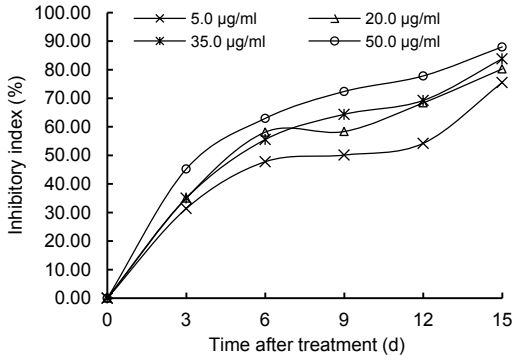

Fig. 4 Resistance of crude *A. alternata* toxin against rose aphids

The rose plants were sprayed with the toxin at different concentrations, and were infested with 10 aphids per plant one day after the treatment to calculate the inhibitory index (II) for rose aphid multiplication using the following formula: $II=(N_c-N_t)/N_c\times100\%$ (N_c is the mean number of aphids on control plants (only water used) and N_t is the mean number of aphids on toxin-treated plants). Six replicates were conducted for each concentration

The maximum of inhibitory index reached 87.99% 15 d after treatment when the toxin was used at 50.0 μg/ml. When comparisons were done among different concentrations during the same period of time, inhibitory index increased generally with the increase of TeA concentration, with the only exception being on Day 6 (inhibitory index at 35.0 μg/ml was lower than that at 20.0 μg/ml). The differences in inhibitory index were significant across the four concentrations on Day 9 (F[3, 20]=4.829, P=0.033) and Day 15 (F[3, 20]=8.430, P=0.003), and also between 5.0 and 50.0 μg/ml on all Days 3, 6, 9, 12, and 15 (F[1, 10]=7.367, 7.747, 8.657, 8.613, and 11.000; P=0.022, 0.019, 0.015, 0.015, and 0.008, respectively).

3.2 HPLC analyses for crude toxin and TeA

TeA was detected in the solution sample of partially purified crude toxin produced by *A. alternata* and its percentage in the crude toxin solid reached 0.1199%. The mean±standard deviation (SD) retention time of TeA in different solutions was (11.13330± 0.000646) min (relative standard deviation (RSD)= 0.01161%; Table 1). With the use of standard pure TeA in solutions, the linear regression equation for TeA was obtained: Y=1502.27325X+0.39274 (F[1, 4]= 572863, P<0.0001; adjusted R^2=0.999994). Here, Y represented the peak area in the chromatograph and X represented the concentration of TeA (μg/ml). With using the HPLC method, the spiked recovery for TeA in the real solution sample of the crude toxin was 99.86% (RSD=0.22%, n=4).

3.3 Toxicity of TeA to rose plants and aphids and resistance of TeA against rose aphids

The diameter of necrotic lesions produced by pure TeA 48 h after the treatment and the leaf area of the necrotic lesions per twig of the rose plants 3 d after the treatment were measured to show the toxicity of TeA to rose plants, and the mortality percentage of the aphids was calculated to test the toxicity of TeA to rose aphids (Table 2). The three tested parameters showed no significant differences among the different concentrations of TeA, and their values did not increase with the increasing of the TeA concentrations (Table 2). In these three bioassays, the toxicity of TeA to rose plants and rose aphids was not found.

The inhibitory index 15 d after infestation is presented in Fig. 5. The significant plant's resistance against rose aphids induced by pure TeA was found in the bioassay at all concentrations of TeA. Inhibitory index did increase with the increasing of the TeA concentrations. When TeA was used at 0.006 μg/ml, the inhibitory index reached 65.18%, and the maximum reached 83.60% at 0.060 μg/ml. The differences in inhibitory index were significant between 0.010 μg/ml and the other three concentrations of TeA (F[3, 20]=13.135, P<0.001), and between 0.024 and 0.060 μg/ml (F[1, 10]=4.998, P=0.049). However, the significant differences were not found between 0.024 and 0.042 μg/ml (F[1, 10]=1.856, P=0.203), or between 0.042 and 0.060 μg/ml (F[1, 10]=1.332, P=0.275).

