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Defensive Mutualism of Endophytic Fungi: Effects of Sphaeropsidin A against a Model Lepidopteran Pest †

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Abstract: Sphaeropsidin A (SphA) is a pimarane diterpene produced by several fungi associated with plants. Following previous evidence of insecticidal properties of SphA, we investigated its contact and oral toxicity against the model chewing lepidopteran *Spodoptera littoralis*. The compound showed no lethal effect when directly sprayed on larvae, while it produced an evident oral toxic effect, associated with sublethal effects. These results demonstrated that SphA might play a defensive role against lepidopteran insects in plants harboring the producing fungus, depending on the extent at which the endophytic strains are able to perform biosynthesis of this and eventually other bioactive metabolites in vivo.

Keywords: endophytic fungi; secondary metabolites; oral toxicity; lepidopteran pests

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1. Introduction

Microbiome associated to plants is more and more regarded as a basic factor regulating their fitness, with reference to the effects of the mutual interactions among and between the holobiont constituents [1–3]. In most instances, symbiotic relationships between fungi and plants are considered with reference to the opposite categories of 'antagonists' and 'mutualists'; nevertheless, in the absence of indications enabling their circumstantial ascription to one or the other, endophytic fungi are often considered as neutral [4]. Studies on host genotype versus symbiotic lifestyle expression revealed that individual isolates of some fungal species could span the symbiotic continuum by expressing either mutualistic or pathogenic lifestyles in different host plants [5,6]. In recent years, the increasing evidence that many fungal pathogens are able to spread endophytically in unrelated plant species has introduced the perspective that they can actually shift between these categories depending on a series of ecological factors [7].

Indeed, recent papers have reported how fungi colonizing plants can either directly or indirectly interfere with arthropod development [8], particularly, in the case of fungi producing bioactive secondary metabolites, this adaptation could be related to the toxic or phagodeterrent effects on pests possibly induced by these products [9,10].

The secondary metabolites, are not essential for the primary metabolic processes but modulate the microorganism interactions with the surrounding environment [11], underline survival functions by modulating competition, parasitism or symbiosis [12]. These natural compounds also exhibit several biological activities, which may offer potential applications in medicine [13,14] and in agriculture as natural biopesticides [15,16].

Mainly described as a secondary metabolite of *Diplodia* species (Dothideomycetes, Botryosphaeriaceae) [17,18], sphaeropsidin A (SphA, Figure 1) is a pimarane diterpene which was previously reported with the number LL-S491β as a product of a strain of *Aspergillus chevalieri* [19]. However, it is produced also by other fungi which are associated as endophytes with plants [20–26]. This compound has displayed larvicidal and phagodeterrent effects against the yellow fever mosquito (*Aedes aegypti*) (Diptera: Culicidae) [27]. Here we have further explored the spectrum of activity of this compound focusing on a herbivore insect, the lepidopteran *Spodoptera littoralis.*

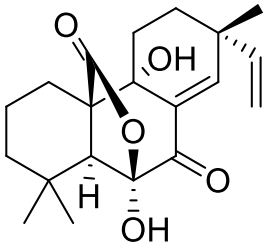

Figure 1. Structure of sphaeropsidin A (SphA).

2. Materials and Methods

2.1. Fungal Strain and Culturing

Diplodia corticola strain (B305) used in this study was previously isolated from *Quercus suber* trees showing canker and dieback symptoms in Algeria. The strain has been identified and characterised, using morphological characters and phylogenetic analysis of molecular data [28]. The nucleotide sequences of B305 are available in GenBank database, under accession numbers MT015626 and MT066136. Liquid cultures of the strain were prepared in Czapek-Dox broth (Oxoid) amended with 2% corn meal in 500 mL Erlenmayer flasks containing 250 mL of the substrate [29], and grown on stationary phase in the dark at 25 °C for 30 days.

