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ORIGINAL PAPER

Impact of biological control agents on fusaric acid secreted from *Fusarium oxysporum* f. sp. *gladioli* (Massey) Snyder and Hansen in *Gladiolus grandiflorus* corms

Walid Nosir · Jim McDonald · Steve Woodward

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Abstract Fusaric acid (FA) (5-*n*-butylpuridine 2-carboxyl acid), a highly toxic secondary metabolite produced by *Fusarium oxysporum* strains, plays a significant role in disease development. The abilities of three *F. oxysporum* f. sp. *gladioli* (Massey) Snyder and Hansen isolates (G010; 649-91; and 160-57) to produce FA in infected *Gladiolus* corm tissues was evaluated in vitro in relation to the presence of two biological control agents, *Trichoderma harzianum* T22, and *Aneurinobacillus migulanus*. Pathogenicity tests were used to differentiate between the abilities of the *F. oxysporum* strains to secrete FA. FA was identified using LC/MS and quantified using HPLC. Isolate G010 was significantly more virulent ($P < 0.01$) on *Gladiolus grandiflorus* corms; it secretes 1.8 μM FA/g fresh weight corm into inoculated *Gladiolus*. Moreover, G010 was the only isolate that produced FA among the three examined isolates. There was a correlation between the corm lesion area and the FA secretion ability of *F. oxysporum* f. sp. *gladioli* ($P < 0.001$; $r^2 = 0.96$). No FA was detected in PDA cultures of *F.oxysporum* f. sp. *gladioli* isolates. The presence of *T. harzianum* T22 appeared to prevent FA secretion into the corms. In the presence of *A. migulanus*, however, the amount of FA secreted into the corm tissues increased. These results support the use of *T. harzianum* as an effective biological control agent against *F. oxysporum* f. sp. *gladioli*.

Keywords Fusaric acid · *Trichoderma harzianum* · *Aneurinobacillus migulanus* · *Fusarium oxysporum* f. sp. *gladioli* · *Gladiolus*

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W. Nosir (✉) · J. McDonald · S. Woodward
Department of Plant and Soil Science,
Institute of Biological and Environmental Sciences,
University of Aberdeen, Cruickshank Building,
St. Machar Drive, Aberdeen AB24 3UU, UK
e-mail: waleedsabry2000@yahoo.com

W. Nosir
Department of Horticulture, Faculty of Agriculture,
University of Zgazig, Zgazig City, Egypt

Introduction

Gladiolus hybridus hort. is an important bulbous ornamental plant. It is used extensively throughout Mediterranean countries at festivals and saints' days, and ranks highly in the cut flower trade (costing up to $0.25/spike) due to its colorful spikes that reach 60–90 cm in height; numerous funnel-shaped flowers are clustered on one side of the stem [1, 4]. *Gladiolus* is cultivated commonly for cut flower production, frequently as a protected crop. The glasshouse or polytunnel environment provides excellent conditions for flower production, also make conditions more favorable for disease to develop.

F. oxysporum f. sp. *gladioli* (Massey) Snyder and Hansen causes leaf yellowing in *Gladiolus* and also infects corms of *Crocus*, *Freesia*, bulbous *Iris*, *Ixia*, and some other iridaceous plants. The disease constitutes one of the major problems in the commercial production of these species. The pathogen survives in soil and plant debris for several years as chlamydospores and is easily spread by conidia during storage conditions. Numbers of propagules are highest in the surface 20 cm of the soil [25]. Isolates of *Fusarium oxysporum* f. sp. *gladioli* were found to vary greatly in their pathogenic capabilities [26]. Diseases affecting *Gladiolus* production include dry rot, brown rot, basal

