*Metarhizium robertsii* produces indole-3-acetic acid, which promotes root growth in *Arabidopsis* and enhances virulence to insects

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**Abstract**

The plant root colonizing insect-pathogenic fungus *Metarhizium robertsii* has been shown to boost plant growth, but little is known about the responsible mechanisms. Here we show that *M. robertsii* promotes lateral root growth and root hair development of *Arabidopsis* seedlings in part through an auxin [indole-3-acetic acid (IAA)]-dependent mechanism. *M. robertsii* or its auxin-containing culture filtrate promoted root proliferation, activated IAA-regulated gene expression and rescued the root hair defect of the IAA-deficient *rh6* *Arabidopsis* mutant. Substrate feeding assays suggest that *M. robertsii* possesses tryptamine (TAM) and indole-3-acetamide tryptophan (Trp)-dependent auxin biosynthetic pathways. Deletion of *Mrtdc* impaired *M. robertsii* IAA production by blocking conversion of Trp to TAM but the reduction was not sufficient to affect plant growth enhancement. We also show that *M. robertsii* secretes IAA on insect cuticle. Del*Mrtdc* produced fewer infection structures and was less virulent to insects than the wild-type, whereas *M. robertsii* spores harvested from culture media containing IAA were more virulent. Furthermore, exogenous application of IAA increased appressorial formation and virulence. Together, these results suggest that auxins play an important role in the ability of *M. robertsii* to promote plant growth, and the endogenous pathways for IAA production may also be involved in regulating entomopathogenicity. Auxins were also produced by other *Metarhizium* species and the endophytic insect pathogen *Beauveria bassiana* suggesting that interplay between plant- and fungal-derived auxins has important implications for plant–microbe–insect interactions.

**INTRODUCTION**

Numerous plant-associated microbes inhabit soil and root ecosystems and are capable of exerting beneficial, neutral or detrimental effects on their host plant [1]. Symbiotic microbes frequently enhance root proliferation [2, 3]. This rhizogenesis is regulated by a plant hormone, auxin indole-3-acetic acid (IAA), which is best known for its role in plant cell elongation, division and differentiation [4, 5]. Rhizobacteria and a few fungi produce IAA, which promotes plant root development and yield [6–10]. The rice blast fungus *Magnaporthe oryzae* produces IAA in its early stages of infection [11]. Reciprocally, fungi recognize plant hosts with chemical signals including plant IAA and these may induce the morphogenetic changes that enable invasion [12, 13].

Several IAA biosynthetic pathways have been proposed [14–16]. The tryptamine (TAM) pathway is initiated by the decarboxylation of tryptophan (Trp) to TAM and proceeds via indole-3-acetaldehyde (IAAld) to IAA through the activities of amine-oxidase or IAAld dehydrogenase in *Bacillus cereus* and *Azospirillum*, respectively [17, 18]. The rate-limiting step for the TAM pathway in plants is probably catalysed by a flavin monooxygenase-like protein (YUCCA) mediating the conversion of TAM to N-hydroxyl-tryptamine [19]. Precursors, enzymes and intermediates for these pathways have been found in *Colletotrichum*, *Ustilago* and *Saccharomyces* species [20–23], but the molecular and genetic basis of IAA biosynthesis in fungi is unclear. Double mutation of IAAld dehydrogenase *Δiad1Δiad2* blocked conversion of TAM to IAA in *Ustilago maydis* [24], suggesting...
that IAAld is the mutual terminal intermediate of indole-3-pyruvic acid (IPA) and TAM pathways in this fungus.

The ubiquitous insect-pathogenic fungus *Metarhizium robertii* is a biocontrol agent used worldwide to control various agricultural pests [25, 26]. As a common inhabitant of soils, *M. robertii* establishes mutualistic interactions with plants as plant root colonizers [27, 28]. *M. robertii* is capable of boosting plant growth by killing insects [29], increasing nutrient absorption by roots [30] and providing protection against plant pathogens [31]. In addition, *Metarhizium* stimulates tap-root growth and root hair development in switchgrass (*Panicum virgatum*) [32]. However, the mechanistic bases of these interactions are just beginning to be defined [25, 28, 33, 34].

