

## The Efficiency of *Trichoderma harzianum* and *Aneurinobacillus migulanus* in the Control of Gladiolus Corm Rot in Soil-Less Culture System

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**Abstract: Problem statement:** Gladiolus is cultivated commonly for cut flower production, frequently as a protected crop. The glasshouse or polytunnel environment, providing excellent conditions for flower production, also make conditions more favorable for disease to develop. **Approach:** The pathogen *Fusarium oxysporum* f. sp. *gladioli* causes wilt diseases in a wide range of economically important plants and can have devastating effects on crop production. *Trichoderma harzianum* and *Aneurinobacillus migulanus* were tested separately and in combination for controlling *F. oxysporum* f. sp. *gladioli* activity in soilless culture using Perlite as the substrate. **Results:** The efficiency of both of antagonists against corm rot was evaluated based on vegetative and root growth parameters and on flowering parameters. *T. harzianum* was more effective than *A. migulanus* in disease suppression and also enhanced plant growth, increased flower production and quality. *A. migulanus* enhanced plant growth when tested alone. The mixture of antagonists reduced the efficiency of *T. harzianum*. Numbers of *T. harzianum* CFU in the substrate and on corms increased following application compared with treating with both antagonists. No *T. harzianum* was detected in the substrate by 120 day after planting, however, *A. migulanus* CFU significantly decreased on corms when inoculated in combination with *T. harzianum* and *F. oxysporum* f. sp. *gladioli*. However, *A. migulanus* CFU was not detected in the substrate of the same combination. SEM and Glasshouse results suggested that suppressive mechanisms of *T. harzianum* and *A. migulanus* differed. *T. harzianum* appeared to operate through a combination of antibiosis and substrate competition, whereas *A. migulanus* produced an electron-dense substance which may have inhibited the penetration of host tissues by *F. oxysporum* f. sp. *gladiolus*. Greater growth of *T. harzianum* was observed when inoculated alone or with *F. oxysporum* f. sp. *gladioli* hyphae. **Conclusion:** It was concluded that *T. harzianum* provided a more efficient and effective control of *F. oxysporum* f. sp. *gladioli* corm rot of *Gladiolus* when inoculated without *A. migulanus*.

**Key words:** Gladiolus, *Trichoderma harzianum*, *A. migulanus*, *F. oxysporum* f. sp. *gladioli* soilless culture, CFU

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### INTRODUCTION

Soil-borne plant pathogens are responsible for severe damage in vegetable and cut flower production. Gladiolus corm rot caused by the fungal pathogen *Fusarium oxysporum* f. sp. *gladioli* is a serious problem in gladiolus production, causing huge financial losses to growers (Ramani *et al.*, 2006). For example, Raiz *et al.*

(2007) reported 100% disease incidence and 20% plant mortality, with reductions in shoot and root biomass of 63 and 100%, respectively, when *Gladiolus grandiflorus* corms grown in a pot culture system were inoculated with *F. oxysporum* f. sp. *gladioli*.

Gladiolus corm rot symptoms usually appear in the generation following the introduction of infected corms. Successful suppression of *F. oxysporum* requires not

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only the prevention of the pathogen from infecting a particular crop, but also the interruption of the pathogen life cycle, which may begin from spores in the substrate used for cultivation. Chemical fungicides alone did not give satisfactory control of *F. oxysporum* f. sp. *gladioli*, because the pathogen is protected in the xylem tissues, which is difficult for many chemical fungicides to penetrate (Ramani *et al.*, 2006). Compared with fungicides, biological control agents have different modes of action including nutrient competition, antibiotic production and production of enzymes that degrade fungal cell wall and induction of host resistance (Williams *et al.*, 2003).

Introduction of two or more Biocontrol Agents (BCA) to the rhizosphere, assuming that each has different ecological requirements, may facilitate disease control without affecting the efficacy of a single antagonist under different conditions and may result in increased efficiency (Guetsky *et al.*, 2001). Most biological control studies deal with one antagonist, although attempts to apply more than one antagonist have been reported previously. Elad *et al.*, (1993) found that, increasing the number of antagonists helped in disease control efficiency. For example, the combining of four bacterial antagonists and conidia of *Trichoderma* sp. on strawberry reduced *Botrytis cinerea* spore germination markedly (Benitez *et al.*, 2004).

The efficiency of biological control agents in mixtures was related to complementary modes of action of combined organisms (Pierson and Weller, 1994). Using a mixture of *Fluorescent pseudomonas* spp. strains decreased *Gaeumannomyces graminis* var. *tritici* infection on wheat by 70% compared to the control (Whipps, 2001). Use of yeast (*Pichia guilhermondii*) or a bacterium (*Bacillus mycoides*) against *B. cinerea* on strawberry gave disease control of 38-98% when applied separately, but in mixture suppressed infection by 80-90%. Application of more than one BCA, therefore, is suggested as a useful means of increasing disease suppression (Guetsky *et al.*, 2001).

A mixture of *Fluorescent pseudomonads* and non-pathogenic isolates of *F. oxysporum* were effective in reducing the density of pathogenic *F. oxysporum* populations in soils, despite the BCA isolates being ineffective when used separately (Lemanceau and Alabouvette, 1993).

