Transgenically Enhanced Expression of Indole-3-Acetic Acid Confers Hypervirulence to Plant Pathogens

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ABSTRACT

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Fusarium oxysporum and *F. arthrosporioides*, pathogenic on *Orobanche aegyptiaca*, were transformed with two genes of the indole-3-acetamide (IAM) pathway leading to indole-3-acetic acid (IAA) to attempt to enhance virulence. Transgenic *F. oxysporum* lines containing both the tryptophan-2-monooxygenase (*iaaM*) and indole-3-acetamide hydrolase (*iaaH*) genes produced significantly more IAA than the wild type. IAM accumulated in culture extracts of *F. oxysporum* containing *iaaM* alone. *F. arthrosporioides* containing only *iaaM* accumulated IAM

and an unidentified indole. Some transformants of *F. oxysporum* expressing only the *iaaM* gene also produced more IAA than the wild type. Sub-threshold levels (that barely infect *Orobanche*) of transgenic *F. oxysporum* expressing both genes and of *F. arthrosporioides* expressing *iaaM* were more effective in suppressing the number and size of *Orobanche* shoots than the wild type on tomato plants grown in soil mixed with *Orobanche* seed. Stimulating an auxin imbalance enhanced pathogen virulence by affecting the host in a manner similar to low doses of auxin herbicides such as 2,4-dichlorophenoxy acetic acid.

Additional keywords: formulation, polymerase chain reaction, transformation.

The *Orobanche* spp. (broomrapes) are obligate flowering parasites attacking cultivated plants causing severe damage (23). Two fungal pathogens of *Orobanche*, *Fusarium oxysporum* and *F. arthrosporioides*, were isolated from young diseased shoots and have been studied to evaluate their mycoherbicidal potential for the control of *Orobanche* (1,2). Methods were developed to preserve the mycelia of *F. oxysporum* and *F. arthrosporioides* in the formulation Stabizeze that allowed storage for more than a year with rapid infection of *Orobanche* upon rehydration (1). Formulated *F. oxysporum* and *F. arthrosporioides* reduce the occurrence of *O. aegyptiaca* tubercles by 50 to 100% at very high inoculation levels on in vitro and greenhouse-cultivated tomato plants (1,2). Strategies are needed to improve the mycoherbicidal organisms so this potential can lead to effective, consistent, field use at lower inoculum levels. One strategy to improve the capability of the fungi to kill *Orobanche* spp. is by transgenically enhancing virulence.

The auxins (i.e., indole-3-acetic acid [IAA]) control plant growth, differentiation, structural organization, and the shift from vegetative to reproductive growth (15). Exogenous IAA can lead to cellulase-catalyzed cleavage of hemicellulose (13), resulting in wall loosening (33,36) and membrane leakage, thereby stimulating the loss of water and nutrients (4,5). Synthetic auxins such as 2,4-dichlorophenoxy acetic acid (2,4-D) affecting the same receptors are fully herbicidal (9). Many microorganisms associated with plants as symbionts or parasites directly alter the auxin content of the host; the auxin imbalance benefits the microorganisms (15,34). The apices of *Euphorbia cyparissias* infected by *Uromyces pisi* contained 10 times more auxin than the level found in healthy apices (17). Pathogen strain virulence directly correlated with the levels of IAA, suggesting that IAA has a positive function in the infection process (11). Plants preinfected with a patho-

gen caused auxin production, which then overcame the hypersensitive defense response of plants subsequently infected by an incompatible pathogen (26). Chitinase production by plant tissues as a defense against pathogens was blocked by auxin, enhancing the virulence of the pathogens (30). Bacterial pathogens causing crown and root gall diseases all produce IAA, and mutants deficient in IAA production are not tumorigenic (31). Full virulence was restored when these mutants were transformed with genes for IAA synthesis (7). Pathogenic strains of *Erwinia herbicola* use the indole-3-acetamide (IAM) pathway for the production of IAA, whereas nonpathogenic strains were devoid of this pathway (22). The IAM pathway (Fig. 1) is uncommon in plants, with some possible exceptions (18,19,25,29). Bacterial pathogens having the IAM pathway may override the regulation of IAA production by the host by using precursors from the host or synthesized by the pathogen (22).