In this study, we reveal that *M. robertii* produces IAA through Trp-dependent pathways. The fungal IAA elicited *Arabidopsis* growth promotion, root ramification and root hair development in a similar manner to plant IAA. More surprisingly, we found that *M. robertii* also produces IAA on insect cuticle. We identified an auxin biosynthesis-related gene, *Mrtdc*, homologous to the Madagascar rosy periwinkle (*Catharanthus roseus*) tryptophan decarboxylase, which functions in conversion of Trp to TAM. Deletion of *Mrtdc* impaired *M. robertii* IAA production but not sufficiently to affect plant growth enhancement. However, Δ*Mrtdc* produced fewer infection structures and was less virulent to insects than the wild-type, whereas spores harvested from cultures containing IAA or exogenous application of plant IAA increased appressorial formation and virulence. We propose a novel role for auxin signalling in mediating *Metarhizium* growth and entomopathogenicity.

**METHODS**

**Living materials and culture conditions**

*M. robertii* ARSEF 2575, *Metarhizium robertsii* ARSEF 23, *Metarhizium acridum* ARSEF 324, *Metarhizium anisopliae* ARSEF 939, *Metarhizium brunneum* ARSEF 820 and *Beauveria bassiana* ARSEF 252 wild-type strains [United States Department of Agriculture/Agricultural Research Service (USDA/ARS) collection] were grown and maintained on potato dextrose agar (PDA; Fluka) at 27 °C. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* C58C1 were used for DNA cloning and fungal transformation. *Arabidopsis thaliana* eco-type Col-0 seeds were purchased from LEHLE SEEDS. *Arabidopsis thaliana* transgenic line BA::GUS and a mutant line *rhd6* were obtained from *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, USA). The BA::GUS transgenic line, consisting of auxin-responsive domains of PS-IAA4/5 promoter and GUS reporter, was used for β-glucuronidase (GUS) expression analysis [35]. The auxin-deficient *rhd6* mutation in *Arabidopsis* [36] was used to test the impact of *Metarhizium* on root hair formation. Seeds were surface-disinfected according to Sauer and Burroughs [37], and germinated and grown on agar plates containing 0.2% or 1% MS medium (Murashige and Skoog Basal Salts Mixture; Sigma-Aldrich) with or without addition of (+/−) 1% sucrose. Plates were placed vertically in a plant growth chamber with a 14:10 h light:dark cycle, light intensity of 300 μmol m⁻² s⁻¹, and temperature of 22 °C.

**Substrates feeding assay and auxin determination**

For the production of auxins, strains were grown in Sabouraud dextrose broth (SDB; Difco) for 30 h, and 0.25 g fresh mycelia (about 30 mg dry weight) was transferred to 100 ml of 0.2% MS broth supplemented with 0.001% Trp (Trp-culture) or M100 medium containing 1% *Manduca sexta* cuticle. The fungal cultures collected at 2, 4 and 8 h were filtered through a 0.22 µm filter for subsequent inoculation experiments and auxin determination. For substrate feeding assays, 0.25 g fresh mycelia was grown for 4 h at 27 °C in 100 ml 0.2% MS medium supplemented with 100 μM of each of the following indolic intermediate: tryptamine (TAM), indole-3-acetaldehyde (IAAld), tryptophol (TOL), indole-3-acetamide (IAM) and indole-3-lactate (ILA). Fungal inoculum grown in 0.2% MS medium was tested in parallel as control (Trp-free culture).

For IAA determination, the fungal culture was filtered and the supernatant was concentrated by lyophilization. Extraction of IAA by methanol and subsequent quantification by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS) were performed according to the methods of Sirrenberg et al. [38]. To estimate the amount of auxin compound produced by *M. robertsi*, a calibration curve was constructed using IAA standard (Sigma-Aldrich).

**Inoculation experiments**

Fungal filtrates were added into 0.2% MS agar medium (10%, v/v) for plant growth. *M. robertsi* 2575 spore suspensions (1×10⁶ spores ml⁻¹) were inoculated 5 cm from *Arabidopsis* seeds on agar plates or suspended in the 0.2% MS agar medium to produce a spore density of 1×10⁴ spores ml⁻¹. *Arabidopsis* seeds with *M. robertsi* filtrate or fungal inoculum were maintained in a growth chamber for 14 days. *Arabidopsis* seedling growth was determined by measuring the whole seedling fresh weight, primary root length and the number of lateral roots. To measure the root hair development, the wild-type *Arabidopsis* and *rhd6* mutant were grown on 0.2% MS agar medium containing 0.5% (v/v) *M. robertsi* 2575 extracts or 0.1 μM IAA standard for 10 days. An equivalent quantity of methanol was added to medium in the controls. Root hairs of *Arabidopsis* seedlings were photographed under a dissection microscope. The number and length of root hairs were measured using the ImageJ program [National Institutes of Health (NIH)].