The aim of the work reported here was to investigate the efficiency of *T. harzianum* and *A. migulanus* separately or together in controlling *F. oxysporum* f. sp. *gladioli* under soil-less culture condition.

## 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. Corms were subsequently rinsed in 3 changes of sterilized distilled water.

**Irrigation system:** *Gladiolus* were cultivated in an open soil-less system using 1.5 L plastic pots (17 cm) placed 25×25 cm apart on the glasshouse bench and filled with Perlite, previously autoclaved for 1 h at 121°C. The nutrient solution was pumped with a submerged pump at a rate of 1700 L h<sup>-1</sup> and passed through a UV unit (12 L m<sup>-1</sup>, 30 jm h<sup>-1</sup>, Filpumps, Aberdeen, UK). The drip irrigation nozzles and all piping were sterilized in 20% NaOCl for 20 min prior to use. Irrigation was timed to operate 5 times during daylight hours, running for 1 min every 2 h. Each experiment comprised 20 replicate plants per treatment organized as a complete block design. The plants were grown in controlled glasshouse conditions at 22°C.

**Preparation of antagonist and pathogen inocula:** *Trichoderma harzianum* isolate T22, obtained from (CBS), was cultured on PDA. Petri dishes were sealed with Parafilm and incubated at 22°C, with routine sub-culturing at 15 day intervals. Spore suspensions were obtained by flooding 7 day old cultures 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 1700×g in a Thomson-MSE Mistral bench top centrifuge for 10 min, the spore pellets rinsed twice in sterile distilled water, with repeated centrifuging and spore concentrations adjusted to 8×10<sup>6</sup> mL<sup>-1</sup> using repeated haemocytometer counts.

The isolate of *Aneurinobacillus migulanus* was obtained from laboratory stocks and maintained on nutrient agar (NA; Oxoid, Basingstoke, Hants, UK) at 35°C, with routine sub-culturing 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 soya broth (TSB; Oxoid, Basingstoke, Hants, UK) 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 1700×g for 10 min, resuspended in 15 mL quarter-strength Ringers solution (Sigma, UK) and washed 3 times in fresh Ringers solution by re-

centrifuging, as described above. Spore densities were estimated as colony forming units on NA following by serial dilutions to  $5 \times 10^8$ .

*F. oxysporum* f. sp. *gladioli* was isolated from the husks of purchased *Gladiolus* corms and maintained on Potato Dextrose Agar (PDA; Oxoid, Basingtoke, Hants, UK) at 22°C, with routine sub-culturing at 15 day intervals. Subcultures were prepared by inoculating fresh PDA in 9 cm diam. Petri dishes with 1 cm diam. disks of colonized PDA plus mycelium, cut from the edge of an actively growing, 7 day old colony.

**Antagonist inoculation:** Corms were submerged in *T. harzianum* or *A. migulanus* spore suspensions for 30 min. For the interaction treatments, corms were suspended in the antagonist suspension and blotted dry on sterilized Whatman, No 3 filter paper under aseptic conditions in a laminar flow cabinet. The combination between *T. harzianum* and *A. migulanus* was prepared by mixing equal volumes of antagonist suspensions in a 2000 mL beaker, immersing surface sterilized corms in the mixed suspension for 30 min and inoculating with the pathogen, as described below. Control corms were immersed in sterilized distilled water for the length of same time.

**Pathogen inoculation:** The *Gladiolus* corms inoculated with antagonists were subsequently inoculated with *F. oxysporum* f. sp. *gladioli* by removing a 10 mm diam., 5 mm deep piece of tissue from the corm and replacing it with a plug of inoculated PDA + fungal mycelium of the same dimensions.

**Data collection:** Vegetative characters, including leaf numbers and area, were recorded at 10 day intervals. Corm and rooting characteristics recorded included root length, corm dry weight and lesion area on the corm. The lesion area was measured by calculated the length and the width of the infected area; the infected areas were defined by the softness and rotting of the tissue. Flower spike length, dry weight of the inflorescence and days from inoculation to flowering were also measured. After obtaining the fresh weights, samples were dried at 70°C to constant weight.

**Re-isolation of antagonists:** *T. harzianum* selective medium was prepared according to method of (Yedidia *et al.*, 2000). *Aneurinobacillus migulanus* selective medium was composed according to (Edwards and Seddon, 2001).

#### **Fungal and bacterial colonization in soil and corms:**

The extent of colonization of corms by BCAs or *F. oxysporum* f. sp. *gladioli* was examined at 30 days intervals after treatment and planting. Inoculated plants were uprooted and the Perlite attached to the roots carefully removed. One g of Perlite was placed into a 30 mL plastic sterile universal tube containing 10 mL 50 mM phosphate buffer, pH 7.0. The tubes were vortexed for 1 min at maximum speed, placed on a rotary shaker at 150 rpm for 30 min and the suspensions diluted to  $10^6$ . Aliquots of suspension (0.1 mL) were plated on the Trichoderma selective medium and *A. migulanus* selective medium. Colonies isolated from the suspensions were considered as the external rhizosphere population. To determine corm colonization, 1 g corm tissue from the inoculated sites were rinsed, dried briefly with study towels, weighed and homogenized using a mortar and pestle containing 10 mL 50 mM phosphate buffer, pH 7.0. The homogenate was serially diluted to  $10^4$ . Aliquots (0.1 mL) of the diluted suspension were placed on the selective media for each microorganism. *T. harzianum* was counted on Trichoderma selective media and *A. migulanus* on Bacillus selective medium every month to monitor the efficiency of colonisation by BCAs. Data from fungal and bacterial population density counts were  $\log_{10}$  transformed before analysis.

