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RESEARCH ARTICLE

Destruxin A production by *Metarhizium brunneum* strains during transient endophytic colonisation of *Solanum tuberosum*

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ABSTRACT

Metarhizium spp. are known to produce destruxin A (dtx A) and can act as endophytes. Data regarding the fate and behaviour of secondary metabolites in the environment are necessary for registration. Endophytic colonisation and dtx A production on potato plants were monitored at 24, 48, 72, 96 and 120 h after inoculation with *Metarhizium brunneum* strains (BIPESCO5 and EAMa 01/58-Su). Both strains were recovered from leaves, stem, tuber and root fragments of fungal-challenged potato plants. Although a similar colonisation was observed for both strains, there were differences in percentages in different parts of the plants, with the higher values occurring in the leaves at 96 h for EAMa 01/58-Su (83.3%) and BIPESCO5 (81.6%), and the lower ones, 10–13.3%, observed in tubers and roots at 72, 96 and 120 h post-inoculation for both strains. For strain EAMa 01/58-Su, dtx A was quantified at 24 h (2.49 ± 1.7 and 2.0 ± 1.4 µg/kg, respectively), and the same concentration was found in both tuber and root at 96 h (2.5 ± 1.7 µg/kg); for BIPESCO5, the concentrations differed in tuber at 24 h and in root at 48 h (6.8 ± 4.8 and 2.1 ± 1.4 µg/kg, respectively). The concentration of dtx A in plant tissues was very low compared to the colonisation levels, suggesting that dtx A production by the fungus may be temporary and that the compound might degrade rapidly.

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BIPESCO5; EAMa 01/58-Su; endophytic fungi; metabolites

1. Introduction

Metarhizium spp. are well-known fungal biological control agents (FBCAs) and are extensively distributed worldwide (Zimmermann, 2007). Members of this genus are often used as an alternative means of pest control because they can potentially reduce the harm caused by the use of chemical pesticides in agriculture. Although progress has been made in the development of FBCAs, few products have reached the market (Strasser, Hutwimmer, & Burgstaller, 2011). One reason for this is that the registration period is very long (over 10 years), which causes many problems for market access and discourages companies from investing in the development of new microbial products (Strauch, Hermann,

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Hauschild, & Ehlers, 2011). *Metarhizium* spp. are also known to produce secondary metabolites. These compounds present an obstacle for registration and commercialisation because they need to undergo risk assessment prior to registration (Strasser et al., 2011, thereby increasing production costs.

Destruxin A (dtx A), one of the major secondary metabolites produced by the genus *Metarhizium*, is important for insecticide activity (Hu, Ren, An, & Qian, 2007) and cytotoxicity (Vey, Matha, & Dumas, 2002) and plays critical roles in pathogenesis (Hu, An, Jin, Freed, & Ren, 2009; Pedras, Zaharia, & Ward, 2002) and in damaging and suppressing innate insect immunity (Pal, St. Leger, & Wu, 2007). Due to its pathogenic and cytotoxic properties, dtx A is of great interest within the population because it could enter the food supply and present a risk to humans. Thus, data regarding the fate and behaviour of secondary metabolites in the environment could alleviate fear among the population about the safety of FBCAs (Strasser, Vey, & Butt, 2000).

Several studies have evaluated the production of dtx A in different culture media (Wang, Skrobek, & Butt, 2004) but more information is required regarding detection and quantification in real samples, such as insects and plants. Recently, destruxin production for *Metarhizium* strains BIPESCO5, EAMa 01/58-Su, ARSEF 23 and ART 2825 was determined with an improved method of ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), which has shown high precision in the detection and quantification of dtxs in four culture media (CM, MM, CN2 and OSM) representing different stress conditions. Fifteen dtxs were detected, with dtx A and B being the most abundant, even if significant differences among strains in dtxs production were detected, and for each strain, dtxs' production was highly dependent on the culture medium (Ríos-Moreno et al., 2016).