rot and yellows. The major symptoms caused by *Fusarium oxysporum* f. sp. *gladioli* (Massey) Snyder and Hansen occur during the flowering period when infected plants develop wilting, damping-off and yellowing symptoms, as well as basal stem rot and corm rot. The pathogen survives in infected corms and in the soil as mycelium, chlamydospores, macroconidia and microconidia [6]. Plants may be infected in the cultivation system, when germinating spores or mycelia enter the roots directly or through wounds. The pathogen is probably introduced into new growing regions via contaminated corms [7]. The virulence of *F. oxysporum* f. sp. *gladioli* (Massey) Snyder and Hansen isolates varies with both fungal and host genotype [12]. Pathogenic and nonpathogenic *Fusarium* strains produce fusaric acid (FA; 5-butylpicolinic acid) [2, 27, 29]. FA is toxic to prokaryotes and eukaryotes [5, 32]. In addition, FA was shown to be involved in fungal defence against biological control strains of *Pseudomonas* spp. by repressing the production of antifungal metabolites [31, 33].

Fusaric acid produced by fungi in the genera *Fusarium* plays a key role in plant death; it alters the permeability of the plant plasma membrane and causes solute leakage. It also chelates copper and other ions that could be involved in maintaining membrane integrity, and affects membrane potentials and ATP levels in tomato root hair cells [2, 27]. In addition, FA is also able to inhibit plant polyphenol oxidase and peroxidase, enzymes involved in defence-related browning reactions [15]. FA was first isolated from *F. heterosporum* Nees as a compound that inhibited the growth of rice seedlings. The involvement of FA in pathogenicity has not been clearly established, although some data suggest it plays a role in cell death [22]. Little information is known about FA production in vitro or in vivo by the fungus or about the toxic effects of this toxin towards different plant tissues [10].

The aims of the present investigation were to evaluate differences in the production of FA by three isolates of *F. oxysporum* f. sp. *gladioli*, and the effects of co-inoculation of corms with the biological control agents *Trichoderma harzianum* and *Aneurinobacillus migulanus* alone or together against fusaric acid mycotoxin secreted by *F. oxysporum* f. sp. *gladioli*.

Materials and methods

Plant material

Gladiolus corms (variety: "Big flower" GT01 size 14; Tylore Bulb, Co., UK) were surface sterilized by removing the old husk and immersing in 70% aqueous ethanol for 1 min followed by 20% NaOCl for 20 min before rinsing under running tap water for 6 h. The corms were subsequently rinsed in three changes of sterilized distilled water.

Fungal cultures

Pathogenity tests were conducted using three *F. oxysporum* f. sp. *gladioli* strains. Two strains (649-91, 160-57), obtained from Centraalbureau voor Schimmelculturen (CBS), The Netherlands, were isolated from infected soils in The Netherlands and Germany, respectively. Freeze-dried spores were suspended in 1 ml sterilized distilled water and 0.1 ml of suspension used to inoculate fresh potato dextrose agar (PDA; Oxoid, Basingstoke, UK) in 9 cm diameter Petri dishes. The third isolate was obtained from the husks of the purchased corms. The three isolates were used in inoculations of *Gladiolus* corms to evaluate virulence.

Preparation of pathogen and antagonist inocula

The third isolate of *F. oxysporum* f. sp. *gladioli*, G010, was maintained on PDA (Oxoid) at 22°C and routinely subcultured at 15-day intervals. Subcultures were prepared by inoculating PDA with 1 cm diameter disks of colonized PDA plus mycelium, cut from the edge of an actively growing, seven-day-old colony.

Trichoderma harzianum isolate T22, obtained from CBS, was cultured on PDA. Petri dishes were sealed with Parafilm and incubated at 22°C, with routine subculturing at 15-day intervals. Spore suspensions were obtained by flooding seven-day-old cultures on PDA with 5 ml sterile distilled water, gently agitating the surface with a wire loop, and passing the suspension through two layers of washed sterile muslin cloth directly into 50 ml centrifuge tubes. Spores were centrifuged at 1,700×*g* in a Thomson–MSE Mistral bench top centrifuge for 10 min, the spore pellets were rinsed twice in sterile distilled water with repeated centrifuging, and spore concentrations were adjusted to 8×10^6 ml^{-1} using repeated hemocytometer counts.