We tested the hypothesis that increased production of IAA may confer hypervirulence to *Fusarium* on *Orobanche*. To the best of our knowledge, this is the first publication demonstrating that by transgenically enhancing IAA levels, virulence is further increased. In this case, enhancing IAA production by *Fusarium* spp. reduced the severity of *Orobanche* infestation on the host plants by increasing fungal virulence.

MATERIALS AND METHODS

Fungal strains. *F. oxysporum* Schlechtend.:Fr. and *F. arthrosporioides* Sherb. were isolated as single spore colonies from diseased, juvenile, emerging *O. aegyptiaca* Pers. flower stalks from a melon field in northern Israel (2). The fungi were deposited at the Collection Nationale de Cultures de Microorganismes at the Institut Pasteur under accession numbers I-1622 (*F. oxysporum*) and I-1621 (*F. arthrosporioides*). Both fungal strains met Koch's postulates for primary pathogens.

Fungal growth media. All of the fungal strains were maintained on potato glucose agar (PDA) or potato glucose broth (PDB) (Oxoid, Basingstoke, England).

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Protoplast isolation. Fungal protoplasts were axenically isolated. Both *F. oxysporum* and *F. arthrosporioides* were cultured on PDA containing 50 µg of chloramphenicol per ml in petri plates and incubated at 25°C under fluorescent lighting for a 12-h photoperiod. Washed spores of *F. oxysporum* and *F. arthrosporioides* from 2-week-old cultures were filtered through 74-µm mesh Nytex nylon monofilament screens (Swiss Bolting Cloth, Zurich, Switzerland) to remove mycelia, sedimented, and adjusted to a spore density of 10^6 per ml in the above medium. The spore suspensions were germinated on a shaker for 20 to 22 h until small mycelial clumps were formed. The mycelial suspensions retained on 74-µm mesh screens were transferred to digestion medium: sterile filtered 0.7 M NaCl, 10 mg of glucanase per ml, 10 mg of Fluka Driselase (ex *Basidiomycetes* spp.), and 50 µg of Sigma chitinase per ml (ex *Streptomyces griseus*). Flasks with 5 g of mycelia and 25 ml of digestion medium were incubated at 30°C for 1.5 to 2.5 h at 70 rpm. The protoplasts released were separated from mycelia and cell debris by filtration through a 74-µm mesh screen, which passed the protoplasts and retained the undigested material. The protoplasts were centrifuged at $1500 \times g$ for 5 min, and the pellet was resuspended in 10 ml of unbuffered 0.7 M NaCl and recentrifuged. The protoplast pellet was then washed in 10 ml of sterile STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, and 50 mM CaCl_2). Aliquots of protoplasts were counted in a hemacytometer cell and adjusted to a density of 10^8 protoplasts per ml of STC.

Plasmids. The tryptophan-2-monooxygenase (*iaaM*) gene (1,663 bp) was amplified by polymerase chain reaction (PCR) from the vector pCP3 (37) and cloned into the *SacI* and *KpnI* sites of pWCG2 (27). Next, a *XbaI/EcoRV* fragment containing the *Aspergillus nidulans* trpC promoter was introduced at the *SmaI/XbaI* site upstream of the *iaaM* gene. The resulting plasmid pWCG2pM (8,261 bp) contains the hygromycin B phosphotransferase resistance (*hph*) gene driven by the *Cochiobolus heterostrophus* P1 promoter and the *iaaM* gene driven by the *A. nidulans* trpC promoter.