**Tissue processing for Scanning Electron Microscopy (SEM):** Corm samples were prepared according to the method modified from Yedidia *et al.* (2000). The plant tissues were collected from the glasshouse experiment after 72 h from the infection

**Statistical analysis:** Glasshouse experiments and biological assays were organized in complete block designs. The experiments were repeated twice, but as the results were homogenous the results represents the mean of the two seasons. Statistical analyses were conducted using the general linear model procedures of SPSS version 16. 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**

**Vegetative characters:** Inoculation of corms with *T. harzianum* resulted in significantly greater leaf areas and number (Fig. 1 and 2;  $p < 0.001$ ) in developing plants, with 80 and 42.1% increases, respectively, compared to untreated control corms, at the end of vegetative growth stage.

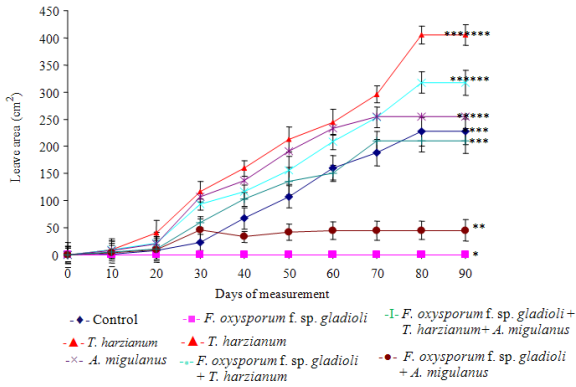


Fig. 1: Effects of BCAs on gladiolus leaves area, data represents the mean  $\pm$  SD. \*: Means  $\pm$  SE are significantly different ( $p < 0.05$ ,  $n = 20$ )

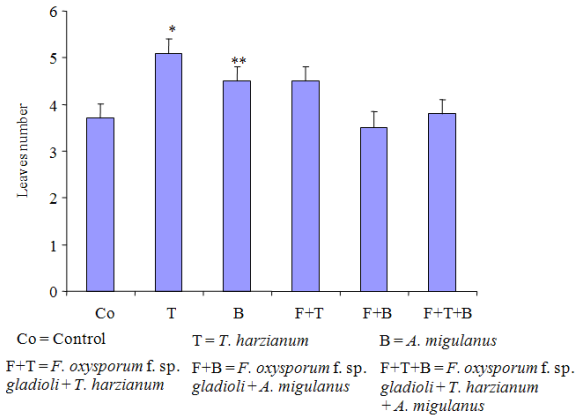


Fig. 2: Effects of BCAs on gladiolus leaves number, data represents the mean  $\pm$  SD. \*: Means  $\pm$  SE are significantly different ( $p < 0.05$ ,  $n = 20$ )

*A. migulanus* treatment also significantly ( $p < 0.001$ ) increased plant growth; leaf area and number increased by 45.4 and 26.31%, respectively.

*T. harzianum* treatment also significantly ( $p < 0.001$ ) increased leaf area (13.6%) and number (26.3%) in corms inoculated with *F. oxysporum* f. sp. *gladioli*. In contrast, corms treated with *A. migulanus* followed by inoculation with *F. oxysporum* f. sp. *gladioli* produced significantly fewer leaves (75%), compared with controls. Using the mixture of antagonists (*Trichoderma* and *Aneurinobacillus*) in the *F. oxysporum* f. sp. *gladioli* inoculated corms lead to a significant decrease ( $p < 0.001$ ) in leaf area and number, compared with corms treated with *A. migulanus* or with *T. harzianum* alone.

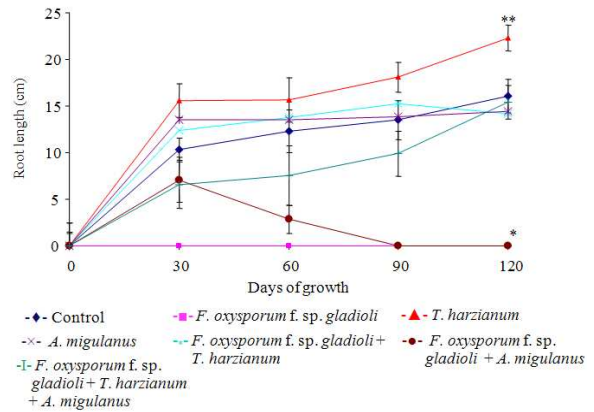


Fig. 3: Effects of BCAs on gladiolus root length; data represents the mean  $\pm$  SD. \*: Means  $\pm$  SE are significantly different ( $p < 0.05$ ,  $n = 20$ )