**Gene disruption, quantitative PCR analysis and enzymatic activity assay**

An *Mrtdc* disruption vector pPK2BargfpDMrtdc was constructed to knock out the *M. robertsi* *Mrtdc* gene. The 5′-end and 3′-end of *Mrtdc*, cloned by PCR, were inserted into a modified master Ti vector pBarGFP using the *XbaI* and *BglII/EcoRV* sites, respectively. The disruption mutant
(ΔMrtdc) was obtained by Agrobacterium tumefaciens-mediated transformation [39, 40].

To quantify gene expression in response to Trp, wild-type M. robertsii and ΔMrtdc were grown in SDB for 30 h, and mycelia were transferred to 0.2 × MS medium containing 0.001 % Trp. To observe the time course of Mrtdc expression, RNA was harvested from mycelia for qPCR analysis as described previously [41].

Tryptophan decarboxylase (TDC) was assayed by immunquantification of tryptamine (TAM). TAM was extracted from fungal cells according to the method of Sangwan et al. [42]. The fungal TAM extract was subjected to immunometric assay using a tryptamine ELISA kit according to the manufacturer’s instructions (Antibodies-online).

**Immunolocalization of auxin**

To immunolocalize auxin in M. robertsii, the spores of gfp-tagged M. robertsii 2575 were germinated at 27 °C on: (1) a glass coverslip supplemented with 0.2 × MS medium plus 0.001 % Trp for 12 h, (2) onion epidermis for 20 h, and (3) locust wing for 20 h. The samples were immunofluorescent stained for auxin using IAA monoclonal antibody (produced in mouse; Sigma-Aldrich) detected by CY-3-conjugated secondary antibody (goat anti-mouse IgG antibody, Cy3 conjugate; EMD Millipore) as described by Tanaka et al. [11]. Controls lacked primary or secondary antibody.

**GUS assays**

For GUS histochemical analysis, seedlings treated with M. robertsii Trp-culture filtrate or 0.1 µM IAA standard for a period of 0–2 h were transferred to GUS-staining buffer [100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM K$_2$Fe(CN)$_6$, 0.5 mM K$_3$Fe(CN)$_6$, and 0.1 % (v/v) Triton X-100] containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc; Sigma-Aldrich) and incubated at 37 °C for up to 10 h. The stained seedlings were cleared in 80 % ethanol, p-Chlorophenoxyisobutyric acid (20 µM PCIB; MP Biomedicals) was added to the fungal filtrate or IAA standard solution before incubation with seedlings. PCIB blocks auxin receptors [43].

**Fungal growth and virulence bioassay**

Spores were inoculated in M100 medium, basal salt medium (0.1 % KH$_2$PO$_4$, 0.025 % Na$_2$SO$_4$, 0.05 % KCl, 0.0125 %, MgSO$_4$$\cdot$7H$_2$O, 0.00625 % CaCl$_2$, and 0.3 % NaNO$_3$) at a concentration of 1 × 10$^7$ spores ml$^{-1}$ or on locust wings (100 µl containing 1 × 10$^6$ spores ml$^{-1}$) concomitantly with IAA or 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich) (1–1000 µM for IAA and 1–100 µM for 2,4-D). Spores harvested from 14-day-old PDA plates containing IAA (0, 1, 10 or 100 µM) were used for virulence bioassays by topical application of spore suspensions onto G. mellonella larvae (Petco) or by injection of spores into Manduca sexta larvae haemocoele (Carolina Biological) as previously described [41]. For all experiments, the overall data were statistically analysed in the SPSS 19 program with Tukey’s honest significant difference test and Student’s t-test.

**RESULTS**

**M. robertsii promotes lateral root growth and root hair development in Arabidopsis**

Arabidopsis (ecotype Col-0) seeds and M. robertsii spores were placed 5 cm apart on 0.2 × MS agar medium containing 1 % sucrose. Fourteen days post-inoculation, Arabidopsis seedlings grown with M. robertsii showed a substantial increase in root proliferation and shoot growth (Fig. 1a, b). Supernatants of M. robertsii cultures containing tryptophan (Trp) reproduced the growth promoting effect of mycelium (Fig. 1c, d). Thus, the biomass of Arabidopsis seedlings and lateral root number were increased 1.6-fold (P<0.05) and 3.4-fold (P<0.01), respectively, in the presence of M. robertsii Trp-culture filtrate compared to controls without culture filtrate (Fig. 1e, g). M. robertsii Trp-free culture filtrates had a negligible effect on Arabidopsis seedling growth, suggesting a derivative of Trp is responsible for plant growth enhancement.

