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Research Article

Exogenous Cellulase Contributes to Mycoherbicidal Activity of *Fusarium arthrosporioides* on *Orobanche aegyptiaca*

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This paper investigates an association between the tubercle size of *Orobanche aegyptiaca*, tubercle death, and days to tubercle death in relation to cellulase-assisted mycoherbicide. *Fusarium arthrosporioides* killed 56% of tubercles when applied with cellulase compared to 35% when no cellulase was added. Death was inversely correlated with days over the two fungal treatment types. *O. aegyptiaca* tubercle size significantly correlated with the two other infection parameters studied. For *F. arthrosporioides*, only 9% (r^2) of the variation in days to death was explained by variation in tubercle size, whereas with cellulase it reaches 14%. In this study, mycelia of *F. arthrosporioides* did not show apparent damage to the tomato roots.

1. Introduction

Orobanche spp. (broomrapes) are holoparasitic flowering plants, penetrating roots of susceptible hosts, removing water, minerals, and sugars. *Orobanche* attacks several crops causing enormous yield losses [1]. *Orobanche* generally maintains a close relationship with the host and so it is unreasonable to attack it using herbicides because the latter may adversely affect the nontarget host. Despite research on *Orobanche* spp. for over three decades, yield losses still abound because there is no sustainable method for controlling the parasite. Several weed biocontrol agents are not sufficiently virulent for field release [2]. Since hydrolytic enzyme plays an important role in the pathogenicity of plants by facilitating fungal penetration through the host cell wall [3, 4], it may be worthwhile to attempt coinoculation of enzyme and fungi. A series of experiments were conducted using *Fusarium arthrosporioides,* a biological control agent that infects *Orobanche* without affecting the roots of tomato. Experiments using mycoherbicidal organisms plus pectinase enzyme [5] indicate that the enzymes enhance the weed control of a pathogenic fungus. The role of cellulase for plant pertains to modifing cellulose assemblies that are the dominant component of the plant cell wall [6]. Here, cellulase has been used to improve 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 *O. aegyptiaca* tubercle size best described the relationship between tubercle death and biological control treatment. The addition of cellulase alone or in mixtures enhanced the virulence of *F. arthrosporioides* on *O. aegyptiaca*.

2. Materials and Methods

2.1. Fungal Inoculum Production. F. arthrosporioides was cultured on potato glucose agar (PDA, Pronadisa) in Petri dishes incubated at 25°C. Subcultures were grown in 100 ml potato glucose broth (PDB, Pronadisa) in 250 ml Erlenmeyer flasks. Chloramphenicol (50 mg l^{-1}) was added to the broth to restrict possible bacterial contamination. The cultures were left on a rotary shaker (Brunswick Scientific) at 150 rpm for 48 h. *F. arthrosporioides* mycelia 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 (7×10^6 propagules ml^{-1}) were estimated after serial dilution and plating.

2.2. Plant Material. About 13 mg *O. aegyptiaca* seeds in small bags formed of Miracloth were wetted and surface sterilized in 80% ethanol for 1 min and in a mixture of 1% sodium hypochlorite in 0.01% aqueous Tween 20 for 10 min. The seeds were rinsed three times in sterile distilled water. Surface sterilizing seeds ensure that the fungal infection on the seeds is from deliberate infection.

2.3. Semiaxenic Polyethylene Bag Experiments. The pathogenicity of *F. arthrosporioides* was tested in the semiaxenic polyethylene bag system of Parker and Dixon [7]. About 13 mg of dry surface-disinfected 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 three or four 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 mls 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. Fourteen-hour photoperiods were provided by a photosynthetically active light intensity of 65 $\mu E/m^2/s$ (LI-COR, Inc., photometer, Model LI-188B) produced by six 40 W cool white fluorescent tubes suspended 35 cm above the benches. Two mls of 5-$\mu g\,ml^{-1}$ 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 was in such a way that the tubercle numbers and sizes were almost the same. All the roots were inoculated using an atomizer with suspensions of chopped mycelia. The virulence of the fungus was determined with and without various concentrations of cellulase (Cellulysin, ex *Trichoderma viride*, 10 U mg^{-1} Calbiochem-Behring Corp., La Jolla, CA 92037). The effect of cellulase concentration (10 to 20 U ml^{-1}) on tubercle death was similarly determined at a constant inoculum level. Control plants were mock-inoculated with either sterile distilled water containing 0.01% Tween 20 or 4 to 20 U ml^{-1} of cellulase 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 to 20 U ml^{-1}). This work was carried out under containment.

2.4. Fungal Reisolation from Infected Tubercles. Some putatively infected tubercles removed 2 days after spray inoculation were surface-sterilized by immersion in 1% sodium hypochlorite in 0.01% aqueous Tween 20. The tubercles were shaken in three changes of sterile distilled water and following Koch's postulates; the infecting fungus was isolated by plating on PDA, retested, reisolated, and compared to the strain used. *F. arthrosporioides* is distinguished from other *Fusarium* spp or fungal contaminants by the appearance of the reddish-brown aerial hyphae and pigmentation [8].

2.5. Data Collection and Statistical Analyses. Data on *O. aegyptiaca* tubercle infection were collected at 24 h intervals after spray inoculation, for 8 to 11 days. 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 three 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. Pearson correlation analyses were performed to quantify the strength of association between tubercle size, tubercle death, and number of days to tubercle death. All analyses were performed using SAS statistical package [9].