The isolate of *A. migulanus* was obtained from laboratory stocks and maintained on nutrient agar (NA; Oxoid) at 35°C, with routine subculturing at 15-day intervals. Cultures of *A. migulanus* were prepared by transferring approximately 1 ml of cell suspension from a 24 h old liquid culture in tryptic soy broth (TSB; Oxoid) to 20 ml fresh TSB in 250 ml conical flasks. Flasks were incubated at 37°C on a rotary shaker at 150 rpm for 24 h. The suspension was centrifuged at 1,700×*g* for 10 min, resuspended in 15 ml quarter-strength Ringer's solution (Sigma, UK), and washed three times in fresh Ringer's solution by re-centrifuging, as described above. Spore densities were estimated

as colony forming units on NA following serial dilutions to 5×10^8.

Pathogen inoculation

Gladiolus corms were inoculated with *F. oxysporum* f. sp. *gladioli* by removing a piece of tissue 10 mm in diameter and 5 mm deep from the side of the corm and replacing it with a plug of inoculated PDA + fungal mycelium of the same dimensions. This method was modified from Cappelli and Minco [7]. The lesion area was calculated based on the height and width of the lesion. The lesion criteria were determined by the rotting and softening of the corm.

Antagonist inoculation

T. harzianum and *A. migulanus* were inoculated into the corms according to a modified version of the method of Elad et al. [17]. Ten corms were prepared for each treatment. The corms were submerged in *T. harzianum* and *A. migulanus* spore suspensions for 30 min. For the interaction treatments, the corms were suspended in the antagonist suspension and blotted dry on sterilized filter paper under aseptic conditions in a laminar flow cabinet. A combination of *T. harzianum* and *A. migulanus* was prepared by mixing the same volume of each suspension of antagonist in a beaker, immersing the surface-sterilized corms in the mixed suspension for 30 min, and inoculating with the pathogen, as described above. Control corms were immersed in sterilized distilled water for the same duration. Inoculated corms were incubated at 25°C.

Fusaric acid extraction and quantification

Fusaric acid extraction from the infected *Gladiolus* corms was performed using a method modified from [13, 27]. *Gladiolus* corm tissue (10 g) was collected from the infected sites 30 days after inoculation. The sampled tissues were frozen in liquid nitrogen and homogenized with a chilled pestle and mortar. The homogenate was suspended in 5 ml phosphate buffer, pH 2.5. Three volumes of 30 ml ethyl acetate were added and the mixture was sonicated for 30 min. The organic phase was adjusted to pH 3.5 using 1 M HCl and dried using a rotary evaporator (Buchi R-200 rotavapor, Switzerland) at 40°C. The resulting pellet was dissolved in 1.0 ml of methanol and kept frozen at −20°C until analysis.

FA was also estimated in cultures of the three *F. oxysporum* f. sp. *gladioli* isolates on PDA. The agar was chopped into small pieces using a sterilized scalpel and suspended in 10 ml phosphate buffer; pH 2.5. The buffer extract was extracted as described above.

LC/MS

Secondary metabolites in the extracts were analyzed using a liquid chromatography–mass spectrometry (LC/MS) system equipped with a C18 HD analytical column (250 mm × 4 mm) supplied by Agilent (USA). LC/MS high-resolution mass spectral data were obtained using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (an Accela PDA detector, PDA autosampler and pump). Compound identification was based on comparing the MS data with that of authentic FA (Sigma–Aldrich).