2.2. Isolation of SphA from Crude Extract

The culture broth and mycelia were homogenised in a mixer with 350 mL of MeOH (1% NaCl). Subsequently, the suspension was centrifuged for 40 min at 7000 rpm and 10 °C. The pellet was resuspended in 150 mL of a mixture H_2O:MeOH (9:11 *v*/*v*, 1% NaCl) and submitted to a second homogenization followed by centrifugation. Supernatants were collected and MeOH was evaporated under reducted pressure obtaining an aqueous solution for the subsequent extraction (3 times) with ethyl acetate at native pH (=6.0). The organic phases were combined, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure yielding crude extract as brown oil (156.7 mg). The organic extract was purified by column chromatography (CC) on silica gel (40 cm × 1.5 cm i.d.) eluted with $CHCl_3$/*i*-PrOH (19:1, *v*/*v*), originating 8 homogeneous fractions (A: 3.7 mg, B: 6.7 mg, C: 40.3 mg, D: 15.2 mg, E: 9.1 mg, F: 15.9 mg, G: 2.3 mg, H: 32.4 mg), the last of which was collected by eluting with methanol. Fraction C was purified by TLC on silica gel eluted

with *n*-hexane/EtOAc (6:4, *v*/*v*) to obtain SphA (35.4 mg, white crystalline solid, R_f 0.70, in the same chromatographic conditions).

2.3. General Experimental Procedures

Optical rotation of SphA measured in MeOH on a Jasco polarimeter (Tokyo, Japan). ^{1}H NMR spectrum was recorded at 400 MHz in deuterated chloroform ($CDCl_3$) on Bruker (Karlsruhe, Germany) spectrometer and the same solvent was used as internal standards. Thin Layer Chromatography were performed on silica gel plates (Kieselgel 60, F254, 0.25 Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation (253 nm), or by spraying first with 10% H_2SO_4 in methanol followed by heating at 110 °C for 10 min. Chromatography was performed on silica gel column (Merck, Kieselgel 60, 0.063–0.200 mm).

2.4. Bioassays on Spodoptera littoralis

Larvae of *S. littoralis* (Lepidoptera, Noctuidae) were reared on artificial diet at 25 ± 1 °C and 70 ± 5% RH, with 16:8 h light-dark period as previously described [30] and used in two different bioassays hereafter described.

2.4.1. Topical Application

Newborn larvae were allowed to grow on the artificial diet until they moulted in 2nd and in 5th instar. The 2nd instar larvae were collected and tested in 4 replicates of 25 larvae each (n = 100), while 5th instar larvae (n = 16) were singly treated as described below. The larvae were kept on sterile filter paper in Petri dishes and were directly sprayed with a water/ethanol 50% (*v*/*v*) solution containing SphA at the concentration of 0.4 μg/cm^2, using a fine perfume atomizer. Control larvae were identically treated with a water/ethanol 50% (*v*/*v*) solution (C_{EtOH}) and with water alone (C_{water}). After treatment, the experimental larvae were kept, with a piece of diet (1 cm^2), in 4-well plastic rearing trays (RT32W, Frontier Agricultural Sciences, Pitman, NJ, United States) closed by perforated plastic lids (RTCV4, Frontier Agricultural Sciences). Larval mortality was daily recorded for six days for 2nd instar larvae and until pupation for 5th instar larvae. All bioassays were carried out in duplicate, under the same rearing conditions reported above.

2.4.2. Oral Administration

Newly molted 5th instar larvae, obtained as described above, were anesthetized on ice and 2 μL of a water/ethanol 50% (*v*/*v*) solution, containing SphA at the concentration of 0.02 μg/μL, were poured into the foregut lumen of the larvae by means of a Hamilton Microliter syringe (1701RNR 10 ll, gauge 26 s, length 55 mm, needle 3). Control larvae were treated as described above. The treatment was repeated for 3 consecutive days, for a total amount of 0.12 μg/larvae of SphA. After treatment, larvae were singly isolated in the bioassay tray as described above. Larval development and larval mortality were recorded until pupation: larval weight, pupal weight and the adults fertility were also recorded. The bioassays were carried out in duplicate, under the same rearing conditions reported above.