Members of the genus *Metarhizium* can act as endophytes by interacting with plant roots and promoting the growth and colonisation of the rhizosphere (St. Leger, 2008). *Metarhizium* spp. was shown to be capable of colonising the roots of cabbage (Hu & St. Leger, 2002), and it significantly increased the root length and weight of corn plants (García, Posadas, Perticari, & Lecuona, 2011) and colonised the roots of soybean (Khan et al., 2012). It was isolated from the roots in crops of oat, cabbage, beans and switchgrass (Sasan & Bidochka, 2012; Steinwender et al., 2015) as well as rhizosphere, strawberry, blueberry, grape and Christmas tree (Behie, Jones, & Bidochka, 2015).

Various entomopathogenic fungi, including *Metarhizium brunneum*, were isolated from the leaf surface in different systems of Mediterranean crops (Garrido-Jurado, Fernandez-Bravo, Campos, & Quesada-Moraga, 2015). Batta (2013) showed that *M. anisopliae* strain 150 can be successfully re-isolated from the leaves, petioles and stems at 2, 3 and 4 weeks after inoculation of *Brassica napus* plants. Members of the genus *Metarhizium* have also been reported in tomato, wheat, beans, corn and sweet sorghum (Liao, O'Brien, Fang, & St. Leger, 2014; Montzoukas, Chondrogiannis, & Grammatikopoulos, 2015). More recently, the use of GFP-transformed *M. brunneum* EAMa 01/58-Su strain together with histological studies have allowed detecting transient endophytic colonisation of melon plants by *M. brunneum* after foliar application and its contribution to the control of *Bemisia tabaci*. Interestingly, mortality with fungal outgrowth was not detected in dead nymphs fed on *M. brunneum* EAMa 01/58-Su colonised melon leaves, whereas dtx A was present in 43% of the cadavers (Garrido-Jurado et al., 2016). Indeed, it has been demonstrated that transient endophytic colonisations of plants improve the

outcome of foliar applications of *M. brunneum* EAMa 01/58-Su strain against *Spodoptera littoralis* larvae, with spray application of entomopathogenic fungi on alfalfa, tomato and melon plants causing additional larval mortality due to a temporal colonisation of the leaves and subsequent ingestion of those leaves by the larvae. Indeed, fungal outgrowth was not detected in any of the dead larvae feeding on *M. brunneum* colonised leaves, with traces of dtx A were detected in 11% of the cadavers (Resquín-Romero, Garrido-Jurado, Delso, Ríos-Moreno, & Quesada-Moraga, 2016). These studies highlight a major question in understanding the association between entomopathogenic fungi and plants is whether they produce metabolites in plants (Vega et al., 2009), but there are few studies on the presence of destruxins (dtxs) in plants.

Dtxs A, B and E were detected in cowpea plants inoculated with *M. robertsii* (Golo et al., 2014), and dtx A was quantified in melon and tomato plants sprayed with *M. brunneum* (Garrido-Jurado et al., 2016; Resquín-Romero et al., 2016). Alternatively, an extraction procedure based on QuEChERS (quick, easy, cheap, effective, rugged and safe) enabled the quantification of dtxs in strawberry and corn (Taibon, Sturm, Seger, Strasser, & Stuppner, 2015), and Carpio et al. (2016) proposed a reliable method based on a modified version of QuEChERS to detect dtxs in potato plants.

In this study, we monitored the endophytic colonisation in different parts of potato plants by strains of *M. brunneum* and assessed the dtx A production on different days in potato plants cultivated under laboratory conditions.

