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Full Length Research Paper

Improved mycoherbicidal activity of *Fusarium arthrosporioides*

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Fusarium arthrosporioides **plus cellulase was evaluated on tomato root systems to ascertain whether cellulase, a cell wall degrading enzyme, could accelerate fungus infection of** ***Orobanche aegyptiaca*** **tubercles. Chopped mycelia alone (1.3 × 10^6 and 5.4 × 10^6 propagules ml^{-1}) killed 17 and 37% of** ***Orobanche*** **tubercles, respectively while in the presence of cellulase (10 Uml^{-1}) *Orobanche* tubercles mortality increased to 37 and 78%. Cellulase treatment alone was ineffective. Only a hypersensitive reaction (9% death) resulted in the absence of cellulase. The findings add to the commercial value of *F. arthrosporioides* as a potential mycoherbicide when sufficiently virulent.**

Key words: Mycoherbicide, cellulase, *Fusarium.*

INTRODUCTION

Orobanche are holoparasitic flowering plants, penetrating roots of susceptible host's, removing water, minerals and sugars. *Orobanche aegyptiaca* Pers. (Egyptian broom-rape) attacks most vegetable crops and grain legumes causing enormous yield losses (Parker and Riches, 1993) . *O. aegyptiaca* attach to the host by penetrating the hosts tissue, but stays outside the host cell membrane. Functional exchange takes place within the haustorial complex drawing nutrients out of its host. Report of lower amino acid concentrations in roots of parasitized carrot suggesting changes in amino acid composition by broomrape parasitism (Nandula et al., 2000) is a typical example. *Fusarium arthrosporioides* infection on broomrapes is not sufficiently virulent to compete with chemical herbicides. A hydrolytic enzyme plays an important role in the pathogenicity of plants by facilitating fungal penetration through the host cell wall (Lebeda et al., 2001; Wanjiru et al., 2002). A series of experiments were conducted using *F. arthrosporioides,* a biological control agent that infects *Orobanche* spp. (broomrapes) without affecting the roots of tomato. Experiments using mycoherbicidal organisms plus pectin-nase (Babalola, 2007) indicate that pectinase enhances the weed control of a pathogenic fungus.

Here, cellulase has been used to enhance the virulence of *F. arthrosporioides* on tomato plants infested with *O. aegyptiaca*. A semiaxenic polyethylene bag system was used that allowed easy visual observation of the fungal infection of the tubercles. It was demonstrated that the addition of cellulase enhanced the virulence of *F. arthrosporioides* on *O. aegyptiaca*.

F. arthrosporioides mycelia at 150 rpm for 48 h were harvested on Miracloth® (Calbiochem, La Jolla, CA), rinsed with distilled water to remove remaining spores and excess medium and harvested by vacuum filtration. The washed hyphae were chopped at 6,000 rpm for 2 min with a homogenizer (IKA T18 basic Ultra-Turrax® USA), resuspended in sterile water and the propagule concentrations of chopped mycelia were estimated after serial dilution and plating.

About 13 mg of dry surface-disinfected *O. aegyptiaca* seeds (up to 1,500) were sprinkled on wet Whatman® GF/A glass- fiber sheets (Whatman Int. Ltd., Maidstone, England) in each bag. The *O. aegyptiaca* seeds were conditioned for a 7 day period on the wet glass-fiber sheets. A tomato seedling with 3 or 4 expanded leaves and washed roots was fixed inside each polyethylene bag containing conditioned *O. aegyptiaca* seeds. The plant roots in each bag were moistened by capillary action with forty ml of modified Hoagland's solution in the base of each bag. Modified Hoagland's solution was replenished as needed. The polyethylene bags were then hung on metal frames wrapped with black plastic. Tomato plants were grown at a constant temperature of 25°C in a growth chamber. 14 h photoperiods were provided by a photosynthetically active light intensity of 65 µE/m^2/s (LI-COR, Inc., photometer, Model LI-188B) produced by six 40 W cool white fluorescent tubes suspended 35 cm