In additional experiments, to mimic the rhizospheric environment, Arabidopsis seeds were planted on 0.2 × MS agar medium containing a suspension of 1 × 10$^7$ M. robertsii spores ml$^{-1}$. M. robertsii increased the number of root hairs 1.25-fold (P<0.01) compared to agar medium containing no spores (Fig. 2a–c), suggesting M. robertsii could promote plant root growth in the rhizosphere by increasing root hair formation. However, M. robertsii inhibited growth of seedlings if the spore density in the medium was >1 × 10$^5$ spores ml$^{-1}$. We speculate that this is due to nutritional and spatial competition as M. robertsii at higher spore concentrations rapidly grew over the surface of the agar.

Auxin (IAA) is one of the major triggers regulating lateral root and root hair formation [44, 45]. We next tested if auxins in M. robertsii culture filtrates could reproduce the effect of plant IAA on root hair development. Auxin-like compounds extracted from M. robertsii Trp-culture filtrate (using methanol) boosted root hair formation by Arabidopsis seedlings significantly compared to the control (methanol solvent) (Fig. 2d–f, P<0.01). Furthermore, M. robertsii extracts and 0.1 µM IAA standard rescued the root hair defect of the auxin-deficient Arabidopsis rhd6 mutant (Fig. 2d–f, P<0.01). These results suggest that auxin production by M. robertsii is involved in promoting lateral root growth and root hair development.

**M. robertsii secretion induces auxin-inducible gene expression in Arabidopsis**

The BA-::GUS transgenic line, which has been used to study auxin-regulated gene expression in Arabidopsis [35], was grown on 1 × MS agar medium for 10 days. Seedlings were treated with 0.1 µM IAA or M. robertsii Trp-culture filtrate for a period of 0–2 h and stained with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-
Fig. 1. Effects of *M. robertsi* inoculation and culture filtrates on the growth of *Arabidopsis* seedlings. (a) *Arabidopsis* wild-type Col-0 was grown for 14 days on 0.2× MS agar medium containing 1% sucrose. (b) *M. robertsi* spores were simultaneously inoculated 5 cm from the *Arabidopsis* seeds. Scale bar, 2.5 cm. (c) Control *Arabidopsis* wild-type Col-0 was grown on 0.2× MS agar medium containing no fungal filtrate or *M. robertsi* Trp-culture filtrate. (d) The results are representative of 24 seedlings. Scale bar, 1 cm. The biomass of seedlings and root growth were measured after 14 days. (e) Seedling biomass. (f) Primary root length. (g) Number of lateral roots per seedling. Means are calculated from three replicates with eight seedlings each, and error bars represent SD. Experiments were repeated three times. An asterisk indicates means statistically different at the 0.01(**) or 0.05 (*) level.
Both authentic IAA and *M. robertsii* filtrate increased auxin-induced GUS expression at the root tip. *p*-Chlorophenoxyisobutyric acid (PCIB) inhibits auxin action in plants [43]. Concomitant application of PCIB (20 µM) with IAA or fungal filtrate inhibited the GUS expression of *BA::GUS* seedlings. These observations suggest that *M. robertsii* induces auxin-regulated gene expression in plants.
Identification and immunolocalization of IAA in *M. robertsi*

We used high performance liquid chromatography-tandem mass spectrometry (HPLC-MS) to identify and quantify auxin compounds from *M. robertsi*. An HPLC retention time of 16.658 min was observed for authentic IAA (Fig. S1a, available in the online Supplementary Material). The full scan spectrum corresponding to this retention time revealed the predominant ion for IAA is m/z 176 (Fig. S1b–d). The HPLC-MS analysis confirmed the presence of IAA in *M. robertsi* secretion. *M. robertsi* produced IAA starting from 2 h in the presence of Trp and by 8 h the estimated IAA concentration was 0.5–1 µM (Table 1), but no IAA product was detected in Trp-free cultures, suggesting that IAA biosynthesis in *M. robertsi* is Trp-dependent. This finding is in accordance with previous reports describing Trp as the primary precursor for IAA biosynthesis in plants and auxin-producing microbes [15, 46].