2. Materials and methods

2.1. Chemical and reagents

The malt extract agar (MEA) and Sabouroud dextrose agar (SDA) CAF 500 culture media were supplied by Biolife (Milan, Italy). Tween 80 (Panreac, Barcelona, Spain) was used to prepare fungal suspensions. Sodium hypochlorite (NaOCl) was supplied by Scharlau (Barcelona, Spain). The reagents for dtx A extraction, sodium chloride (NaCl), magnesium sulphate ($MgSO_4$) and formic acid (FA) were supplied by Panreac (Barcelona, Spain). Methanol (MeOH), tri-sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) were obtained from Merck (Darmstadt, Germany), and disodium hydrogen citrate sesquihydrate ($C_6H_6Na_2O_7 \cdot 1.5H_2O$) was obtained from Acros (NJ, USA). Acetonitrile (MeCN) was supplied by Fisher scientific (Loughborough, UK). Dtx A was obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Instrumentation

All water was purified in a Millipore Elix 10 (Bedford, MA, USA), and samples and reagents were weighed in a precision balance explorer OHAUS (Barcelona, Spain) and analytical balance Gram st-2205 (Barcelona, Spain). Potato plants were sprayed with an aerograph 27095 (China) and lyophilised with a LyoQuest-Ht 40 (Telstar technologies, Spain).

A vortex mixer (VELP scientific, New York, USA), an Eppendorf concentrator plus (Fisher Scientific, Madrid, Spain), ultrasonicator (P Selecta, Barcelona, Spain) and centrifuge 6–16 K (Sigma, Germany) were used in a dtx A procedure. Dtx A determination was

carried out using an Agilent Technologies 1200-HPLC tandem mass spectrometry Q Trap AB Sciex 5500 (AB SCIEX, Darmstadt, Germany) with electrospray ionization (ESI). A Phenomenex C18 (150 mm Kinetex × 2.10 mm, 2.7 µm) column was used for the separation, and the data were collected using the Analyst® Software version 1.6.2 with MS/MS in MRM mode (AB SCIEX).

2.3. Fungal strains

Two *M. brunneum* strains were evaluated: (1) EAMa 01/58-Su (formally *M. anisopliae*) from the culture collection at the Department of Agricultural and Forestry Sciences and Resources of the University of Cordoba (Spain) originally isolated from the soil of a wheat crop at Hinojosa del Duque (Cordoba, Spain) in 2001 deposited with accession number CECT 20764 in the Spanish collection of culture types (CECT) located at the University of Valencia (Spain) and (2) BIPESCO5 from the BIPESCO Team Innsbruck culture collection originally isolated in Austria from *Cydia pomonella* L. (Lepidoptera: Tortricidae), in 1967. The fungi were sub-cultured on Petri plates of MEA for 15 days to obtain conidia. Conidia were harvested with a sterile spatula, suspended in sterile water containing 0.1% (w/v) Tween 80 and passed through a piece of cheesecloth to remove mycelium mats. The conidial concentration was quantified in a Malassez chamber and adjusted at 10^8 conidia mL^{-1}, followed by sonication for 5 min to homogenise the conidial suspensions.

2.4. Plant material

Pieces of potato tuber with one sprout that weighed between 6 and 8 g were sterilised by immersion in 70% ethanol for 2 min, followed by 2% NaOCl for 5 min and by rinsing in sterile deionised water three times. Disinfected tubers were planted in pots (70 × 70 × 60 mm) with approximately 300 g of soil substrate (Floragard, Oldenburg, Germany) which had been sterilised by autoclaving three times every 24 h at 121°C for 45 min. The pots were kept at 22°C, 16:8 photoperiod and were monitored regularly and watered.

2.5. Fungal inoculation of potato plant

Thirty days after planting, the potato plants were inoculated by spraying with 10^8 conidia mL^{-1} fungal suspension (2 mL per plant). During spraying, the pots were covered with aluminium foil to avoid run-off. Control plants were sprayed with 0.1% (w/v) Tween 80 in sterile water. Five plants were removed from the pots every 24 h over 120 h and washed with water. Plants were divided into leaves, stem, tuber and root, which were disinfected by submerging in 70% ethanol for 2 min, 5 min in 5% NaOCl, followed by two rinses in sterile water for 2 min. The samples were dried on filter paper in airflow chambers, frozen at −80°C and lyophilised until sample treatment. The final water rinse was placed in selective culture medium SDA CAF 500 to determine the effectiveness of the disinfection. The plates were kept in an incubator at 25°C for 12 days, and no fungal growth was recorded in any of the rinsed water samples.