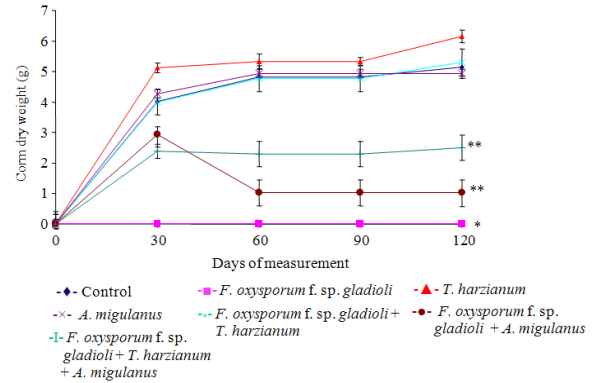


Fig. 4: Effects of BCAs on gladiolus corm dry weight, data represents the mean  $\pm$  SD. \*: Means  $\pm$  SE are significantly different ( $p < 0.05$ ,  $n = 20$ )

**Rooting characters:** Treatment with *T. harzianum* significantly enhanced all rooting characters measured (Fig. 3-5,  $p < 0.001$ ). Plants in this treatment had the longest roots and the highest corm dry weights; these growth parameters were 45.1, 30% higher than the equivalents in control plants, respectively. In contrast, no differences were found in the corms treated with *A. migulanus* compared with the control. Moreover, *T. harzianum* significantly protected plants from *F. oxysporum* f. sp. *gladioli* infection ( $p < 0.001$ ), compared with corms treated with *F. oxysporum* f. sp. *gladioli* alone (Fig. 5).

*A. migulanus*, however, had no biological control effect against the pathogen. Corms treated with *A. migulanus* and inoculated with *F. oxysporum* f. sp. *gladioli* began to form roots normally, but by 30 days after inoculation, all rooting parameters decreased linearly until 60 days from planting.

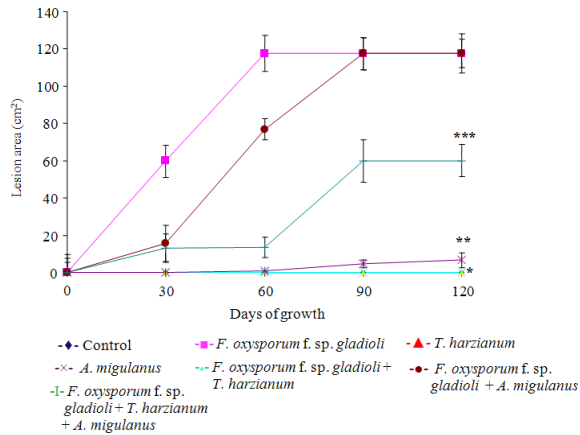


Fig. 5: Effects of BCAs on gladiolus corm dry weight, data represents the mean  $\pm$  SD. \*: Means  $\pm$  SE are significantly different ( $p < 0.05$ ,  $n = 20$ )

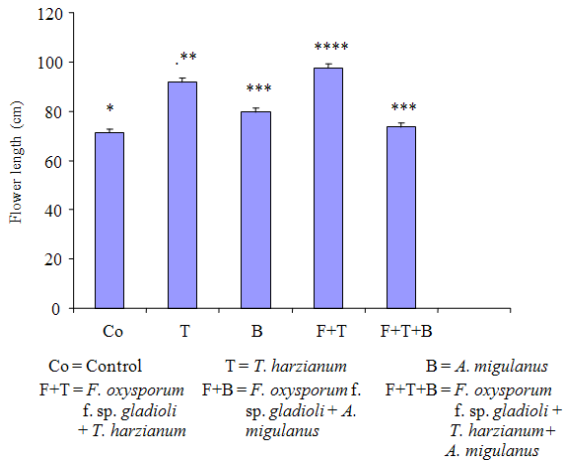


Fig. 6: Effects of BCAs on gladiolus flower dry weight. Data represents the mean  $\pm$  SD. \*: Means  $\pm$  SE are significantly different ( $p < 0.05$ ,  $n = 20$ )

After that time, no growth was recorded and the plants died. In corms treated with *A. migulanus*, rooting decreased significantly ( $p < 0.001$ ). When corms were treated with the mixture of antagonists and inoculated with the pathogen, biological control potential of *T. harzianum* was reduced compared with the *T. harzianum*-*F. oxysporum f. sp. gladioli* treatment.

Treating the pathogen-inoculated corms with antagonistic organisms had a significant effect on root length and corm dry weight, when compared with controls ( $p < 0.001$ , Fig. 3-5). No lesions were detected in the inoculated corms treated with *T. harzianum* at the first harvest, although lesions were present by 60 days after inoculation. *A. migulanus* significantly reduced lesion areas on inoculated corms compared with corms inoculated with *F. oxysporum f. sp. gladioli* alone.