Table 1. Quantification of IAA from *M. robertsi* by HPLC-MS

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IAA production (µM)</th>
<th>0.2× MS</th>
<th>+Trp</th>
<th>+ Cuticle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>ΔMrtdc</td>
<td>wt</td>
<td>ΔMrtdc</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>21.2±0.7</td>
<td>17.4±0.3**</td>
<td>12.5±1.4</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>25.4±1.9</td>
<td>22.6±2.1*</td>
<td>17.3±2.1</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>63.9±5.6</td>
<td>55.3±8.1</td>
<td>36.5±3.1</td>
</tr>
</tbody>
</table>

*M. robertsi* has a bifunctional lifestyle as an endophytic insect pathogen, and insect cuticles contain high levels of aromatic amino acids. We found that *M. robertsi* produced IAA during growth on *Manduca sexta* cuticles (Table 1). We also tested if other entomopathogenic fungi species could produce IAA. The IAA product was detected in culture filtrates of *M. anisopliae* 939, *M. brunneum* 820 and *B. bassiana* strain 252 (Fig. S2).

IAA immunolocalization was used to locate *M. robertsi*-derived IAA. Spores of gfp-tagged *M. robertsi* were germinated on a coverslip supplemented with 0.2× MS medium ±0.001 % Trp, on onion epidermal layers (a representative plant surface) and on locust wings. Auxins were not detected on ungerminated spores, but germings on glass, plant epidermis and insect cuticle produced an IAA immune signal in the mucilaginous layer surrounding hyphae (Fig. 4), indicating secretion. No red signal was detected in negative controls (lacking primary or secondary antibody) or in 0.2× MS medium lacking Trp.

Characterization of the IAA biosynthesis pathways in *M. robertsi*

Substrate feeding assays were performed to identify the IAA biosynthetic pathways in *M. robertsi*. We
monitored substrate utilization and identified IAA products resulting from growth on tryptophan (Trp), and various indolic intermediates of different pathways including tryptamine (TAM), indole-3-acetaldehyde (IAAld), tryptophol (TOL), indole-3-acetamide (IAM) and indole-3-lactate (ILA). IAA production utilizing Trp, TAM or IAM suggests that *M. robertsii* possesses two IAA biosynthetic pathways, namely TAM and IAM (Table S1 and Fig. 5).

To identify genes involved in IAA synthesis, we BLAST searched the *M. robertsii* genome using well-characterized IAA biosynthetic pathway genes from bacteria and plants as queries (Table S2). *In silico* analysis predicted a number of homologous IAA biosynthesis-related genes in the *M. robertsii* genome. One hypothetical gene, *Mrtdc*, homologous to the tryptophan decarboxylase (TDC) in the TAM pathway, was identified. MrTDC (accession no. EFZ04091) showed an identity of 38 % (*7e-113*) with the *C. roseus* TDC (accession no. CAA47898). The ORF of *Mrtdc* is 1727 bp long, coding for a putative 499 amino acid protein with a predicted molecular weight of 54.7 kDa, and is interrupted by one 227 bp intron.

**Fig. 5.** The IAA biosynthesis-related gene (*Mrtdc*) identified in this study. The schematic diagram of IAA biosynthetic pathways in *M. robertsii* is adapted from the analogous pathways identified in bacteria [15]. Two possible pathways, TAM and IAM, were identified in *M. robertsii* by substrate feeding assays (in boldface). Other putative pathways established in bacteria or plants, and genes corresponding to the enzyme reactions in intermediate steps that have not been demonstrated in *M. robertsii*, are shown in grey.

Growth of *M. robertsii* in Trp-free medium resulted in low-level constitutive expression of *Mrtdc*. A time course for *Mrtdc* expression in Trp-cultures showed an increase commencing at 30 min, with a peak at 4 h that then declined (Fig. 6a). To investigate the involvement of *Mrtdc* in *M. robertsii* IAA biosynthesis, the *Mrtdc* disruption mutant (∆*Mrtdc*) was produced by gene replacement.