To evaluate colonisation, 10 fragments were randomly taken, leaves and root (about 1 cm^2), stem and roots (about 10 mm in length) from each group of plant inoculated

and control were placed in selective medium SDA. The plates were sealed with parafilm and kept in an incubator at 25°C for 12 days. Colonisation was evaluated by counting the number of pieces that showed fungal outgrowth. For each strain, there were three replicates, five plants each and the full experiment was repeated twice using new inoculum and a new batch of plants.

2.6. Dtx A extraction

The extraction of dtx A (at 24, 48, 72, 96 and 120 h after treatment) from potato was carried out following the QuEChERS-based sample treatment proposed by Carpio et al. (2016). A portion of plant parts (0.1 g of leaves, stem, tuber or root) and 8 mL of distilled water were each placed in 50 mL screw cap test tubes with conical bottom and shaken by vortex for 10 s. Then, 5 mL of 5% FA in MeCN was added and shaken by vortex for 2 min. A mixture of four salts (4 g $MgSO_4$, 1 g NaCl, 1 g $C_6H_5Na_3O_7{\cdot}2H_2O$ and 0.5 g $C_6H_6Na_2O_7{\cdot}1.5H_2O$) was added, and the mixture was shaken by hand for 1 min and by vortex for 2 min and subsequently centrifuged at 6000 rpm for 5 min. Next, 2 mL of the upper MeCN layer was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2-µm filter and analysed using HPLC-MS following the conditions reported by Carpio et al. (2016) with some modifications.

HPLC separations were performed in a C18 column (Phenomenex, 150 mm Kinetex × 2.10 mm, 2.7 µm) using a mobile phase consisting of 0.01% aqueous FA solution (solvent A), and MeOH (solvent B) at a flow of 0.25 mL/min. The eluent gradient profile was as follows: 0 min, 5% B; 15 min, 65% B and 90% B 15.50 min. The eluent was returned to 5% B after 0.5 min and maintained for 2 min to allow column equilibration. The column temperature was set at 35°C, and the injection volume was 10 µL. The MS/MS was working with ESI in positive mode.

The analytical method, previously described by Carpio et al. (2016), showed precision values below 8.5%; and in all cases, recoveries higher than 91%. Calibration was done with external standard. The calibration curve was obtained using five concentrations of dtx A between 0.1 and 10 µg/kg. The calibration curve was $y = 135{,}061x + 2765$ ($R^2 = 0.9997$), and the limit of instrumental quantification was of 0.1 µg/kg, corresponding to 1 µg/kg sample, considering dilution 1/10.

3. Results

Both strains were recovered from leaves, stem, tuber and root fragments of the fungal-challenged potato plants. Although similar colonisation was observed for both strains, there were differences in the colonisation percentages and times in different parts of the plants (Figure 1). Colonisation data were analysed by ANOVA, and showed significant differences in the stem at 72 h ($P = .0219$). Colonisation in leaves was slightly higher than that in stems and the values obtained were significantly higher than the percentage colonisation in tubers and roots. Colonisations in the leaves by EAMa 01/58-Su and BIPESCO5 at 24 h were 78.3 and 76.6%, respectively, and the maximum colonisation occurred at 96 h for EAMa 01/58-Su (83.3%) and BIPESCO5 (81.6%). There was a tendency for a decrease in colonisation at the end of the trial.