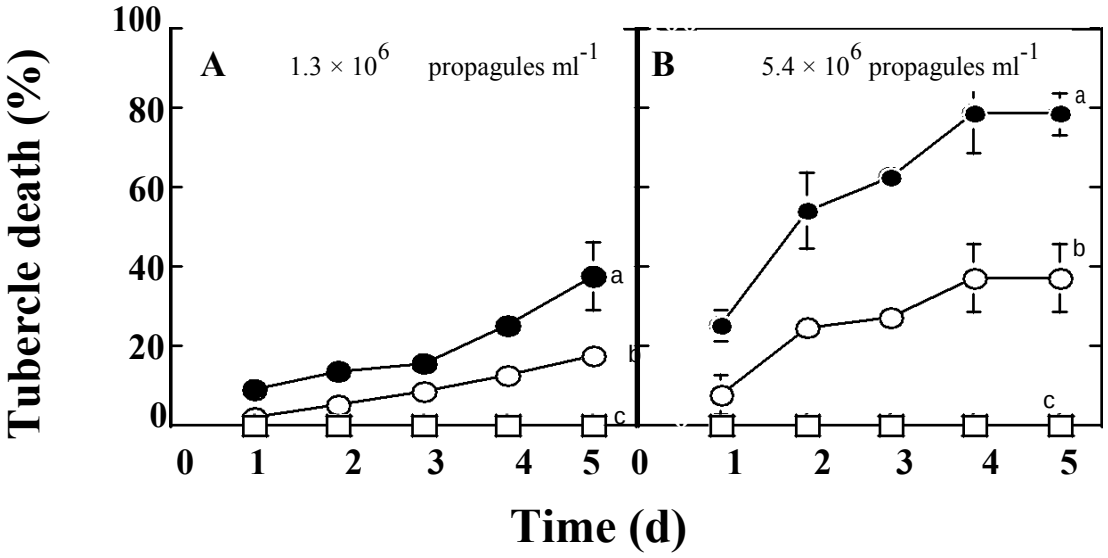

Figure 1. Cellulase enhances virulence of *F. arthrosporioides* on *O. aegyptiaca* tubercles attached to tomato roots. The infection kinetics of chopped *F. arthrosporioides* mycelia), and *F. arthrosporioides* mycelia plus cellulase (10 Uml^{-1}) mix on *O. aegyptiaca* tubercles. (A) 1.3 X 10^{6}; (B) 5.4 X 10^{6} propagules ml^{-1}. The controls, which gave the same responses, were tubercles mock-inoculated with water, or cellulase (10 Uml^{-1}). The trial was conducted twice, each treatment with three replications.

above the benches. 2 ml of 5 µg ml^{-1} GR-24 (synthetic germination stimulant) were added to each bag with a pipette to augment the tomato root exudates. This spreads by capillary action. The *O. aegyptiaca* seeds germinated, attached to tomato roots, and formed small tubercles during the following 2 weeks.

Allocation of treatment to *Orobanche*-infested tomato plants were in such a way that the tubercle numbers and sizes were almost the same. The virulence of the fungus was determined with and without various concentrations of cellulase (Cellulysin, ex *Trichoderma viride*, 10 Umg^{-1} Calbiochem-Behring Corp., La Jolla, CA 92037). The effect of cellulase concentration (10 - 20 Uml^{-1}) on tubercle death was similarly determined at a constant inoculum level. Thereafter, the virulence of the fungus with the enzyme was determined in combination at varying ratios. Control plants were mock-inoculated with either sterile distilled water containing 0.01% Tween 20 or 4 - 20 Uml^{-1} of single or combined enzyme preparations but without fungal mycelia. Tubercles on the tomato plants infested with *O. aegyptiaca* were counted and the diameters were measured with a ruler, with the assumption that the tubercles are perfectly spherical. The treatments consisted of *F. arthrosporioides* or *F. arthrosporioides* plus cellulase (4 - 20 Uml^{-1}). This work was carried out under containment.