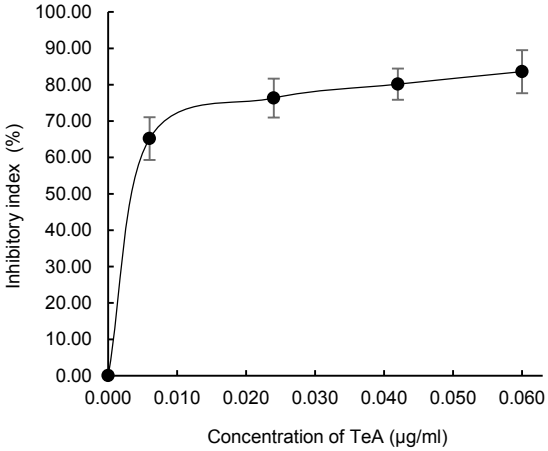

Fig. 5 Resistance of pure TeA against rose aphids
The methods for bioassay and calculation of the inhibitory index (15 d after infestation) were the same as those described in Fig. 4, with the only exception being the different TeA concentrations. The values are expressed as mean±SD. Six replicates were performed for each concentration

Table 1 HPLC analyses of TeA in different solutions

Solution	Peak No.	Retention time (min)[a]	Peak area (mV·s)	Conc. of TeA (μg/ml)[b]	TeA in different solids (%)[b]
Crude toxin (100.00 μg/ml)	23	11.13223	180.58956	0.1199	0.1199
Pure TeA (0.50 μg/ml)	2	11.13434	742.96641	0.4943	98.8628
Pure TeA (0.10 μg/ml)	2	11.13449	148.91348	0.0989	98.8617
Mixture (100.00 μg/ml crude toxin+0.10 μg/ml pure TeA)	23	11.13213	329.11030	0.2188	0.2185

[a] RSD=0.01161%; Four replicates were conducted for each solution. [b] The concentration or percentage of TeA in the solid substance corresponding to their respective solutions

Table 2 Toxicity of pure TeA to rose plants and aphids[a]

Concentration of TeA (μg/ml)	Diameter of necrotic lesions (mm)[b]	Leaf area of necrotic lesions (mm^2)[c]	Mortality percentage of aphids (%)
0	1.63±0.18	13.75±3.62	1.62±0.39
0.006	1.53±0.18	15.32±3.59	1.89±0.44
0.024	1.43±0.23	17.82±5.26	1.49±0.48
0.042	1.80±0.41	14.52±4.14	1.57±0.37
0.060	1.69±0.29	16.23±5.01	1.92±0.58
F[4, 25]	1.733	0.807	1.091
P	0.174	0.532	0.382

[a] All data were presented as the mean±SD, and one-way ANOVA was used to compare the differences of means. For all three tested parameters, no significant differences were found among the different concentrations (0.000 μg/ml as control, only water used). The methods used were the same as those described for the bioassays for toxicity of the crude toxin (Figs. 1–3). Six replicates were performed for each concentration for all three bioassays. [b] The diameter was measured 48 h after the treatment. [c] The leaf area of the necrotic lesions per twig was measured 3 d after the treatment

3.4 HPLC analyses for TeA in TeA-treated rose plants

Under the HPLC method, TeA was detected both on the surface and in the inner rose plants treated with TeA for 3 d (Fig. 6). After the treatment, the concentrations of TeA from the surface and the inner portion of the rose twigs decreased significantly with the increasing of time. Forty minutes after the treatment (Day 0), the concentrations from the surface and inner plants were 15.35 and 26.92 ng/g fresh weight, respectively, but on Day 7, only 0.04 ng/g fresh weight on the surfaces and 0 ng/g in the inner portion of the twigs were detected (Fig. 6). There were significant differences in concentrations between Day 0 and Days 1, 3, 5, and 7 (for surface: F[4, 10]=384.204, P<0.001; for the inner portions: F[4, 10]=74.360, P<0.001) and between Day 1 and Days 3, 5, and 7 (for surface: F[3, 8]=84.396, P<0.001; for the inner portions: F[3, 8]=40.084, P<0.001), and the same was true between the concentrations of TeA on the surfaces and in the inner portions on Day 0 (F[1, 4]= 15.672, P=0.017).

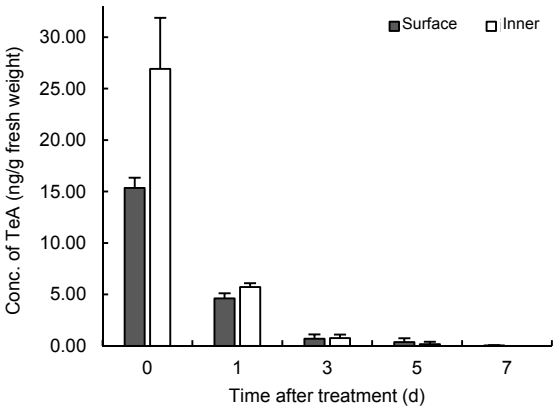

Fig. 6 Concentration of TeA in rose plants treated with TeA for 3 d

After the treatment, TeA was separately extracted with methanol from the surface and the inner portion of the plants for HPLC analyses. The values are expressed as mean (SD), and three replicates were performed for each treatment