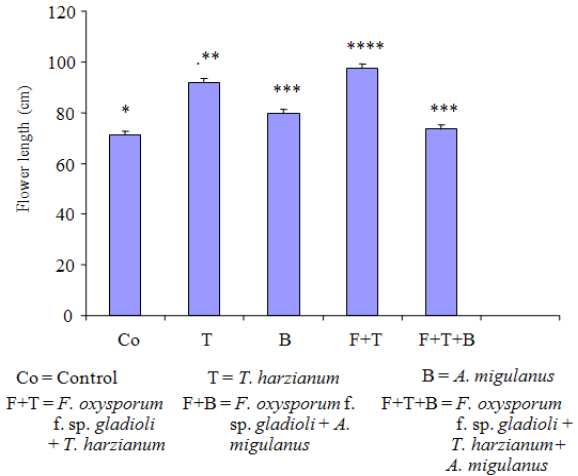


Fig. 7: Effects of BCAs on Gladiolus spike length. Data represents the mean  $\pm$  SD. \*: Means  $\pm$  SE are significantly different ( $p < 0.05$ ,  $n = 20$ )

Using a combination of *T. harzianum* and *A. migulanus* was not effective in reducing lesion size compared to the treatment with *T. harzianum* alone.

**Flower production:** *T. harzianum* treatment significantly enhanced flowering production (Fig. 6 and 7;  $p < 0.001$ ). Plants in this treatment had the highest flower spike dry weight and height, 36.84 and 25.64% higher in control plants, respectively. Flower spike dry weight and height were also significantly higher ( $p < 0.001$ ) in the *A. migulanus* treatment, 21.6 and 9.33%, higher than the control plants, respectively.

Flowers of plants treated with *T. harzianum* and *F. oxysporum f. sp. gladioli* were also of significantly higher weight and height ( $p < 0.001$ ) compared with the control.

In contrast corms treated with *A. migulanus* followed by inoculation with *F. oxysporum f. sp. gladioli* died before reaching the flowering stage. Using the mixture of antagonists (*Trichoderma* and *Aneurinobacillus*) and inoculating with *F. oxysporum f. sp. gladioli* lead to a significant decrease ( $p < 0.001$ ) in the flower weight and height compared with corms treated with *T. harzianum* or *A. migulanus* alone.

**Re-isolation of antagonists:** The population of *T. harzianum* in the substrate was maintained at approx. 5-8  $\log_{10}$  CFU per g in inoculations with both the antagonist alone, or in combination with *F. oxysporum f. sp. gladioli* (Fig. 8A). Greater fluctuations in the *T. harzianum* population occurred in the substrate, however, in treatments with both *T. harzianum* and *A.*

*migulanus* ( $p < 0.001$ ). The population increased from days 30-60, followed by a decrease, until, by day 150 after inoculation, no *T. harzianum* CFU were recovered. On the corm surface, the *T. harzianum* populations increased from day 30-150 after inoculation in treatments with either the antagonist alone, or in combination with *F. oxysporum* f. sp. *gladioli* (Fig. 8B). In contrast, CFU of *T. harzianum* were significantly lower ( $p < 0.001$ ) on the surfaces of corms also treated with *A. migulanus*.

No *A. migulanus* was recovered from the substrate of corms treated with the combination of bacterium, *T. harzianum* and *F. oxysporum* f. sp. *gladioli* (Fig. 9A). At 30 days after treatment, significantly higher CFU of *A. migulanus* were present in the substrate around corms treated with the bacterium and *F. oxysporum* f. sp. *gladioli*, than in the *A. migulanus* treatment ( $p < 0.01$ ). From 60 days onwards, however, the populations of *A. migulanus* declined slowly and no significant differences were found in substrates around corms inoculated with the bacterium alone, or in combination with the pathogen ( $p > 0.05$ ).

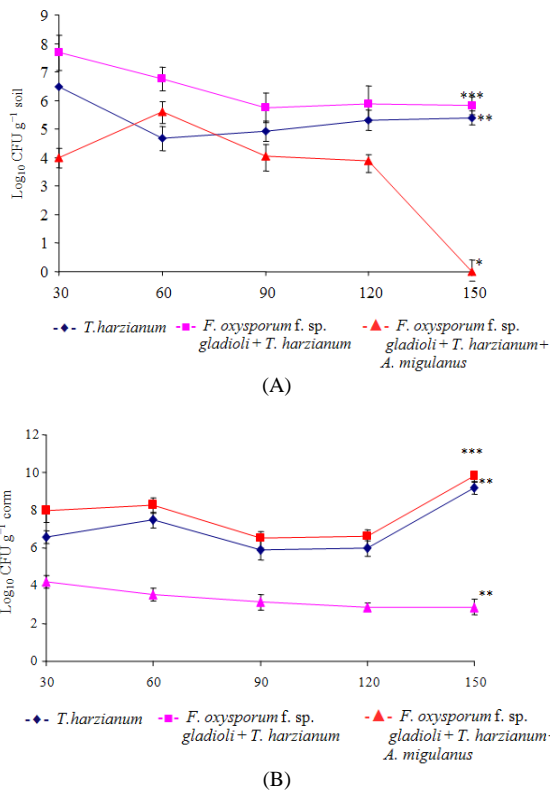


Fig. 8: Effects of BCAs on *T. harzianum* Log<sub>10</sub> CFU g<sup>-1</sup> fresh weight; (A) soil; (B), corm, data represents the mean ± SD. \*: Means ± SE are significantly different ( $p < 0.05$ )

In contrast, *A. migulanus* CFU were obtained from the corm surface in all combinations, from days 30-90 after treatment (Fig. 9B). There were significant differences between CFU recovered from all treatments at 30 days after inoculation ( $p < 0.01$ ). After that time, however, cfus obtained erduced, particularly in the *A. migulanus* alone and *A. migulanus* with *F. oxysporum* f. sp. *gladioli* treatments; by 120 days after inoculation, no *A. migulanus* CFU were obtained in these treatments.