The deletion of *Mrtdc* in the mutant was confirmed by PCR using the genomic DNA as template (Fig. S3). Reverse transcriptase PCR (RT-PCR) confirmed there was no *Mrtdc* expression in ∆*Mrtdc* (data not shown). The *in vivo* TDC enzymatic activity was determined by measuring the accumulation of TAM in extracts of wild-type *M. robertsii* and ∆*Mrtdc* (Fig. 6b). During 8 h growth with Trp, TAM accumulation was greatly reduced in ∆*Mrtdc* relative to wild-type, indicating that MrTDC is functional in conversion of Trp to TAM in *M. robertsii*. The low TAM accumulation in ∆*Mrtdc* implies *M. robertsii* has additional less efficient mechanisms for TAM synthesis from Trp derivates.

Relative to the wild-type, IAA production by ∆*Mrtdc* with Trp was decreased by 18 % (*P<0.01*) after 2 h and by 11 % (*P<0.05*) after 4 h. The reduction in IAA did not occur when ∆*Mrtdc* was supplied with exogenous TAM (Table S1, *P<0.05*), bypassing the need for tryptophan decarboxylase, but did occur when Trp was replaced with insect cuticle. Compared to the wild-type, ∆*Mrtdc* produced 26 % (*P<0.05*) and 20 % (*P<0.05*) less IAA after 2 h and 4 h, respectively, on cuticle. However, by 8 h growth on either Trp or insect cuticle, the wild-type and ∆*Mrtdc* produced similar levels of IAA (Table 1). These data suggest that *Mrtdc* is involved in the biosynthesis of IAA by converting Trp to TAM, and the alternative IAM biosynthetic pathway partially complements the TAM pathway deficiency in ∆*Mrtdc*.
Exogenous IAA enhances *M. robertsii* growth and virulence

IAA does not increase germination of *M. robertsii* spores in a basal salt medium (Table S3), indicating IAA is not a readily utilized nutrient source. IAA at 10 μM or 100 μM significantly increased germination in M100 medium (Table S3, *P*<0.05), whereas 1 μM and 1 mM IAA had no effect, unlike other fungi where growth is inhibited by 500 μM IAA [12]. We determined if exogenous IAA applied with spores to locust cuticle affected differentiation of virulence structures (appressoria). Twelve hours post-inoculation, germination was increased by 22% (1 μM IAA), 25% (10 μM) and 28% (100 μM), and appressorial formation was increased by 29% (1 μM IAA), 38% (10 μM) or 41% (100 μM) (Table 2, *P*<0.05). Appressorial formation terminates hyphal growth, and concomitant with early formation of infection structures, hyphal growth was reduced by IAA. The maximal 49% reduction observed in hyphal growth (*P*<0.05) occurred with 100 μM IAA 16 h post-inoculation. The synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) is a herbicide causing uncontrolled, unsustainable growth in broadleaf plants. Appressorial formation was increased in the presence of 1 μM 2,4-D, but was inhibited by concentrations >10 μM (Table S4, *P*<0.05), showing that as with plants 2,4-D is more potent than IAA. A relatively high concentration of IAA (100 μM) was required to increase spore germination and appressorial formation by the locust specialist *M. acridum* (Table S5, *P*<0.05), which may be related to this strain being a poor root colonizer.

Since exogenous IAA results in early formation of infection structures, we investigated its impact on virulence to caterpillars. Concomitant topical application of IAA (1–1000 μM) with *M. robertsii* spores did not change virulence against *G. mellonella* (Fig. S4). However, suspending *M. robertsii* spores in 1 μM IAA decreased time to kill when injected into the haemocoel of *Manduca sexta* larvae (Fig. S5). Assuming the fifth instar larvae had a blood volume of ~1.5 ml we estimated the concentration of IAA following injection to be 0.03 μM. IAA caused the caterpillars to darken, presumably by activation of the prophenoloxidase system, and delayed pupation time by 20–24 h confirming a physiological impact on the caterpillars.

We also grew *M. robertsii* on PDA medium supplemented with IAA (1–100 μM) to determine if this would change pathogenic properties. Spores from these plates produced 15% (1 μM), 38% (10 μM) and 33% (100 μM) more appressoria on locust wings than spores from un-supplemented plates (Table 2, *P*<0.05). Median lethal time (LT50) assays showed that spores from PDA + 10 μM IAA reduced time to death by 16% compared to the IAA-free treatment (Fig. 7, *P*<0.01). Virulence was also enhanced by 100 μM IAA, but no significant effect was found with 1 μM IAA.