HPLC

The HPLC apparatus comprised a Series 1100 LC pump from Agilent (USA) and an LC 90 UV spectrophotometer (Jasco International Co. Ltd) at room temperature. Samples (100 µl) were eluted in a linear gradient of (20–80% acetonitrile) and acidified water with 0.1% trifluoroacetic acid (Sigma–Aldrich) over 30 min. Fusaric acid was detected by monitoring absorbance at 270 nm using an MD-910 multiwavelength detector (Jasco International Co. Ltd.). The retention time of FA was 13 min at a flow rate of 1 ml min^{-1}. FA was quantified based on a series of standard concentrations prepared with synthetic FA (Sigma–Aldrich) in methanol in the range 10–100 µg ml^{-1}. HPLC linear regression curves (absolute amount of standard against the chromatographic peak area integrated from valley to valley) were calculated from three injections of different amounts of standard. Fusaric acid was measured from the corms inoculated with the pathogen 30 days after inoculation and from the inoculated PDA plates 15 days after the inoculation, as no amount was detected before this period.

Statistical analysis

The experiments were organized into complete block designs. Statistical analyses were conducted using the general linear model procedures of the SPSS software package (v.15; SPSS Inc., Chicago, IL, USA). The experiment was repeated three times with ten replicates each time. Experiments were analyzed using analysis of variance (ANOVA). Significance was evaluated at $P < 0.05$ for all tests. Mean separation was tested using the Tukey HSD test.

Results

Pathogenicity test

The three isolates of *F. oxysporum* f. sp. *gladioli* differed significantly in virulence towards *Gladiolus* corms (Figs. 1, 2;

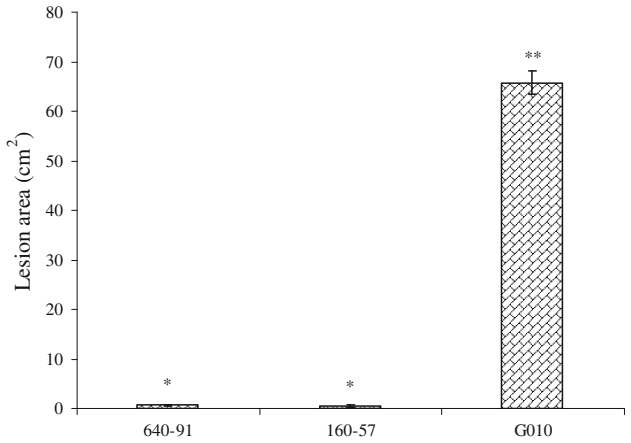

Fig. 1 Effect of different *F. oxysporum* f. sp. *gladioli* isolates on *Gladiolus* corm lesion area (cm^2). *Mean ± SE are significantly different ($P< 0.05$, analysis of variance (ANOVA), $n= 10$ replicates).

$P < 0.001$). Isolate G010 was significantly more virulent ($P < 0.01$) than isolates 640-91 or 160-57 towards *Gladiolus* tissues.

Effects of *T. harzianum* T22 and *A. migulanus* on *F. oxysporum* f. sp. *gladioli* in the infected corm tissue

T. harzianum T22 completely suppressed *F. oxysporum* f. sp. *gladioli* hyphae growth, and no lesions were detected on the infected corms with *F. oxysporum* f. sp. *gladioli*. However, the co-inoculation between *A. migulanus* and *F. oxysporum* f. sp. *gladioli* did not significantly reduce the lesion area. On the other hand, the corms treated with both antagonists prior to infection with *F. oxysporum* f. sp. *gladioli* showed significantly reduced lesion areas compared with the corms inoculated with *F. oxysporum* f. sp. *gladioli* alone after 40 days of inoculation (Fig. 3; $P < 0.001$).