**Scanning electron microscopy:** In corms inoculated with *F. oxysporum* f. sp. *gladioli* alone, pathogen hyphae penetrated through the epidermis and into the parenchymatous tissues of the corm (Fig. 10A). Within the corm, hyphae of *F. oxysporum* f. sp. *gladioli* were abundant, apparently growing intercellularly.

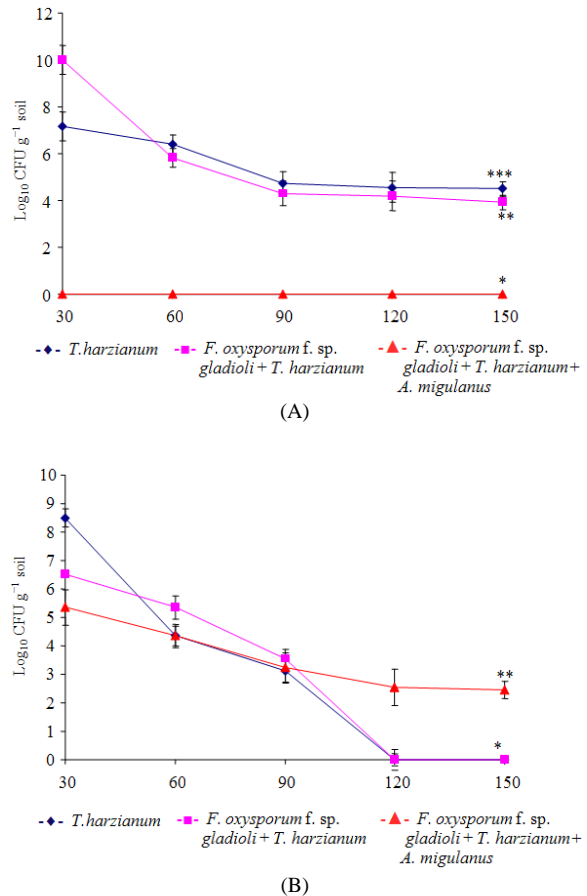


Fig. 9: Effects of BCAs on *A. migulanus* Log<sub>10</sub> CFU g<sup>-1</sup> fresh weight (A) soil; (B) corm; data represents the mean ± SD. \*: Means ± SE are significantly different ( $p < 0.05$ )