The indole-3-acetamide hydrolase (*iaaH*) gene (1,385 bp) was amplified by PCR from the vector pCP3 and cloned into the *NcoI* and *HindIII* sites of pAN52-1 plasmid between the *gdpH* promoter

and the trpC terminator (24). The resulting plasmid was cut with *BglIII* and *HindIII* and the fragment containing the *gpdH-iaaH-trpC* cassette was ligated into the *BamHI/HindIII* sites of pC8HS. The resulting plasmid pWh52-1 contains the P1-*hph* cassette and the *iaaH* gene driven by *A. nidulans gpdH* promoter and terminated with the *A. nidulans trpC* terminator.

Transformation. The transformations of the *Fusarium* spp. were based on the method of Turgeon et al. (35) using fungal protoplasts with polyethylene glycol (PEG) and CaCl_2 to mediate the transformation. Protoplasts (10^7 in 100 µl of STC) were incubated at 4°C with 25 µg of plasmid DNA for single transformations or 12.5 µg of DNA each in the cotransformations. The tubes were gently rolled and tilted back and forth for 5 min at 4°C. Sterile PEG/ CaCl_2 medium (60%, wt/vol, PEG molecular weight 3,500 to 4,000 in 10 mM Tris-HCl, pH 7.5, and 50 mM CaCl_2) was added in three aliquots (200, 200, and 800 µl) at 5-min intervals. The protoplasts were incubated on ice for an additional 20 min and diluted with 1 ml of STC medium to reach a calculated density of 4.35×10^6 protoplasts per ml.

Preliminary dose response curves demonstrated that *F. oxysporum* and *F. arthrosporioides* wild-type fungal cultures were totally inhibited by 65 and 25 µg of hygromycin per ml of medium, respectively (6). Aliquots (200 µl) of putatively transformed protoplasts were mixed into 20 ml of molten (45°C) sterilized regeneration medium (PDB with 20% sucrose, 1.6% Difco agar, and 50 µg of chloramphenicol per ml). Three hours later, each plate was overlaid with 10 ml of (45°C) molten regeneration medium containing three times more hygromycin than totally inhibited growth of the nontransformed cultures. The sealed petri plates were incubated at 28°C, and hygromycin-resistant colonies became visible on the selection plates after 3 to 7 days.

The putative transformants were picked, subcultured onto slants with PDA with 65 or 25 µg of hygromycin per ml for *F. oxysporum* and *F. arthrosporioides*, respectively, and kept at 4°C after growth reached 7-cm in diameter. The cultures were subcultured on hygromycin-containing medium at least six times and remained stable for more than 1 year.

Fig. 1. The biosynthesis pathways leading to indole-3-acetic acid (IAA). Either or both the indole-3-acetamide (IAM) (21) and the indole-3-pyruvate acid (IPA) (32) biosynthesis pathways of IAA are found in different microorganisms. The IPA pathway is considered the major IAA synthesis pathway in plants. Source modified from Magie et al. (21).

Detection of IAA and related indoles. Methods for the culturing of the fungi and the extraction and detection of IAA from the culture medium were based on Robinson et al. (27), as adapted for *F. oxysporum* and *F. arthrosporioides*. Mycelia were cultured in PDB supplemented with 5 mM filter-sterilized L-tryptophan (in foil-covered flasks to prevent indole photodegradation) until the fungal growth reached stationary phase. The hyphae were separated from the medium by centrifugation, and the supernatant was brought to pH 3.0 with HCl and mixed with an equal volume of ethyl acetate. The medium and ethyl acetate were vortex mixed at the top speed for 45 s and placed at -20°C overnight to freeze the aqueous phase and form ice crystals around impurities at the interface. The ethyl acetate upper phase was rapidly poured through Miracloth to remove ice crystals. The ethyl acetate was evaporated in vacuo and the dried residues were resuspended in 500 µl of methanol containing 1% butylated hydroxytoluene to prevent auto-oxidation of indoles.