Effects of *T. harzianum* T22 and *A. migulanus* on FA produced by *F. oxysporum* f. sp. *gladioli*

FA was not detected in PDA cultures of *F. oxysporum* f. sp. *gladioli* isolates; however, isolate G010 secretes 1.8 μM FA/g fresh weight corm into inoculated *Gladiolus* (Fig. 4). FA was not found in corms inoculated with isolates 640-91 and 160-57. Co-inoculation with *F.oxysporum* f. sp. *gladioli* and *T. harzianum* completely suppressed fusaric acid secretion by isolate G010; however, inoculation of the infected corms with *A. migulanus* increased fusaric acid secretion into the corms from 1.8 to 3.5 μM/g fresh weight (Fig. 5). When corms were treated with both *T. harzianum* and *A. migulanus* prior to inoculation with *F. oxysporum* f. sp. *gladioli*, no FA was detected. There was a relationship between the corm lesion area and the FA secretion ability of *F. oxysporum* f. sp. *gladioli* ($P < 0.001$; $r^2 = 0.96$). The experiment was repeated three times with eight replicates each time.

Discussion

Pathogenicity test

This work is the first to demonstrate that inoculation with *T. harzianum* suppresses fusaric acid production in *Gladiolus* by *F. oxysporum* f. sp. *gladioli*, indicating a possible mechanism for the inhibition of *F. oxysporum* f. sp. *gladioli* by *T. harzianum* involving the prevention of fusaric acid (an important virulence factor) production. Pathogenicity factors are required by plant pathogens to cause disease. G010 was the only isolate of *F. oxysporum* f. sp. *gladioli* from which FA was detected among the three isolates tested. These findings agree with the previous report by De Weert [13], who found significant differences between concentrations

Fig. 2 Symptoms observed on *Galdiolus* corms 30 days after inoculation with *F. oxysporum* f. sp. *gladioli* isolates. *Left to right*: G010, 640-91 and 160-57

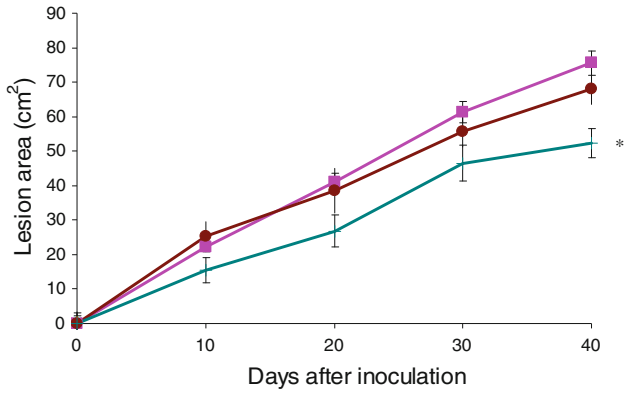

Fig. 3 The efficiency of the suppressive actions of *T. harzianum* T22 and *A. migulans* against *F. oxysporum* f.sp *gladioli*, evaluated via *Gladiolus* corm lesion area; *filled squares*, *F. oxysporum* f. sp *gladioli*; *filled circles*, *F. oxysporum* f. sp *gladioli* + *A. migulanus*; *bars*, *F. oxysporum* f. sp *gladioli* + *T. harzianum* T22 + *A. migulanus*. * Means ± SE are significantly different ($P < 0.05$, analysis of variance (ANOVA), $n = 10$ replicates).

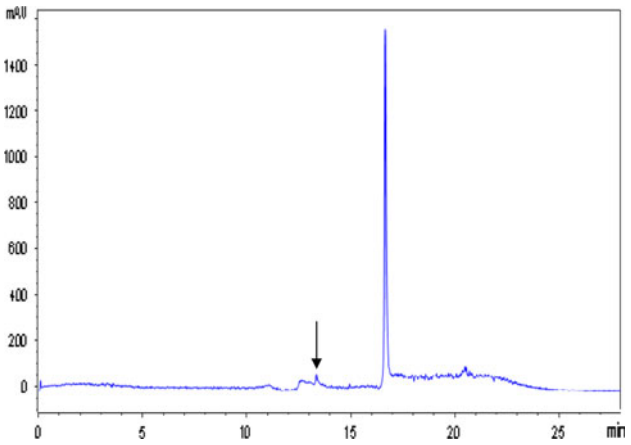

Fig. 4 HPLC chromatogram of extract from *Gladiolus* corms inoculated with *F. oxysporum* f. sp. *gladioli* isolate G010. *Arrow*, fusaric acid

of FA secreted by three different isolates of *F. oxysporum* f. sp. *radicis-lycopersici*.