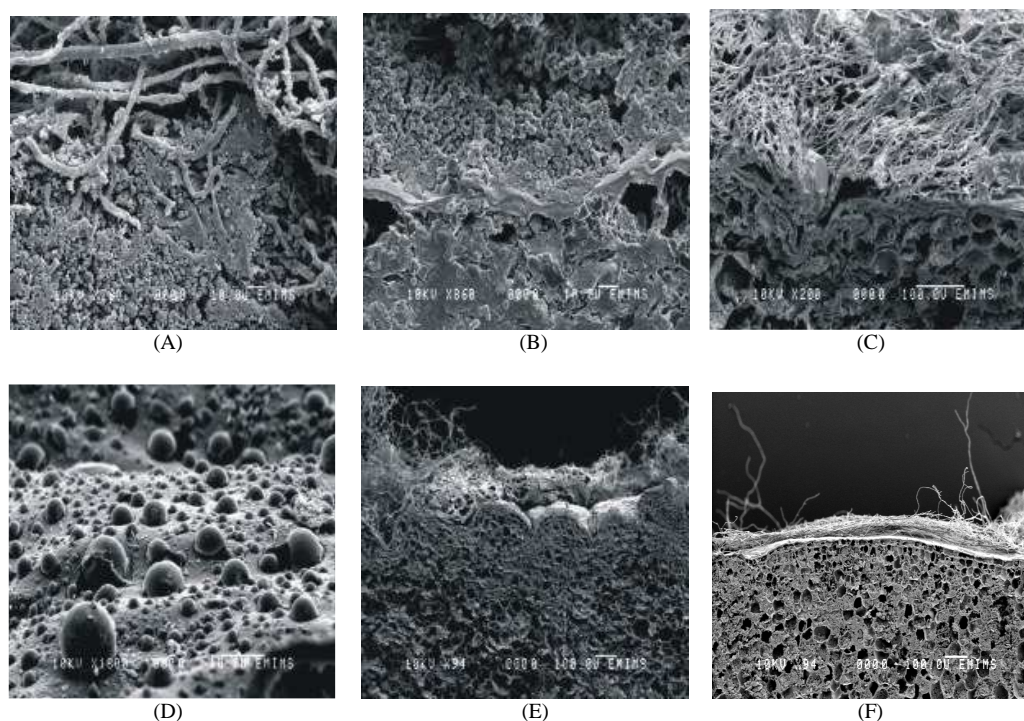


Fig. 10: Scanning electron micrographs cross sections of Gladiolus corms grown in soilless culture 72 h after inoculation with *T. harzianum* and *A. migulanus* as antagonists for *F. oxysporum* f. sp. *gladioli*, (A): Infected corms with *F. oxysporum* f. sp. *gladioli* showing the colonization of fungal hyphae between the cells ( $\times 780$ ); (B): *T. harzianum* spores and dense compound covering the epidermal cells of the infected corm with *F. oxysporum* f. sp. *gladioli* ( $\times 860$ ); (C): *T. harzianum* hyphae covering the epidermal cell of uninfected Gladiolus corms ( $\times 200$ ); (D): *A. migulanus* dense compound covering the epidermal surface ( $\times 1800$ ); (E): Showing infected corm with *F. oxysporum* f. sp. *gladioli* + *A. migulanus* and the fungal hyphae coiling away from the corm surface; (F): Infected corm with *F. oxysporum* f. sp. *gladioli* and treated with *T. harzianum* and *A. migulanus*, Fusarium hyphae still growing but not completely in contact with the epidermal cells and dense compound covering the epidermal surface ( $\times 94$ )

In combined inoculations with *T. harzianum* and *F. oxysporum* f. sp. *gladioli*, abundant sporulation of the BCA was observed on the surface of the corm (Fig. 10B) and no penetration of the host tissues had occurred. Vigorous growth of hyphae over the surface of corms treated with *T. harzianum* alone (Fig. 10C), with only scattered patches of conidia observed. Inoculation with *A. migulanus* leads to the accumulation of electron dense globules over the whole surface of the corm. Hyphae of the pathogen were present on corms treated with both *A. migulanus* and *F. oxysporum* f. sp. *gladioli*, but no penetration of host tissues was visible (Fig. 10D and E). Hyphae of the pathogen were present above the dense layer covering on the corm surface in the treated corms with the mixture of antagonists (*Trichoderma* and *Aneurinobacillus*) and inoculating with *F. oxysporum* f. sp. *gladioli* (Fig. 10F).

## DISCUSSION

Using BCAs to suppress fungal pathogens is considered an environmentally friendly alternative to chemicals usage. The hypothesis of this study is to see if *T. harzianum* and *A. migulanus* strains are able to suppress *F. oxysporum* f. sp. *gladioli* separately or in mixture under soilless culture conditions. Biological control efficiency depends upon the establishment and maintenance of a threshold population of antagonists on planting material or in soil (Whipps, 2001).

The findings of this study show that, the use of *T. harzianum* alone was the most efficient treatment in the suppression of *F. oxysporum*. Using a mixture of both of antagonists increased different plant growth characters; however, the efficiency was reduced in comparison with the *T. harzianum* treatment.

The results obtained in this study are comparable with those obtained in previous work by Sharma and Chandel (2006) and Singh *et al.* (1999). When *T. harzianum* and *T. viride* were used to control *F. oxysporum* f. sp. *gladioli* under field conditions, disease reductions of by 8.9 and 11.1% respectively were obtained following 3 months of application under storage conditions. However, using glasshouse conditions increased the efficiency of both biological control agents. Further work by Weller *et al.* (1988) found the application of BCAs in the field increased the plant growth and number of marketable flowers. Raj and Upmanyu (2006) also reported the efficiency of *T. viridi* in controlling *F. oxysporum* f. sp. *gladioli* under field conditions was increased by increasing *T. viridi* spore concentration. *T. harzianum* was the most effective treatment in enhancing Gladiolus corm and flower yield and improving the plant health compared with all other treatments. In other study, mixing *T. harzianum* with Neem cake as a natural product has been shown to enhance the antagonistic efficiency and increased plant growth (Sharma and Chandel, 2006). The mixture of *T. harzianum* and *Pseudomonas fluorescens* (PS07) as a dressing treatment enhanced Gladiolus growth by 15-28% and decreased the infection significantly (Khan and Mustafa, 2005). Mishra *et al.* (2000) used *T. virens* to control *F. oxysporum* f. sp. *Gladioli*. Their results showed *T. virens* to be an effective treatment but that using a combination of biological and chemical treatments at different times increased the efficiency in controlling the pathogen.

Using CFU can be predictor technique in prediction disease development over the growing season. A relationship was found between *F. oxysporum* and disease suppression by introducing *T. harzianum* into the soil. Raaijmakers *et al.* (1999) and Ram *et al.* (2004) found a negative relationship between *Fluorescent pseudomonas* density in the rizosphere and disease incidence. Our results agree with what previously reported about population densities of *Fluorescent pseudomonads* which show a decrease with time. There is also a gradual decrease in the antagonist population density to approximately  $10^3$  CFU  $g^{-1}$  soil during 3-4 months. Bowers and Locke (2000) indicated a significant reduction of the *F. oxysporum* f. sp. *chrysanthemi* population density in the soil by 97.5% after 3 days of inoculation.