2.5. Statistical Analysis

Differences in larval weights were analyzed by One-Way ANOVA followed by the Tukey-Kramer Honestly Significant Difference (HSD) multiple range test ($p < 0.05$). Differences in survival rate were compared by using Kaplan-Meier and long-rank analysis. Data were analyzed using GraphPad Prism version 6.01 (GraphPad software; San Diego, CA, USA).

3. Results and Discussion

SphA (Figure 1) used in this study was obtained as white crystals (35.4 mg) from culture of *D. corticola* B305. In particular, the organic extract was subjected to a chromatographic purification process as described in detail in Section 2.2. This compound was identified on the basis of spectroscopic (^{1}H NMR) and optical rotation data previously determined [13].

Topical application of SphA did not affect the survival rate of both 2nd (Log-Rank test: $p = 0.9437$) (Figure 2A) and 5th instar larve (100% survival) (Figure 2B). These latter achieved the same weigth before pupation (One Way ANOVA. $p = 0.7536$) (Figure 2C) and when they attained the pupal stage (One Way ANOVA: $p = 0.6772$) (Figure 2D).

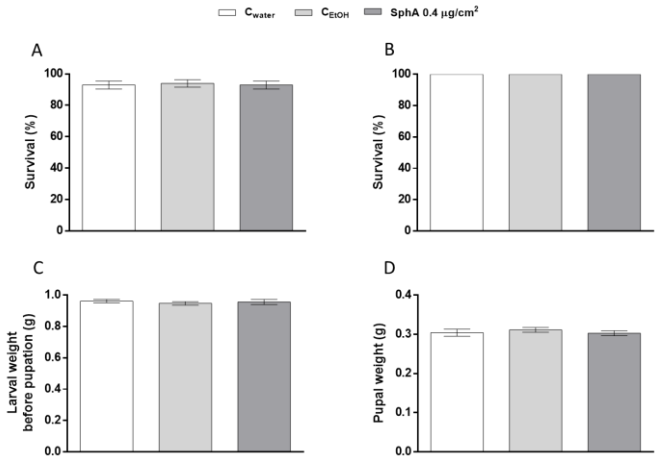

Figure 2. Effect of SphA topical application on *S. littoralis* larvae. SphA sprayed at the concentration of 0.4 µg/cm^2 on *S. littoralis* larvae did not affect the survival rate of 2nd instar (**A**) (Log-Rank test: $\chi^2 = 0.1159$, $p = 0.9437$, dF = 2) and 5th instar larvae (**B**), as well as the larval weight before pupation (**C**) (One Way ANOVA: $F_{(2, 93)} = 0.2838$, $p = 0.7536$) and the pupal weight (**D**) (One Way ANOVA: $F_{(2, 93)} = 0.3914$, $p = 0.6772$). Values are reported as means ± SE.

Experimental larvae orally treated with SphA showed a very strong reduction of the survival rate, which was significantly lower compared to controls (Log-Rank test: $p < 0.0001$) (Figure 3A). The larval mortality started from the last administration of SphA (day 3) and increased over the time until pupation (Figure 3A) with a recorded pupal survival rate of about 67% (for the controls 100%). A significant difference was recorded also for the larval weight before pupation (One Way ANOVA: $p < 0.0001$) (Figure 3B). SphA-treated larvae also showed a modified bodily appearance, and they were smaller than controls (Figure 4). Moreover, despite no alteration of the development time was observed, the pupal weight of the SphA-treated larvae resulted lower than controls (One Way ANOVA: $p < 0.0001$) (Figure 3C). All the adults obtained survived, without differences in their longevity, and no differences in their fecundity was observed (One Way ANOVA: $p = 0.8695$) (Figure 3D).