4 Discussion

Our data shows that the multiplication of the rose aphid, *M. rosivorum*, is intensively inhibited by the toxin extracted from the culture filtrates of *A. alternata* when the toxin is applied to the rose plant, *R. chinensis*. Obviously, it can be deduced that the rose aphids will also be affected by this fungus if the rose plants are directly infected by *A. alternata* because the toxin produced by *A. alternata* will exist in the plants. It has also been reported previously that many leaf-chewing or sap-sucking insects can be detrimentally affected by different fungi on their shared host plants (Xiao *et al*., 2002; Rostás *et al*., 2003; Yang *et al*., 2013). For example, the adults of the Australian weevil, *Oxyops vitiosa*, consumed lower proportions of leaf tissues of *Melaleuca quinquenervia* infected by the Neotropical rust fungus, *Puccinia psidii*, and the females preferentially oviposited on the rust-free leaves (Rayamajhi *et al*., 2006). This interaction is a typical type of plant-mediated interaction between an insect and a fungus in the tripartite system consisting of fungus, insect, and their shared host plant. However, within such a system, it is difficult to ascertain whether the detrimental effects of

the fungus infection on insects are induced or not. Some mycelia or toxins of fungi can still remain in the plants used in the bioassays, resulting in the effects on insects. It is also difficult to investigate the toxicity of the toxins in the plants on insects.

Therefore, *Alternaria* toxin was extracted from the culture filtrates and used for the bioassays in the present study. To exclude the possibility that the toxin itself caused the above inhibitory effect, the toxicities of the toxin to excised and live rose plants and to rose aphids were tested. The results showed that this toxin was nontoxic to the plants and the insects at low concentrations (≤100.0 μg/ml) and possessed the intensive inhibitory effect on the multiplication of rose aphids at lower concentrations (≤50.0 μg/ml). Thus, the *Alternaria* toxin possessed the potential to be developed for biocontrol of rose aphids. According to these results (Figs. 1–4), such a plant-mediated inhibitory effect should be induced by the *Alternaria* toxin and might be an indirect plant-mediated interaction between these two natural enemies of rose plants under natural conditions. The intensity of toxicity is intensively related to species and concentration of toxins (Berestetskiy, 2008), and the same is true for the level of plant resistance to insects.

Although more experiments might be needed to ascertain that the toxin is non-harmful to rose aphids, we think that, based on the above results, the rose plants have obtained the *Alternaria* toxin-induced resistance to rose aphids in our study, involving a series of plant defense responses. The toxin of a fungus can induce plant defense responses of resistance-related metabolites, including different peptides, proteins, oligosaccharides, lipids, benzoquinones, and terpenoids. However, such compounds are commonly believed to enable plants to resist phytopathogen attacks (Montesano *et al*., 2003). Some nonphytopathogenic *Alternaria* toxins have also been developed to control other phytopathogens (Chen *et al*., 2005). Although the application of the *Alternaria* toxin to host plants has had detrimental effects on aphids, this toxin itself is non-harmful to rose aphids, not having acute or contact toxicity or insecticidal activity (Fig. 3). Therefore, the plant's changes induced by the *Alternaria* toxins may be responsible for the intensive inhibitory effect on the aphids. This should be SAR and/or ISR (Kloepper *et al*., 1992; Rostás *et al*., 2003).

Furthermore, the toxins used on the rose plants, instead of infection by fungus itself, may make plants obtain the fungus-induced resistance to rose aphids, especially under the controlled conditions in the present study. Therefore, a proactive defense barrier in plants for controlling insect pests can be built to a certain extent, instead of desinsection after infestation (Zhang, 2003). Thus, the results suggest that toxins possess a potential application for the management of aphids. Such a biologically based strategy may be a new idea for insect control. However, it is still needed to elucidate the active chemicals in *Alternaria* toxins.

According to the results of HPLC analyses in our study, there is TeA, a typical fungal toxin, in the partially purified crude toxin produced by *A. alternata*, and its concentration in the solid crude toxin reached about 0.12% (Table 1). It was reported previously that TeA was obtained from the culture filtrates of *Alternaria* spp. For instance, TeA was isolated from the culture filtrates of *Alternaria tenuis* Auct. (Rosett *et al*., 1957).