The findings of this work agree with those previously reported by Kuzniak et al. and McLean et al. [24, 26], who found that fusaric acid (5-*n*-butylpicolinic acid; FA)—which is produced by *Fusarium oxysporum*—is associated with wilt in banana, cotton, pea, tomato, and other plants, affects membrane permeability (increasing the leakage of potassium and other electrolytes), and inhibits respiration. Fakhouri et al. [20] found that FA is a wilt-inducing toxin; on tomato plants infected with *F. oxysporum* f. sp. *Lycopersici*, the concentration required to induce wilting was approximately 150 ppm, which was higher than the FA concentration found in the infected corms, meaning that the *F. oxysporum* f. sp. *gladioli* strain (G010) used in this research employs

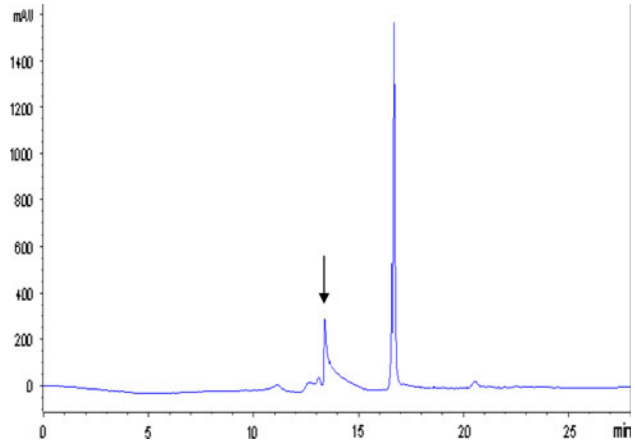

Fig. 5 HPLC chromatogram of extract from *Gladiolus* corms co-inoculated with *F. oxysporum* f. sp. *gladioli* isolate G010 and *A. migulans*. *Arrow*, fusaric acid; no fusaric acid was detected in corms co-inoculated with *T. harzianum* and *F. oxysporum* f. sp. *gladioli*

additional mechanisms to induce wilting symptoms, such as colonizing the infected tissue. This assumption agrees with the findings of Bacon et al. [2], who reported that the wilting symptoms may be the result of the interaction between the fungal hyphae and the infected plant. Therefore, FA may be one of the mechanisms used by *F. oxysporum* species, or synergistic interactions of FA with other naturally co-occurring mycotoxins may cause toxicity.

The results of the present work support those of Venter et al. [32], who also demonstrated a significant correlation between the virulence of *Fusarium oxysporum* isolates and the production of FA in potato during the development of dry rot. The findings of Notz et al. [27] support those of the present work, since they showed that FA secretion into artificial media may differ according to the isolate and the composition of the media. *F. oxysporum* strains 235, 240, and 242 did not produce detectable amounts of FA in any of the studied media. The other strains did produce FA in the media tested. This means that 640-91 and 160-57 could produce FA on other artificial media, or they could be more virulent toward other iridaceous plants.

The previous studies agree with the findings of the present work regarding the differences in FA secretion among *F. oxysporum* f. sp. *gladioli* strains. The failure to detect FA in corms inoculated with isolates 640-91 or 160-57 may be related to the relative virulences of these isolates compared with G010. The time required from inoculation to the production of sufficient FA for detection in the infected host tissues may differ between isolates.