Thirty-microliter aliquots of each of the samples were loaded onto separate lanes on a 20 × 20 cm × 0.25 mm precoated silica gel 60 F₂₅₄ thin-layer chromatographic (TLC) glass plates (Merck, Darmstadt, Germany). Standards (2.5 µl of 10 mM IAA, IAM, or tryptophol) were loaded in separate lanes on the TLC plates. The indoles were separated by chloroform/methanol/water (84:14:1) for approximately 45 min. Indoles were detected on the air-dried plates by spraying until they appeared transparent with Ehmann's reagent (10) containing 0.5% (wt/vol) *p*-dimethylaminobenzaldehyde, 1.5 M HCl, 12.5% ethanol, 7.1 mM FeCl₃, and 7.8 M H₂SO₄. The dark blue staining of the indoles was intensified by heating at 100°C for 10 min. Hydrochloric acid (which causes yellowing) was removed by submerging the plates in water for approximately 15 min. The air-dried plates were scanned, and images were saved in Adobe Photoshop. The various indoles in the samples were compared with the *R_f* values of the indole standards.

Quantification of IAA. The indoles were viewed as quenched spots on unsprayed TLC plates in 254 nm UV light. Marked spots of separated IAA, reference standards, and silica blanks were separately scraped into tubes. One milliliter of absolute ethanol was added to each tube to dissolve IAA from the silica by vortex mixing at the medium setting for 1 min. The supernatants were removed and centrifuged twice. The absorbance of supernatants was measured at 225 nm against blanks. The absorbance of each of the samples was recorded and converted to nanograms of IAA based on a series of standards.

PCR analysis of gene introgression in chromosomal DNA. PCR was used to amplify *iaaM* and *iaaH* gene fragments in the genomic DNA of putatively transformed *F. oxysporum* and *F. arthrosporioides* strains to assay for integration into the fungal genome. Genomic DNA was extracted from mycelia according to Daboussi et al. (8). The unique primer pairs chosen from the *iaaM* and *iaaH* gene sequences were 5'-CGGGTAGGTCGCAT-TTCCAGA and 5'-CGTCGGGTGTATCGCAGCAAC (*iaaM*) and 5'-GGATGGCGAGGTGGAGA and 5'-CCCGACAGGAATAGT-AATGGA (*iaaH*). The primers were prepared by the Oligonucleotide and Peptide Sequence Unit at the Weizmann Institute. The optimal annealing temperatures for the primers were determined using the Oligo primer analysis version 5.1 computer software program: 58°C for *iaaM* and 56°C for *iaaH* genes, respectively. Both PCR programs had the double-stranded DNA denaturation at 94°C for 1 min, primer extension at 72°C for 1 min, and annealing for 1 min for 30 cycles of denaturation, annealing, and extension in a Peltier Cycler (PTC-200; MJ Research, Watertown, MA) PCR apparatus. PCR products were analyzed by gel electrophoresis and ethidium bromide staining.

TABLE 1. Production of indole-3-acetic acid (IAA) by various transformants^a

Strain	Strain no.	PCR		ng of IAA/mg of myc	
		<i>iaaM</i>	<i>iaaH</i>	–try	+try
<i>Fusarium oxysporum</i>					
Nontransformed	Wild type	–	–	3	16
<i>iaaM</i> , <i>iaaH</i>	42	+	+	6	62
<i>iaaM</i> , <i>iaaH</i>	76	+	+	4	190
<i>iaaM</i> , <i>iaaH</i>	82	+	+	15	91
<i>iaaM</i>	47	+	–	7	17
<i>iaaM</i>	62	+	–	6	33
<i>F. arthrosporioides</i>					
Nontransformed	Wild type	–	–	9	12
<i>iaaM</i>	5	+	–	9	20
<i>iaaM</i>	14	+	–	8	26
<i>iaaM</i>	20	+	–	11	24

^a Polymerase chain reaction (PCR) analysis was performed on genomic DNA of the putative transformants to confirm the presence of the *iaaM* and *iaaH* transcripts. Expected positive confirmation was marked +, negative results were marked –. Differences in IAA production between all strains of (*iaaM*+*iaaH*)*F. oxysporum* versus wild type. *F. oxysporum* were significant at *P* < 0.05 as determined by Student's paired *t* test (myc = mycelium; +try = 5 mM tryptophan added to the culture medium).