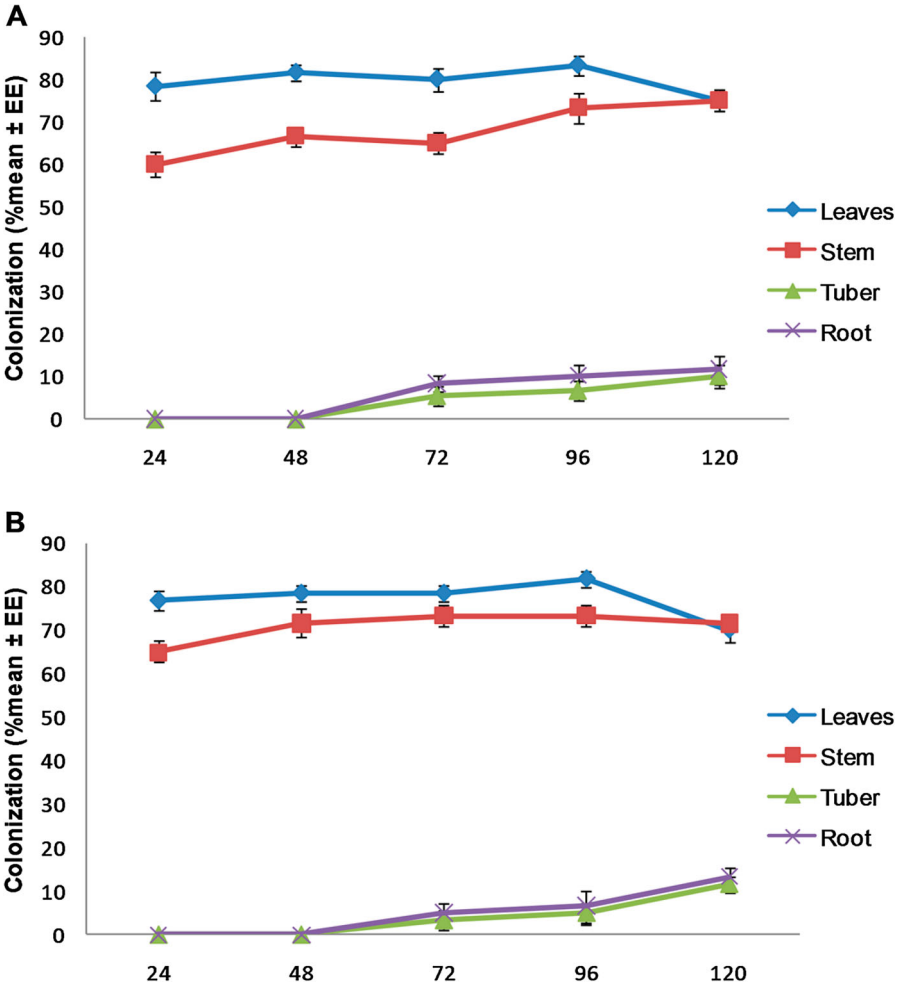

Figure 1. Colonisation percentage (after 24, 48, 72, 96, and 120 h of inoculation) in different parts of potato plants sprayed with a suspension of 10^8 conidia mL^{-1} *M. brunneum* strains: (A) EAMa 01/58-Su and (B) BIPESCO5.

At 24 h, the stems had 60% colonisation by the EAMa 01/58-Su strain, and this number increased to 75% at 120 h. For BIPESCO5, the colonisation of the stems at 24 h was 65%, and the maximum value was 73.3% at 72 and 96 h. At 72 h, colonisation was observed in the tubers and roots for both strains, although the increase in colonisation percentage was not very high. For the EAMa 01/58-Su strain, the colonisation was 10 and 11.6% at 120 h in tubers and roots, respectively, and for BIPESCO5, it was 11.6% in tubers and 13.3% in roots, reflecting slightly more colonisation of the root compared to the tuber (Figure 1).

Table 1 shows the results of dtx A accumulation at a given time in different parts of the potato plant that were sprayed with strains *M. brunneum*. Twenty-four hours after inoculation with the EAMa 01/58-Su strain, dtx A was quantified in the root and tuber at 2.49 ± 1.7 and 2.0 ± 1.4 µg/kg, respectively, and the same concentration was found in the tuber and root (2.5 ± 1.7 µg/kg) after 96 h. Dtx A was only quantified in the tuber at 24 h and

Table 1. Production of destruxin A by strain of *M. brunneum* in potato plants sprayed with a suspension of 10^8 conidia mL^{-1}.