*T. harzianum* population can be an employed mechanism in disease suppression; So increasing *T. harzianum* CFU in the soil or in the corms could be used in *F. oxysporum* f. sp. *gladioli* suppression and lead to competition for nutrients or space as, non

pathogenic *F. oxysporum* showed several modes of action contributing to their biocontrol capacity, competition for nutrient or sites and trigger plant defense (Fravel *et al.*, 2003). The use of BCAs of soil borne plant pathogens in the field has given variable results (De Boer *et al.*, 1999) The combination between *Pseudomonas putida* strains (CS358 with RE8) gave better biocontrol and reduced *Fusarium* wilt of radish CFUs (De Boer *et al.*, 1999). The number of CFUs of *Fusarium* wilt of rye isolated from plant tissue was higher than those of the rhizosphere. Using non-pathogenic *Fusarium* strains reduced disease incidence by 20% and CFU of pathogen isolates decreased by 14% (Jaroszk-Scisel *et al.*, 2008).

In this study, *T. harzianum* and *A. migulanus* were shown to enhance plant growth. Increasing transpiration rate and stomatal conductance would result in increasing leaf longevity (Sharma and Tripathi, 2008). SEM showed the efficiency of *T. harzianum* was related to its ability to colonize the corms epidermal layer and prevent the pathogen hyphae from invading the plant cell wall. *T. harzianum* probably follows more than one mechanism in the suppression of pathogens such as production of cell walls, degradation of enzymes and competition for nutrients. *T. harzianum* grew vigorously in the infected corms and no *F. oxysporum* f. sp. *gladioli* growth was found on or in the corms. This suggests that competition for the colonization area is the primary mechanism by which *T. harzianum* suppresses *F. oxysporum* f. sp. *gladioli*. Therefore, using antagonists enhance to plant growth could help promote disease resistance in plants (Whipps, 2001).

A variety of *Bacillus* spp. has been tested as biocontrol agents on a wide variety of plant species. They are appealing candidates for biological control as they produce endospores that are tolerant to heat and desiccation. The bacterial cultural conditions have an effect on the bacteria survival; that effect will go in parallel with the bacteria efficiency as antagonistic against pathogenic fungi (Weller and Cook, 1983). Therefore, *A. migulanus* efficiency could be affect by the growth conditions and the viability of the cells.

The *A. migulanus* inefficiency observed in this study was probably related to the environmental factors which were not the organism's normal growth conditions. It is known from previous studies that the optimum growth temperature for *A. migulanus* is 35°C (ref) whereas the temperature of the glass house used in our experiments was 22°C. Substrate moisture or the loss of ecological competence could be further reasons for the observed inefficiency (Baker, 1987). Repeated culturing of *Fluorescent pseudomonads* can result in a



loss of field efficacy, loss of cell surface structures, or reduction in antibiotic and siderophore production (Caesar and Burr, 1987).

*Bacillus* sp. strain A13 has been shown to increase the growth of carrots by 48%, oats by 33% and peanut by 37% (Baker, 1987). Abiotic and biotic factors affect the distribution of introduced bacteria and their propagation and survival (Howie and Echandi, 1983).

The reduced efficiency of *T. harzianum* observed when both antagonists were present could be because the Gramicidin S produced by *A. migulanus* could have its negative effect on *T.harzianum*. Other possible reasons are that the inoculation method meant that *T. harzianum* hyphae in contact and adjacent to antibiotic produced by *A. migulanus*. *T. harzianum* spores normally grow faster than *F. oxysporum* f. sp. *gladioli* at this stage *T. harzianum* could have acted as a shield for *F. oxysporum* f. sp. *gladioli* spores. However, this effect may only last for a week after inoculation, since this is the normal time taken for *A. migulanus* to grow and produce its secondary metabolites in the inoculation site. Following this, colonization by *F. oxysporum* f. sp. *gladioli* spores of the corms becomes both faster and more virulent by producing more spores and more hyphen growth. A closer examination of the colonized regions on the root surface revealed the occurrence of penetration zones, mainly at the junctions of adjacent epidermal cell wall, similar to those previously described by Yedidia *et al.* (2000). *A. migulanus* spores were observed to colonize the whole corm surface area although; *F. oxysporum* f. sp. *gladioli* spores germinated and grew over the inoculated sites. This work is the first time that this part of the interaction between *F. oxysporum* f. sp. *gladioli* and *A. migulanus* on Gladiolus corms surface has been reported

The SEM images showed the efficiency of using the mixture of antagonists (*Trichoderma* and *Aneurinobacillus*) in *F. oxysporum* f. sp. *gladioli* suppression and indicated that the suppressive mechanisms of *T. harzianum* and *A. migulanus* were different. *T. harzianum* appeared to operate through a combination of antibiosis and substrate competition, whereas *A. migulanus* produced an electron-dense substance which may have inhibited the penetration of host tissues by *F. oxysporum* f. sp. *gladiolus*.

## CONCLUSION

This study has taken a step in determining the efficiency of *T. harzianum* and *A. migulanus* separately or in mixtures on *F. oxysporum* f. sp. *gladioli*. However, it is possible that other studies will produce different results by using different inoculation methods.

Growing the corms using alternative environmental factors, may lead to a different relationship between the antagonists and the plants. The approach outlined in this study should be replicated with other plants under different ecological conditions.

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