**Mrtdc** disruption impairs *M. robertsii* virulence but not its ability to promote plant growth

The *Mrtdc* deletion mutant Δ*Mrtdc* was as effective as wild type in promoting *Arabidopsis* seedling growth (Fig. S6). We also tested if reduced IAA production affected *M. robertsii*’s virulence determinants. Δ*Mrtdc* and the wild-type were incubated for 24 h on locust wings ± 10 μM exogenous IAA. In the absence of IAA, Δ*Mrtdc* spore germination was reduced by ~11% relative to the wild-type 12 to 24 h post-inoculation, and appressorial formation was reduced by 20% (10 h), 34% (12 h) and 16% (24 h) (Table 3, *P*<0.05). With the addition of IAA, Δ*Mrtdc* and the wild-type produced equal numbers of appressoria over 24 h growth on locust wing (Table 3), demonstrating that exogenous IAA is sufficient to rescue the defective phenotype of Δ*Mrtdc*. Δ*Mrtdc* took 13% longer to kill *G. mellonella* than the wild-type. Growing Δ*Mrtdc* on PDA supplemented with IAA restored Δ*Mrtdc* to the wild-type level of virulence (Fig. 7, *P*<0.05). However, Δ*Mrtdc* spores injected into *Manduca sexta* larvae haemocoel were as virulent as the wild-type,
Table 2. Effects of IAA on *M. robertsii* growth on locust wing

1×10⁶ spores ml⁻¹ *M. robertsii* 2575 suspensions in 1 µM, 10 µM and 100 µM IAA were inoculated onto locust wings and incubated for 24 h. Spore germination, appressorial formation and hyphal growth were measured at 10, 12 and 24 h, respectively. The mean±SD values were calculated from five replicates. An asterisk indicates means statistically different at the 0.05 level.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>1 µM</th>
<th>10 µM</th>
<th>100 µM</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Germination (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>23.7±2.8ab</td>
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<td>24</td>
<td>92.3±1.9a</td>
<td>97.4±2.8a</td>
<td>95.7±0.9a</td>
<td>96.7±0.9a</td>
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<tr>
<td>Appressorial formation (%)</td>
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<tr>
<td>10</td>
<td>2.7±0.3b</td>
<td>4.4±0.4b</td>
<td>4.3±0.3ab</td>
<td>5.3±0.7a</td>
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<tr>
<td>12</td>
<td>17.3±1.2b</td>
<td>22.4±1.1a</td>
<td>23.8±1.5a</td>
<td>24.4±1.3a</td>
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<tr>
<td>16</td>
<td>19.3±1.8b</td>
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<tr>
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<td>31.3±1.1b</td>
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<td>37.7±0.4a</td>
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<td>47.1±0.8a</td>
<td>48.3±0.9a</td>
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<td>Hyphal growth (µm)</td>
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<tr>
<td>12</td>
<td>22.6±1.5a</td>
<td>20.1±0.5a</td>
<td>19.0±1.3a</td>
<td>17.6±1.8a</td>
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<td>60.7±4.5a</td>
<td>33.9±4.1b</td>
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<td>54.8±4.9b</td>
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<td>24</td>
<td>117.0±7.8a</td>
<td>82.6±5.1ab</td>
<td>82.2±6.8b</td>
<td>66.3±4.5b</td>
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</table>

suggesting the defective phenotype only affects events on the cuticle. These studies suggest that deletion of Mrtdc impairs *M. robertsii* virulence, presumably due to reduced IAA production causing ΔMrtdc to produce fewer appressoria on the insect cuticle.

**DISCUSSION**

Microbial IAA is known to have a dose-dependent effect on root growth, with concentrations ≥100 µM being repressive [47]. Thus, the impact of *M. robertsii* on plants in natural conditions may depend on the amount of auxins being produced. The natural occurrence of *Metarhizium* spp. is at most 10⁶ propagules g⁻¹ in turf soils [48], and these levels are most likely to be reached in the vicinity of roots [27]. We found that 10⁴ spores ml⁻¹ in agar was stimulating while higher concentrations of spores reduced plant growth.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>10 µM IAA</th>
<th>wt (%)</th>
<th>ΔMrtdc (%)</th>
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<td>Germination</td>
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<td>34.6±0.8</td>
<td>32.6±2.0</td>
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<tr>
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<td>–</td>
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<td>65.7±1.0</td>
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<tr>
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<td>87.2±2.7</td>
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<tr>
<td>Appressorial formation</td>
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<td>9.1±0.8</td>
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<td>13.7±1.2*</td>
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<td>+</td>
<td>25.0±1.7</td>
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<td>+</td>
<td>42.7±0.7</td>
<td>38.7±2.4</td>
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</tr>
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</table>