	Dtx A (μg/kg = ppb ± SE)							
	BIPESCO5				EMAa 01/58-Su			
Time	Leaves	Stem	Tuber	Root	Leaves	Stem	Tuber	Root
24 h	n.d.	n.d.	n.d.	6.8 (±4.8)	n.d.	n.d.	2.0 (±1.4)	2.49 (±1.7)
48 h	n.d.	n.d.	2.1 (±1.4)	n.d.	n.d.	n.d.	n.d.	n.d.
72 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
96 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.5 (±1.7)	2.5 (±1.7)
120 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Note: n.d., not detected.

the root at 48 h (6.8 ± 4.8 and 2.1 ± 1.4 μg/kg, respectively) when the BIPESCO5 strain was used.

4. Discussion

Strains of *M. brunneum* were successfully established as endophytes in potato plants that had been inoculated by spraying. The removal of plant surface microbes is particularly important in endophyte studies and the experimental protocol used in the present work, based on the chemical treatment developed by Arnold (2007), has been shown to guarantee the complete removal of possible *Metarhizium* propagules from the sampled potato plants, controls and fungal-challenged ones. Besides, this chemical treatment together with the plating of the rinsed cleaning water have been successfully used in several previous endophytic entomopathogenic fungi works (Garrido-Jurado et al., 2016; Landa et al., 2013; Tefera & Vidal, 2009; Vega et al., 2010).

Colonisation remained fairly constant in leaves and stems over the duration of the experiment at approximately 80 and 70%, respectively, in both strains. Similar results were obtained by Resquín-Romero et al. (2016) in alfalfa, tomato and melon plants with *M. brunneum*, where the colonisation percentages in leaves and stems were also quite constant but somewhat lower, remaining between 40% and 70%, respectively. Garrido-Jurado et al. (2016) sprayed melon plants with two strains of *M. brunneum*, one of which was EAMa 01/58-Su, which showed a percentage of colonisation between 40% and 50%; however, the other strain, EAMb 09/01-Su, showed a percentage of colonisation that was similar to that observed by Resquín-Romero et al. (2016) in melon plants. García et al. (2011) evaluated the endophytic colonisation of three *M. anisopliae* strains on tomato plants by applying a conidia suspension directly to the substrate. Of the three strains isolated, only one colonised the leaves 21 days post-inoculation. Akutse, Maniania, Fiaboe, Van Den Berg, and Ekesi (2013) soaked seeds of *Vicia faba* and *Phaseolus vulgaris* with ICIPE30 and S4ST7 strains of *M. anisopliae* and failed to colonise plants. The different findings of these studies suggest that colonisation varies depending on the plant species and strain and possibly on the inoculation method used.

The colonisation of roots and tubers was observed at 72 h after spraying, which is consistent with the data obtained by Garrido-Jurado et al. (2016) and Resquín-Romero et al. (2016), showing that the fungus may move within the plant. Endophytes can live in the intercellular spaces of stems, leaves and roots (Schulz, Boyle, Draeger, Rommert, & Krohn, 2002). There are reports on the ability of fungi to move within the plant. In

opium poppy plants, intercellular spaces may be the easiest means of movement for *Beauveria bassiana* (Landa et al., 2013), and this fungus was shown to grow through the air spaces between parenchymal cells and sometimes within xylem vessels, leading to whole plant colonisation (Quesada-Moraga, Landa, Muñoz-Ledesma, Jiménez-Díaz, & Santiago-Álvarez, 2006; Wagner & Lewis, 2000).