Fig. 2. Presence of polymerase chain reaction products of *iaaM* and *iaaH* in various *Fusarium oxysporum* and *F. arthrosporioides* transformants.

The gels were prepared as 1.5% agarose in Tris-acetate-EDTA (TEA) buffer, and a 1-kb DNA ladder (GeneRuler sm0331; MBI Fermentas, Hanover, MD) was used as the size markers.

Formulation. Fungi were cultured in 500 ml of PDB in Erlenmeyer flasks aerated at 150 rpm at 25°C. The fungi in late exponential phase of growth were harvested onto Miracloth in vacuo after 48 h. The retained mycelia were washed with 200 ml of glass-distilled water to remove the culture medium. The mycelia were fragmented in a blender and formulated in Stabileze according to Amsellem et al. (3).

Soil mixture. The soil consisted of unsterilized loam containing 15% peat and 6 g/kg of fertilizer 19-6-11 (Multicote 4 Extra Control Release; Agrimport Haifa Chem. Ltd., Haifa).

Plant material. Seeds of *O. aegyptiaca* (Pers.) (Egyptian broomrape) were provided by the lab of Y. Kleifeld of the Newe Yaar Research Center, Ramat Ishay, Israel. Seeds were surface sterilized in hypochlorite as previously outlined (2) and mixed into the soil (15 mg/kg of soil = $\approx$ 5,000 seeds per kg of soil).

Tomato (*Lycopersicon esculentum* Mill. cv. M82) seedlings with two to three expanded leaves in speedling insert trays were provided by Hishtil Inc., Ashkelon, Israel. The tomato plants were grown at 25°C under 70 μ Ein/m²/s fluorescent light with a 14-h photoperiod.

Fungal virulence. The virulence of the transgenic fungi versus the wild-type *F. oxysporum* and *F. arthrosporioides* was tested in two experiments. The formulated mycelia were mixed into the soil at inoculum loadings of 80, 400, and 2,000 CFU/g of soil (first experiment) and 2,000 and 3,000 CFU/g of soil (second experiment). The inoculum loadings of the wild type were previously demonstrated as being under the threshold for reproducible infection (6). The formulated mycelia were mixed into the soil and placed separately in 3-liter plastic pots. All inoculant treatments were replicated in three pots in the first experiment and five pots in the second experiment, and the pots were randomized in the greenhouse with 16-h light at 27 $\pm$ 3°C. Each pot initially contained a tomato seedling with three expanded leaves. Both the number and the size of the *Orobanchae* shoots that emerged in pots were evaluated weekly when *Orobanchae* shoots began to emerge. The experiments were terminated when *Orobanchae* began flowering, at about 10 weeks after transplanting, to prevent *Orobanchae* seed production due to biosafety considerations.

Statistical analysis. The data were analyzed using Student's paired *t* test.

RESULTS

Verification of transformation. Plasmid DNA preparations containing the *iaaM* or the *iaaH* genes were used separately to transfer single plasmid DNA or several together to cotransform into *F. oxysporum* and *F. arthrosporioides*. Each plasmid carried the (*hph*) gene as a selectable marker. The presence of the indole biosynthesis genes was confirmed by PCR amplification of gene fragments of *iaaM* and *iaaH* in the genomic DNA of various putative transformants (Fig. 2; Table 1). The presence of either *iaaM*, *iaaH* in single transformants, or both genes in the cotransformants was positively confirmed, except *iaaH* was not confirmed in strain 73, which was a putative cotransformant (Fig. 2; Table 1).