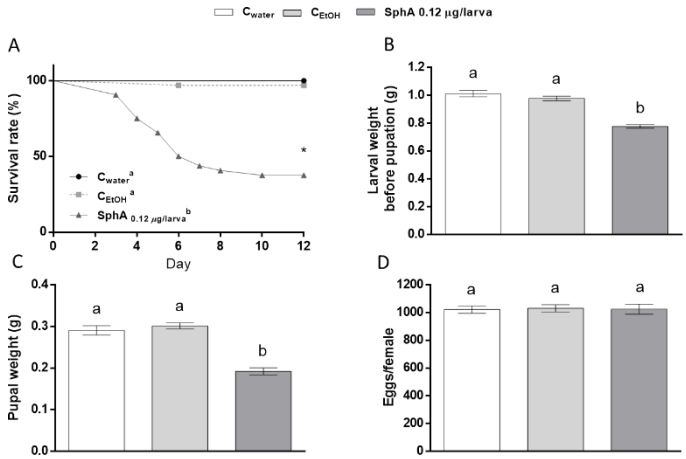

Figure 3. Effect of SphA oral administration 5th instar *S. littoralis* larvae. Orally treated larvae showed a strong reduction of the survival rate compared to controls, decreasing from day three to pupation. (**A**) (Log-Rank test: $\chi^2 = 53.66$, $p < 0.0001$, dF = 2). A significant reduction of the larval weight before pupation (**B**) (One Way ANOVA: $F_{(2, 71)} = 22.14$, $p < 0.0001$) and of the pupal weight (**C**) (One Way ANOVA: $F_{(2, 71)} = 21.41$, $p < 0.0001$) was also observed. No differences were observed in the fecundity of adults obtained from SphA-treated larvae compared with controls (**D**) (One Way ANOVA: $F_{(2, 21)} = 0.1408$, $p = 0.8695$ Asterisk indicate significant differences in the survival curves (Log-Rank test, $p < 0.0001$)). The values in the histograms are means ± SE. Different letters indicate a statistical difference (One Way ANOVA, $p < 0.0001$).

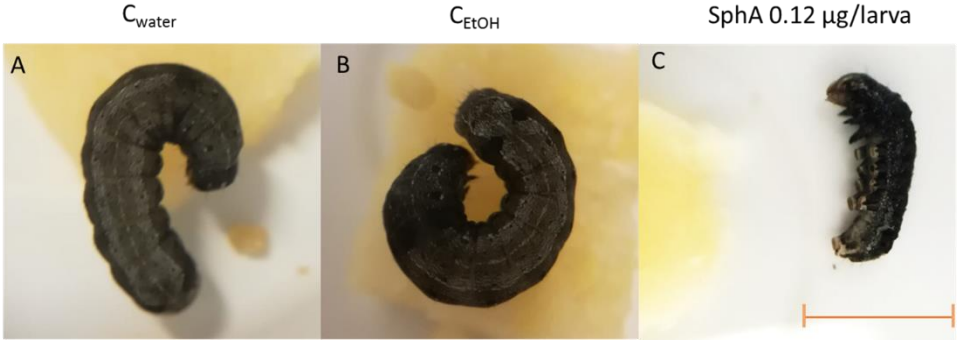

Figure 4. Alteration of *S. littoralis* larval development following oral administration of SphA. Larvae treated with SphA for three days showed a clear reduction of vitality and body size (**C**) No difference in the larval head capsule size indicates that all the larvae are in the same instar. compared to control larvae treated with water (**A**) or EtOH 50% (**B**). Scale bar, 0.5 cm.

Taken together our results indicate that although SphA has no lethal contact activity against *S. littoralis* larvae it showed clear lethal and sublethal effects after ingestion in 5th instar larvae, unequivocally indicating the oral direct toxicity of SphA. Further investigations are needed to better define the insecticidal role of SphA against chewing insects. In case its production by endophytic fungi is demonstrated in planta, SphA might be considered to play a role in the modulation of insect-plant interactions, which is worth of further research efforts aiming to elucidate its mechanism of action and functional role under in vivo conditions.

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