There is a need to determine the fate of secondary metabolites produced by entomopathogenic fungi in plants, but there are few published reports on the detection of dtx compounds in plants. In our study, dtx A was not detected in leaves or stems. Previous studies reported the quantification of dtx A in leaves and stems. Carpio et al. (2016) quantified dtx A in one of four samples of stems and leaves using the same strain. Garrido-Jurado et al. (2016) quantified dtx A at 72 h after inoculation (10.4 ± 0.4 μg/kg) in melon leaves sprayed with the EAMb 09/01-Su strain of *M. Brunneum*. Resquín-Romero et al. (2016) also quantified dtx A in leaves of tomato and melon (0.007 μg/kg and 0.011 μg/kg, respectively) using the EAMb 09/01-Su strain.

Interestingly, dtx A produced by the EAMa 01/58-Su and BIPESCO5 strains was only found in plant tubers and roots. Collemare et al. (2014) extracted metabolites from apoplastic fluids of tomato leaves colonised by *Cladosporium fulvum* and found that dtx A could move via apoplastic fluids because it was observed on parts of the plant that had not yet been colonised. To the best of our knowledge, this is the first time that dtx A, produced by spraying the BIPESCO5 on a plant, has been quantified. Taibon et al. (2015) enriched samples of maize and strawberry with an internal standard of dtxs isolated from BIPESCO5 and then used a QuEChERS-based extraction to demonstrate the accuracy, with values ranging from 83.5% to 105.3%. Carpio et al. (2016) showed recoveries higher than 91%. Golo et al. (2014) monitored the production of dtxs by immersing cowpea and cucumber seeds in a suspension of *M. Robertsii* (ARSEF 2575) or *M. acridum* (ARSEF 324) at 10^6 conidia mL^{-1} and quantified only dtxs A, B and E (1.56 ± 0.29 μg dtx A/g dry weight) in a mixture of stems, roots and leaves of cowpea plants 12 days after inoculation by ARSEF 2575.

Dtx A production by strains BIPESCO5 and EAMa 01/58-Su in potato plants was very low; one possibility is that dtx A production by the fungus is temporary and that the metabolite degrades rapidly. According to Schulz and Boyle (2005), the concentration of secondary metabolites in plant tissues is very low relative to colonisation. Other authors demonstrated that entomopathogenic fungi do not secrete metabolites on plant material in sufficient quantities to pose a health risk (Strasser et al., 2000) and concentrations are low and they cannot be easily detected in the crop or the environment in amounts sufficient to monitor their presence or fate (Strauch et al., 2011 and citation within).

Secondary metabolites contribute positively to their host (Schulz et al., 2002), and there is evidence that the plant uses the endophyte as a second line of defence and a signalling mechanism to enable the endophyte to generate the same or similar defence compounds (Kusari, Pandey, & Spiteller, 2013; Schulz & Boyle, 2005). Insects can be links between endophytic fungi-producing plant bioactive compounds and the host plants (Kusari et al., 2013). This suggests that the production of dtx A in plants may increase if they are attacked by an insect pathogen, but considering the work of Garrido-Jurado et al. (2016), in which a melon plant was exposed to a pest (*B. tabaci*), and of Resquín-Romero et al. (2016), in which tomato and melon plants were attacked by *S. littoralis*,

we detected a similar amount of dtx A. However, endophytes are capable of synthesising the bioactive compounds that are used by plants for defence (Nair & Padmavathy, 2014). Secondary metabolites isolated from *Trichoderma* spp. are directly involved in the activation of plant defence (Vinale et al., 2008), but the extent to which endophytes produce natural products within the plant or the role of secondary metabolites in helping the plant is not well understood (Kusari, Singh, & Jayabaskaran, 2014). Moreover, fungal secondary metabolite production may vary with the biotope in which it grows and to which it is adapted (Schulz et al., 2002), and it is likely that fungi are able to regulate the energetically costly process of secondary metabolite production according to the environmental conditions and specific needs (Tenguria, Khan, & Quereshi, 2011). For this reason, as part of a risk assessment, it would be interesting to determine whether fungi reduce the concentration of dtxs under natural conditions and whether this concentration increases when the plant is attacked by a pest.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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