Overproduction of indoles: IAM. Transformants were cultivated in a medium with tryptophan so that the substrate for IAA production would not be limiting. Culture extracts were TLC fractionated together with indole standards. IAM was not detected in untransformed *F. oxysporum* but it accumulated in the culture extracts of many of the (*iaaM*)*F. oxysporum* transformants (Fig. 3A). IAM accumulated in both the culture medium of (*iaaM*)*F. arthrosporioides* strains and in the wild-type *F. arthrosporioides* (Fig. 3C). An unidentified indole (R_f = 0.63) was detected in culture medium of some (*iaaM*)*F. arthrosporioides* strains that was not detected in the filtrate of wild-type *F. arthrosporioides* (Fig. 3C).

Overproduction of indoles: IAA. Some of the (*iaaM*+*iaaH*)*F. oxysporum* cotransformants had greatly increased production and excretion of IAA, especially transformants 76, 82, and 86 (Fig. 3B). The *iaaH* gene product in transformants of (*iaaM*+*iaaH*)*F. oxysporum* presumably hydrolyzed the IAM to IAA. Some strains of single transformants of (*iaaM*)*F. oxysporum* and (*iaaM*)*F. arthrosporioides* excreted slightly more IAA in the culture medium than the amounts produced by wild-type *F. oxysporum* and *F. arthrosporioides* (Fig. 3A and C). The transformants were cultivated in a medium with and without a supplement of 5 mM L-tryptophan to ascertain if the precursor might be limiting in culture. IAA was quantified by spectrophotometry (Table 1). Both

Fig. 3. Accumulation of indole-3-acetamide (IAM) and enhanced production of indole-3-acetic acid (IAA) in culture filtrates of transformants **A**, (*iaaM*)*Fusarium oxysporum*; **B**, (*iaaM*+*iaaH*)*F. oxysporum*; and **C**, (*iaaM*)*F. arthrosporioides*. Hygromycin-resistant colonies were grown in liquid culture containing 5 mM L-tryptophan. The culture filtrate of each 3-day-old culture was extracted with ethyl acetate and prepared for thin-layer chromatography. Transformant (*iaaM*)*F. oxysporum* 62 was used in a hypervirulence experiment (Fig. 4A), transformant (*iaaM*+*iaaH*)*F. oxysporum* 82 in a hypervirulence experiment (Fig. 4B), and transformant (*iaaM*)*F. arthrosporioides* 5 was used in both hypervirulence experiments (Fig. 4A and B).

transgenic and wild-type *F. oxysporum* and *F. arthrosporioides* strains produced less than 16 ng of IAA per mg fresh weight of mycelium on medium not supplemented with L-tryptophan. Transgenic *F. oxysporum* strains containing *iaaM* but lacking the *iaaH* gene produced up to 78 ng of IAA per mg of mycelium grown in the presence of L-tryptophan. However, transgenic *F. oxysporum* strains having both the *iaaM* and *iaaH* produced twice as much IAA as the best *F. oxysporum* strain containing only the *iaaM* gene on L-tryptophan medium. Transgenic *F. arthrosporioides* having either the *iaaM* or *iaaH* gene produced up to 31 ng of IAA per mg of mycelium when supplemented with L-tryptophan. Strains of (*iaaM*+*iaaH*)*F. oxysporum* 42, 76, and 82 overproduced IAA in several different experiments (Table 1). Some transgenic *F. arthrosporioides* cultures having the *iaaM* gene produced more IAA than wild-type *F. arthrosporioides* grown in the presence of L-tryptophan (data not shown).

Production of tryptophol. Tryptophol is an alternative product of the indole-3-pyruvate acid (IPA) pathway (Fig. 1). The detection of tryptophol in the fractionated samples would imply that the fungi could also use the IPA pathway of IAA synthesis (27). A minor tryptophol TLC spot was seen in TLC separations of all of the *F. oxysporum* and *F. arthrosporioides* culture extracts (Fig. 3A, B, and C). It can be surmised that the IPA pathway may be active in these fungal species.