3. Results and Discussion

Experiments were performed to ascertain whether there is an association between the tubercle size of *O. aegyptiaca*, tubercle death, and days to tubercle death. It was observed that *F. arthrosporioides* —— killed 35% of tubercles with an average size of 23 mm diameter in >7 days. With the addition of 20 U ml^{-1}cellulase, the fungus killed 56% of tubercles 29 mm in diameter in 6 days (Figure 1). The data indicated that *O. aegyptiaca* tubercles are more amenable to biological control when small in size (<5 mm). The tubercles die faster when cellulase (20 U ml^{-1}) is added to the chopped mycelial suspension. The correlation coefficient (r) between death and days to death was highest ($r = -0.95^{**}$) for *F. arthrosporioides* and for *F. arthrosporioides* plus cellulase (-0.92^{**}) followed by the correlations between tubercle size and days to death in *F. arthrosporioides* plus cellulase ($r = 0.38^{**}$) (Table 1). Death was strongly inversely correlated (≥ -0.92) with days over the two fungal treatment types. *O. aegyptiaca* tubercle size significantly correlated with the two other infection parameters studied. For *F. arthrosporioides,* the correlation between tubercle size and days to death was $r = 0.30$, indicating that only 9% (r^2) of the variation in days to death was explained by variation in tubercle size, whereas with cellulase r becomes 0.38, indicating 14% variation in days to death explained by variation in tubercle size. The correlation between *O. aegyptiaca* death (% value) and tubercle size was $r = 0.27$ (*F. arthrosporioides*) and 0.38 (*F. arthrosporioides* plus cellulase), indicating that the fungus plus enzyme mix had a higher factor contributing to the death of *O. aegyptiaca* than the fungus alone (Table 1).

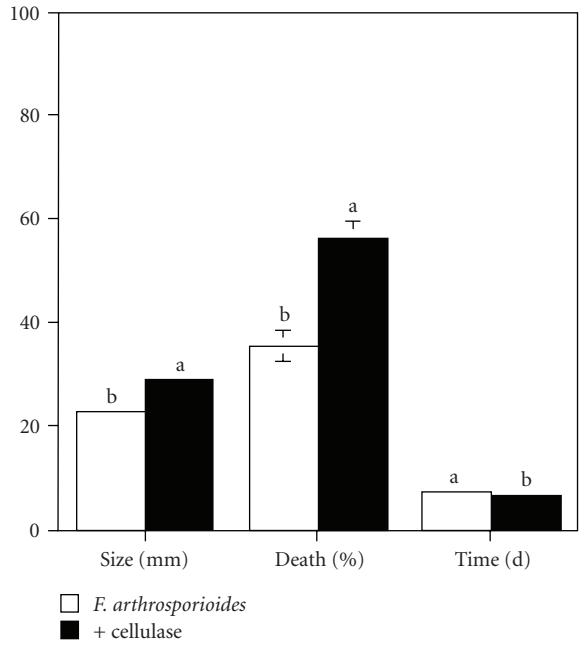

Figure 1: Tubercle size, corresponding percentage death, and number of days to death as observed for *F. arthrosporioides* (7×10^6 propagules ml^{-1}) □ and *F. arthrosporioides* plus 20 U ml^{-1} cellulase ■. Data represent means ± standard error for 278 replicate for *F. arthrosporioides* □ and 282 for *F. arthrosporioides* plus 20 U ml^{-1} cellulase ■. Tubercle size, % death, and the time means with different letters among treatments are significantly different according to Fisher's LSD test.

Table 1: Correlation coefficients (r) across two fungal treatments of *Orobanche aegyptiaca* tubercle size, death, and days to death.

Infection parameter	Treatment[a]	Correlation coefficient (r)		
		Size	Death	Day
Size	Mycelia alone	1.00		
	Mycelia + cellulase	1.00		
Death	Mycelia alone	−0.27**	1.00	
	Mycelia + cellulase	−0.38**	1.00	
Day	Mycelia alone	0.30**	−0.95**	1.00
	Mycelia + cellulase	0.38**	−0.92**	1.00

**Correlation is significant at the 0.01 level; [a]Mycelia = 7×10^6 propagules ml^{-1} of *F. arthrosporioides*; Cellulase = 20 U ml^{-1} cellulase.

Despite the fact that the average tubercle size investigated for *F. arthrosporioides* plus cellulase is higher (29 mm) than that investigated for *F. arthrosporioides* without any enzyme (23 mm), the trend holds that the addition of cellulase to the fungus accelerates the infection of *O. aegyptiaca* tubercles. It had been known that cellulase may not contribute to symptom expression, however, cellulase plays a role in penetration [5, 10] as also observed in this study. There is considerable evidence that cellulase is used by fungi [11] as part of their pathogenesis process. The strong correlations among tubercle size, death, and days to death indicate that the distribution of *O. aegyptiaca*-infested plants in research experiments plays an important role in observed tubercle mortality rate. The results in this study show that the extent to which tubercle size affects tubercle mortality is very high so that the mortality claimed in reported works on *O. aegyptiaca* whose tubercle sizes are largely significantly different may be misleading, as the mortality is relative to the tuber size of the *O. aegyptiaca* used at the study time. In this study, mycelia of *F. arthrosporioides* did not show apparent damage to the tomato roots. The latter fact adds to the commercial value of *F. arthrosporioides* as a potential mycoherbicide when sufficiently virulent. Future studies may want to know why, contrary to the findings associated with enhanced *F. arthrosporioides* infection on *O. aegyptiaca*, *F. oxysporum* pathogenicity on *O. aegyptiaca* was not enhanced by cellulase. Although *F. oxysporum* and *F. arthrosporioides* belong to the same genus, they may have different mycoherbicidal mechanisms.

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