**Table 3. Effect of IAA on the growth of *M. robertsii* auxin biosynthesis deficiency mutant ΔMrtdc on locust wing**

1×10⁶ spores ml⁻¹ *M. robertsii* 2575 wild-type (wt) and ΔMrtdc suspensions ±10 µM IAA were inoculated onto locust wings and incubated for 24 h. Spore germination and appressorial formation were measured at 10, 12 and 24 h, respectively. The mean±SD values were calculated from five replicates. An asterisk indicates means statistically different at the 0.05 level.
but this may have been due to the rapid fungal growth in the artificial agar medium. In studies to date, Metarhizium has been found to have a positive overall impact on plant growth in the field [28, 29]. Co-inoculating plates with seeds and Metarhizium spores provides a system for disentangling the complexity of plant–Metarhizium interactions. The plant root growth promoting properties of the fungus in this system were likely to be due to a combination of chemicals, including possibly CO₂. However, replacing mycelium with culture filtrates enabled us to confirm that IAA production by M. robertsi stimulated Arabidopsis lateral root growth.

To date, five different Trp-dependent pathways have been identified in bacteria. Our results indicate that M. robertsi possesses two Trp-dependent IAA biosynthetic pathways, namely TAM (Trp→TAM→IAAld→IAA) and IAM (Trp→IAM→IAA). A hypothetical gene homologous to the well-characterized IAM-hydrolase from Agrobacterium tumefaciens was identified in the M. robertsi genome. The gene encoding tryptophan decarboxylase has been cloned from C. roseus [35]. BLASTp and genomic BLAST searches confirmed that Mrtdc is a single copy gene in M. robertsi. MrTDC is responsible for conversion of Trp to TAM but it may not be the key enzyme in this pathway. In plants, the rate-limiting step is conversion of TAM to N-hydroxytryptamine, which has not been shown in bacteria and fungi. Most plant-associated microbial growth is inhibited by high concentrations of IAA [49], whereas M. robertsi is resistant to 1 mM IAA. Little is known about how IAA is perceived or metabolized by fungal cells and how it subsequently induces fungal growth and pathogenesis. PCIB blocks plant auxin receptors [43] but had no impact on M. robertsi’s response to IAA (data not shown), suggesting that fungal receptors are functionally different from those in plants.

Metarhizium spp. likely evolved from an endophytic ancestor, and it is postulated that some genes for insect pathogenicity may have been co-opted from genes involved in endophytic colonization [50]. Interestingly, M. robertsi produced IAA in culture on insect cuticles, and immunolocalization confirmed release of IAA by germinating hypha on locust wings. Genes for auxin biosynthesis may therefore have been involved in lifestyle transitions towards insect pathogenicity. Tryptophan is a major component of insect cuticle [51, 52], providing M. robertsi with a Trp-rich substrate to synthesize IAA. Many phytopathogenic microbes including bacteria and fungi produce IAA during infection of their plant hosts [11, 53, 54], and these may block expression of auxin-repressed plant defence genes [55]. Thus, exogenous application of auxins enhances susceptibility to some bacterial pathogens [56]. Paradoxically, the best evidence that IAA induces morphological transitions is from the non-pathogenic yeast Saccharomyces cerevisiae. IAA induces invasive growth and regulates filamentation in S. cerevisiae, which is an important virulence trait of pathogenic fungi [12]. Appressoria are essential infection structures for many plant and insect pathogens, because they enable penetration of host integuments. M. robertsi appressorial formation was enhanced by plant IAA but attenuated by endogenous IAA reduction. This suggests that plant-derived IAA could complement fungal IAA and play an important role in elaboration of invasive structure and virulence.

M. robertsi has a bifunctional lifestyle being both a rhizosphere competent root colonizer and an insect pathogen. Plants withstand attacks by herbivores in part by emitting volatiles that attract insect predators and bolster resistance to future threats [57]. Jasmonic acid (JA) and IAA are particularly important in regulating plant defensive responses [58]. IAA is likely to accumulate at the site of insect feeding [59], which could make it a useful host-related signal triggering infection-related processes in M. robertsi and other entomopathogens. This remains to be confirmed but the data presented here suggest an important role for Metarhizium-derived auxin signalling in plant–microbe-insect interactions, both symbiotic and pathogenic. Our results will yield a better understanding of M. robertsi’s biofunctional lifestyle in the natural ecological habitat.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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