Hypervirulence of transformed strains. The virulence of selected strains of the transgenic fungi on *Orobanche* was tested in two experiments. Tomato plants were grown in soil mixed with *Orobanche* seeds and wild type or selected transgenic *F. oxysporum* and *F. arthrosporioides* strains versus soil with just *Orobanche* and tomato. It was desired to ascertain whether the added genes would have an effect on the previously shown threshold levels for inoculum (2). Therefore, in the first experiment, sub-threshold levels of inoculum were used. In the second experiment, the inoculum levels were nearer the previously shown threshold. The untransformed fungi had no significant reduction in the number and size of *Orobanche* shoots at the sub-threshold inoculum levels used in both experiments (Fig. 4A and B). IAA-overproducing *F. oxysporum* and *F. arthrosporioides* strains at the same inoculum levels decreased the number of *Orobanche* shoots appearing compared with the wild type (Fig. 4A and B). The reduction in both number and size of the emerged *Orobanche* shoots indicated hypervirulence of some transgenic lines (Table 2). Single transformants (*iaaM*)*F. oxysporum* 62 and (*iaaM*)*F. arthrosporioides* 5 were significantly more active than wild-type *F. oxysporum* or *F. arthrosporioides* in reducing both the total number of shoots and the total number of shoots greater than 5 cm (Table 2). The single (*iaaM*)*F. arthrosporioides* transformant 5 was significantly more active than wild-type *F. arthrosporioides* in

Fig. 4. Hypervirulence of transgenic *Fusarium oxysporum* and *F. arthrosporioides*. Each point represents the data from a single pot.

reducing the total number of shoots, but not better in reducing the number of those shoots larger than 5 cm (Table 2). The higher inoculum level used in the second experiment may have allowed *F. arthrosporioides* 5 to exert a significant effect. Cotransformant (*iaaM+iaaH*)*F. oxysporum* 82 was significantly more active in reducing the number and size of shoots than the wild-type *F. oxysporum* and the single transformants (Table 2).

DISCUSSION

The presence of both the *iaaM* and *iaaH* genes cotransformed into the fungus is required for maximal IAA production and maximal suppression of *Orobanche* spp. The production of IAM contributed to the enhanced virulence of both (*iaaM*)*F. oxysporum* and (*iaaM*)*F. arthrosporioides* compared with the wild-type pathogen. Some (*iaaM*)*F. oxysporum* and (*iaaM*)*F. arthrosporioides* transformants were more effective IAA producers than the wild type. Possible explanations for the increased IAA levels found in the culture filtrates of some transformants bearing only the *iaaM* gene might be due to auto-oxidation of IAM to IAA (12), or that this pathway may exist in these species as previously shown for *Colletotrichum gloeosporioides* (27). Wild-type *F. oxysporum* did not accumulate IAM, but strains of (*iaaM*)*F. oxysporum* accumulated IAM (Fig. 3A). *F. oxysporum* strains cotransformed with both *iaaM* and *iaaH* accumulated up to 12 times more IAA than the wild type, because they could enzymatically convert the overproduced IAM into IAA (Fig. 3B; Table 1). Strains of (*iaaM*)*F. arthrosporioides* and wild-type *F. arthrosporioides* all produced some IAA and IAM (Fig. 3C). Many of the (*iaaM*)*F. arthrosporioides* strains accumulated an unidentified indole ($R_f = 0.63$) that was not found in wild-type *F. arthrosporioides* culture filtrates (Fig. 3C). It cannot be disregarded that the unidentified indole produced by (*iaaM*)*F. arthrosporioides* strains might play a role in the increased virulence of (*iaaM*)*F. arthrosporioides* 5 because wild-type *F. arthrosporioides* endogenously produces some IAA and IAM.