Efficiency of BCAs against *F. oxysporum* f. sp. *gladioli*

The ability of a biological control agent to suppress secretion of FA by *Fusarium* during pathogenesis is an interesting finding. Parallel work on the *Gladiolus*– *F. oxysporum*

f. sp. *gladioli* interaction (Nosir, McDonald and Woodward, unpublished) suggested that *T. harzianum* could act as a biological control agent against the pathogen. Several species of *Trichoderma* have attracted much academic and commercial interest as bioprotective agents against fungal pathogens. The mode of action appears to be very complex. In the rhizosphere, biocontrol strains interact with the environment, which includes other microorganisms [11]. *T. harzianum* are well-known biological control agents, but one of the novel findings of this research is the ability of *T. harzianum* to prevent FA secretion in infected *Gladiolus* corms, even when *T. harzianum* was used in combination with *A. migulanus* as an antagonist mixture. El-Hasan et al. [18] reported the abilities of *T. harzianum* T23 and T16 to degrade 50 mg/ml FA by 51.4 and 88.4%, respectively; this could be one of the novel mechanisms used by *T. harzianum* strains in *F. oxysporum* suppression. Cooney et al. [9] found that the presence of 6-pentyl-α-pyrone (6PAP), a major secondary metabolite produced by *T. harzianum* in the culture medium, reduced the mycotoxin deoxynivalenol (DON) from *Fusarium graminearum* by 80%, which indicates the ability of *T. harzianum*T22 to prevent *Fusarium* mycotoxins from being secreted into the infected plants.

Fusaric acid may play a role in the defense of *Fusarium* spp. against attack by potentially parasitic microorganisms. For example, FA was shown using microarray analysis to be involved in the defence of *F. oxysporum* against *Pseudomonas* spp. by interfering with enzyme function and gene expression [31]. In contrast, several microorganisms, including the bacteria *Bacillus cepacia* and *Ralstonia solanacearum*, as well as the nonpathogenic isolate of the fungus *Colletotrichum*, can hydrolyze FA [8, 20]. Moreover, pathogen toxins often display broad-spectrum activity and can suppress the growth of microbial competitors, or pathogens may detoxify the antibiotics produced by some biocontrol microorganisms as a self-defense mechanism against biocontrol agents [8, 21]. The results of the present work support those of [27], who also demonstrated the abilities of a wide range of bacterial and fungal isolates to detoxify FA, which is produced by a great number of *Fusarium* species. Some of the antagonistic *Fluorescent pseudomonad* isolates were resistant to high concentrations of FA (up to 500 ppm). However, the bacterial isolates were unable to degrade or detoxify FA. A nonpathogenic *Colletotrichum* sp. isolate was able to completely detoxify FA after four days of incubation in malt broth medium containing 200 ppm of FA. At higher concentrations of FA (up to 400 ppm), the fungal isolate did not grow and FA was not degraded after ten days of incubation [32]. Duffy and Defago [16] also reported that the presence of different concentrations of nutrients may impact on the ability of *Fusarium* species to produce FA. The addition of Zn reduced FA production by *F. oxysporum* f. sp. *radicis–lycopersici* and produced a concomitant increase in the concentration of the antibiotic 2,4-diacetylphloroglucinol by *Pseudomonas fluorescens*; the biological control effect of *P. fluorescens* also increased.

However, *A. migulanus* alone did not prevent FA production in inoculated corms; this result was supported by the observed poor biological control action of this bacterium against *F. oxysporum* f. sp. *gladioli* in corms. Work on peanuts has also shown that *Trichoderma* spp. may inhibit FA production in roots and leaves of peanut or maize infected with *Fusarium* spp. [21, 23]. The efficiency of *A. migulanus* in the biological control of *Fusarium* is probably affected by temperature, as this species has a high optimum temperature for growth (Nosir, McDonald and Woodward, unpublished). Other factors potentially impacting on biological control by *A. migulanus* include inoculation time and dose rate.

Further work will focus on the expression of genes involved in the synthesis of FA by *Fusarium* spp. in infected plant tissues, and determine interactions between expression and biological control organisms during infection of *Gladiolus* by *F. oxysporum* f.sp. *lycopersici*.

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