MATERIALS AND METHODS

Data on *O. aegyptiaca* tubercle infection were collected at 24 h intervals after spray inoculation, for 8 - 11 days except where stated otherwise. Tubercles were visually scored as healthy (translucent, dense and intact), infected (diseased), or dead (black and soft). All experiments were performed at least twice, with at least 3 replications per treatment. Data are presented for the tubercles present at the time of inoculation. Values are means and standard errors of the means. Analysis of variance was done on tubercle death data and means were compared by Fisher's least-significant-difference (LSD) test, using a probability level of 0.05. Multiple mean comparisons were performed using Student- Newman-Keuls (SNK) multiple range test at P = 0.05. All analyses were performed using SAS statistical package (SAS, 2004).

RESULTS AND DISCUSSION

F. arthrosporioides plus cellulase was evaluated on tomato root systems to ascertain whether cellulase, a cell wall degrading enzyme, could accelerate fungus infection of *O. aegyptiaca* tubercles. Chopped mycelia (1.3 x 10^{6} and 5.4 x 10^{6} propagules ml^{-1}) plus cellulase (10 Uml^{-1}) increased *Orobanche* tubercles mortality compared to mycelia alone (Figures 1A and B). The short root-like structures from the *O. aegyptiaca* tubercles begin to turn brown, and softened gradually in the manner of wet rot in less than 24 h after spray inoculation. Cellulase treatment alone was ineffective but its presence aggravates fungal infection. Symptoms caused by *F. arthrosporioides* and *F. arthrosporioides* plus cellulase on *O. aegyptiaca* tubercles were similar, but the magnitude of infection produced by the latter was significantly higher (Figures 1A and B). *F. arthrosporioides* alone killed 17% of *Orobanche* tubercles when inoculated with 1.3 x 10^{6} propagules ml^{-1} (Figure 1A). The addition of cellulase to