Viable colonies of *F. arthrosporioides* cotransformed with both *iaaM* and *iaaH* genes have not been obtained despite repeated attempts. If selectable markers could be found for (*iaaM*)*F. arthrosporioides* and (*iaaH*)*F. arthrosporioides*, then it might be possible to form (*iaaM+iaaH*)*F. arthrosporioides* heterokaryons. It is equally reasonable to assume that cotransformation could be made. Perhaps there is a lethal antagonism between the genes in this particular species. There may be a seeming contradiction between the in vitro results showing that IAA production is enhanced more when tryptophan is in the medium and the hypervirulence experiments where no exogenous tryptophan was added. Tryptophan may be naturally supplied to the *Fusarium* spp. from

root exudates of the tomato plants attacked by *Orobanche*. Tomato excretes numerous amino acids (14) and tryptophan was detected in tomato root sap (28).

There have been many publications containing reports that IAA is necessary for pathogenicity (20,31), but none showing that transgenically enhanced IAA production leads to enhanced virulence. The results in this report indicate that transgenic *F. oxysporum* and *F. arthrosporioides* overproducing IAA have enhanced virulence, which results in a decrease in the number and size of *Orobanche* shoots (Table 2). The suppression of both the number and size of the shoots implies that the hypervirulent fungi prevent or delay *Orobanche* attachment to tomato or severely inhibit *Orobanche* growth. The best result would be no *Orobanche*. Still, tomato with delayed *Orobanche* attachment will suffer considerably less damage than plants with early attachments. Early attachment translates to a greater impact on crop yield. Neither the wild type (2) nor the hypervirulent fungi appeared to negatively affect the normal growth of the tomato plants (6), however the tomato plants treated with *F. oxysporum* or *F. arthrosporioides* were more vigorous, taller, and had larger fruit compared with those plants grown in mock-inoculated soil (6). Indeed, the transgenic lines of both *Fusarium* spp. colonize tomato roots, growing along them for great distances in the soil, as followed by *gus* or *gfp* in tandem with the other *F. oxysporum* and *F. arthrosporioides* transformants (3,6).

The potential release of hypervirulent fungi in the environment raises safety concerns from the persistence of the fungi in the environment and the possibility of transfer of hypervirulent transgenes from the biocontrol agent to a pathogen of a crop. Failsafe mechanisms have been proposed to prevent spread and mitigate introgression of transgenic hypervirulent biocontrol fungi (16). Asporogenic deletion mutants of the transgenic hypervirulent fungi would prevent the spread. Asporogenic mycelia could be formulated in 'Stabileze' that retained high infectivity after more than a year in cold storage (1,3). Mitigating genes would be added in tandem with the hypervirulent genes to reduce fitness or precluding infection of crops (16). For example, antimelanizing genes would inhibit appressorium formation of crop pathogens (16). There were no appressoria formed during infection of *Orobanche* by the *Fusarium* spp. (6).

The results obtained from the hypervirulence studies may have implications for other systems that use mycoherbicides for weed control.

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TABLE 2. Suppression of emergence and of size of *Orobanche* shoots by transgenic *Fusarium oxysporum* and *F. arthrosporioides* overproducing indole-3-acetic acid (IAA)^a

Treatment	Strain	Genes	Total no. of shoots	Shoots >50 mm
Experiment A				
<i>F. oxysporum</i>	Wild type	...	21.7	8.2
<i>F. oxysporum</i>	62	<i>iaaM</i>	13.6**	5*
<i>F. arthrosporioides</i>	Wild type	...	19.4	5.8
<i>F. arthrosporioides</i>	5	<i>iaaM</i>	14.4*	4.6
Experiment B				
<i>F. oxysporum</i>	Wild type	...	23.6	15.6
<i>F. oxysporum</i>	82	<i>iaaM+iaaH</i>	15.4**	8.9**
<i>F. arthrosporioides</i>	Wild type	...	22.7	14.3
<i>F. arthrosporioides</i>	5	<i>iaaM</i>	12**	7.1**

^a Because there were no significant differences among the results from the different inoculum levels, the data were averaged. * and ** indicate significant differences at $P < 0.01$ or 0.05, respectively, as determined by Student's paired *t* test. Mock-inoculated wild type was used.

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