In the meantime, the toxicity of pure TeA to rose plants and rose aphids was tested with the same methods used for the crude *Alternaria* toxin. The results showed that TeA at a low concentration was non-harmful to rose plants or rose aphids. Further bioassay also demonstrated that TeA possessed an intensive inhibitory effect on rose aphids when it was applied to the plants. Thus, TeA can also induce defense responses in rose plants, similar to those induced by the crude *Alternaria* toxin, and can strongly enhance the resistance of plants to aphids. The inhibitory index of TeA against rose aphids reached 83.60% at 0.060 μg/ml of TeA, while inhibitory index of the crude *Alternaria* toxin reached 88.0% when the concentration of TeA in the crude toxin solution was also about 0.060 μg/ml. Only a slight difference was found between these two values of inhibitory index. Therefore, TeA is one of the most important active components in the crude toxin produced by the fungus *A. alternata*. When the crude toxin was applied to the rose plants, the plant resistance against rose aphids was mainly induced by TeA in the crude toxin. It is also possible that there are other active chemical components produced by *A. alternata* in the crude toxin, which requires further study.

Nevertheless, TeA still has a great potential to be used to control rose aphids in the production of cut

rose flowers. The previously reported results showed that TeA exhibited a calculated 50% lethal dose of 548 μg per egg in the egg embryos of chickens (Griffin and Chu, 1983). TeA is also toxic to several other animal species, e.g. mice, dogs, and monkeys (Davis *et al*., 1977; Ostry, 2008). In the present study, TeA was used on the plants only at a very low concentration (0.060 μg/ml), and was not used on any animals. More importantly, the results in our study show that TeA can be degradable within a short time (about 7 d; Fig. 6). Under natural conditions, the half-life period of TeA in the field experiment is only about 3.22 d (Zhou and Qiang, 2007). Thus, TeA can be safer than common chemical pesticides.

Furthermore, the results from the bioassay for the plant resistance showed that inhibitory index of TeA against rose aphids still had significantly high values 15 d after the treatment with TeA (Fig. 5), suggesting not only that the resistance-related response of rose plants can be induced by TeA, but also that the inhibitory effect of TeA against rose aphids does not result from TeA itself (the same as the effect of the crude toxin). TeA or crude toxin of *A. alternata* can induce system resistance in rose plants, and the ISR can continue to protect the plants for a long time after TeA or the crude toxin is completely degraded. Further studies in the future are needed to elucidate that the changes in resistance-related genes in rose plants can be induced by the treatment with TeA or the crude toxin on rose plants, to demonstrate the ISR with molecular technology.

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Compliance with ethics guidelines

Fa-zhong YANG, Bin YANG, Bei-bei LI, and Chun XIAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：链格孢菌毒素能诱导中国月季产生抗蚜活性的细交链孢菌酮酸

目　的：研究链格孢菌毒素能诱导中国月季植株产生对月季长管蚜的抗性，从而证实寄主植物介导的病虫互作关系的存在，并研究其互作机制。

创新点：证实了一种对寄主植物和害虫均无毒性的真菌毒素能使寄主植物产生对昆虫的诱导抗性。

方　法：马铃薯葡萄糖琼脂（PDA）培养基培养链格孢菌获得毒素粗品，大孔树脂纯化后配制成不同浓度的溶液，喷施到中国月季植株上。处理结束后接种月季长管蚜，与对照相比，计算毒素处理对蚜虫的抑制百分数。用高效液相色谱法（HPLC）结合标准品分析毒素中是否存在细交链孢菌酮酸（TeA），并测定其含量。TeA 同法处理中国月季植株，测定 TeA 对蚜虫的抑制率，并与毒素粗品比较。再通过 HPLC 法测定植物体表和体内残留的 TeA，以证明 TeA 能自然降解完全。

结　论：（1）链格孢菌粗毒素和对照品 TeA 均能使中国月季植株产生对月季长管蚜的系统诱导抗性，显著降低月季长管蚜对中国月季的危害；（2）链格孢菌粗毒素中的主要抗蚜活性成分是 TeA，TeA 有望成为中国月季上具有抗蚜活性的先导化合物；（3）粗毒素和对照品 TeA 对蚜虫和植物均无伤害作用，但能激活植物对虫害的诱导抗性（ISR）和系统获得性抗性（SAR），可直接证实二者间存在着寄主植物介导的间接的病虫互作关系。

关键词：链格孢菌毒素；细交链孢菌酮酸；病虫互作关系；高效液相色谱法（HPLC）；诱导抗性