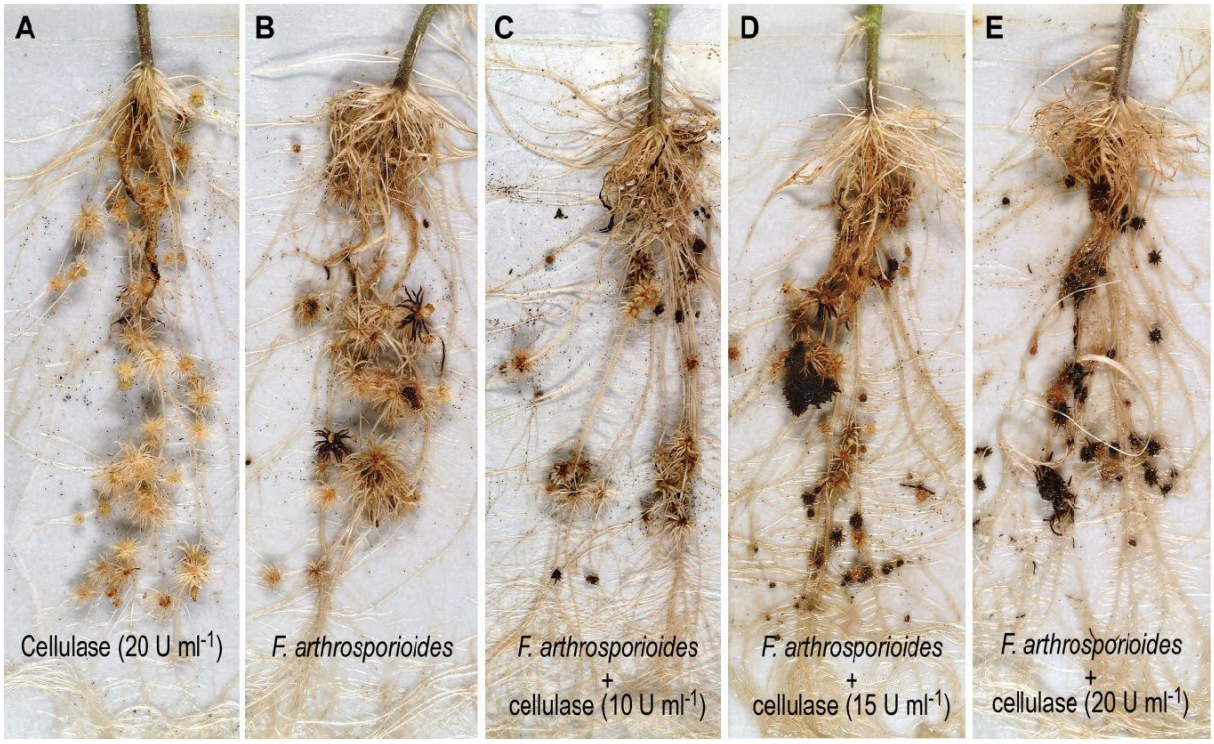

Figure 2. Increasing cellulase concentration added to chopped *F. arthrosporioides* mycelia increases *O. aegyptiaca* tubercle death. Roots of tomato plants infested with *O. aegyptiaca* were sprayed to runoff with (A) 20 Uml $^{-1}$ cellulase as a control treatment; (B) 1.2 x 10 ^{6} propagules ml^{-1} homogenized mycelia alone; (C) mycelia plus 10 Uml^{-1} cellulase; (D) mycelia plus 15 Uml^{-1} cellulase; and (E) mycelia plus 20 Uml^{-1} cellulase. Each plant bore an average of 25±2 healthy tubercles at inoculation. The pictures are from the same experiment. The experiment was carried out three times with a similar trend. The pictures shown were obtained 5 days after fungal inoculation.

this inoculum just before spraying led to 37% tubercle death. Similarly, *F. arthrosporioides* (5.4 x 10^{6} propagules ml^{-1}) combined with 10 Uml^{-1} cellulase killed more *Orobanche* tubercles (78%) than *F. arthrosporioides* alone (37%) (Figure 1B).

The *Orobanche* were at 3 main phases of their life cycle (seed, germination and parasitic phases) at fungal inoculation. Tubercles are usually present at the germination phase and later become parasitic. In this study, the tubercles were mostly at the germination phase. In total, these results demonstrate that exogenous enzyme can contribute to pathogenicity. The mycelia plus enzyme mix killed some *O. aegyptiaca* tubercles, thus they could not form new seeds to replenish the *Orobanche* seed bank in the soil.

This study attempted to elucidate enzyme concentration effect on fungal virulence. A 20 Uml^{-1} cellulase in water is injurious neither to *O. aegyptiaca* tubercles nor to tomato roots (Figure 2A). *F. arthrosporioides* at an inoculum level of 1.2 x 10^{6} propagules ml^{-1} of chopped hyphae killed 25% *O. aegyptiaca* tubercles (Figure 2B). Combinations of *F. arthrosporioides* and cellulase concentration of 10 Uml^{-1} resulted in 33% tubercle death (Figure 2C). In the presence of 15 Uml^{-1} cellulase at the same inoculum level 46% tubercles were killed (Figure 2D). However, 68% death was reached in the presence of 20 Uml^{-1} cellulase (Figure 2E).

Cellulose is the major constituent of the lignocellulosic materials in plants and consists of linear -1,4- linked D-glucose residues responsible for strength and flexibility (De Vries and Visser, 2001). Thus the exogenous cellulase mixed with *F. arthrosporioides* has advantage over the fungi alone in causing tubercle death by breaking the linear - 1,4-linked D-glucose residues. The enzyme substrate range and mechanism of action could partly explain the interaction between *F. arthrosporioides* and the enzyme on *O. aegyptiaca* tubercles. Besides, cellulase was ordinarily not able to injure and kill *O. aegyptiaca* at the concentration used (max. 20 Uml^{-1}), demonstrating that the *O. aegyptiaca* tubercle infection and death observed are due to the addition of a mycoherbicidal organism. Nature has the answer to the physicochemical reactions that occur between fungi and enzyme, for example, grass was degraded with fungal capable of producing cellulases and hemicellulases. The fungal strains were able to grow because of the soluble sugars and proteins, present in the grass cell (Hallesmeersch and Vandamme, 2003). The role of cellulase in the degradation of cell wall material as observed in this study corresponds with the reports of many

authors (Esquerre-Tugaye et al., 2000; Lebeda et al., 2001; Wanjiru et al., 2002; Hallesmeersch and Vandamme, 2003; Chanliaud et al., 2004). The latter fact adds to the commercial value of *F. arthrosporioides* as a potential mycoherbicide when sufficiently virulent.

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