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Antigenic Relationship between Two Bio-Agents and Pathogenic *Fusarium semitectum* Using Two Serological Techniques

Hassan M. Emara[1*] • Mostafa M. El-Shamy[2] • Mahmoud A. Swelim[1] • Mohammed A. Gad[2]

[1] Botany Department, Faculty of Science, Benha University, Benha, Egypt
[2] Wheat Disease Research Department, Plant Pathology Institute, Agriculture Research Center, Giza, Egypt
Corresponding author: * **hassanmemara@yahoo.com**

ABSTRACT

Wheat (*Triticum aestivum* L.) is an important crop cultivated successfully worldwide and in Egypt, which could be infected by *Fusarium* sp. Fungicides are generally used to control this pathogen. Nowadays, *Trichoderma* spp. and *Pseudomonas fluorescens* are used as biocontrol agents. In this study, a serological technique was carried out to explain the interaction between *Trichoderma viride*, *P. fluorescens* and the pathogenic fungus *Fusarium semitectum* using crossed-immunoelectrophoresis (CIE) and double diffusion reaction (DDR). In homologous reactions, the antigenic structures of *F. semitectum*, *T. viride*, and *P. fluorescens* were 7, 5 and 4 precipitin bands in CIE, while 2, 2, and 3 precipitin bands were detected in DDR, respectively. In heterologous reactions, antigens of *T. viride* electrophoresed against antibodies of *F. semitectum* gave two common bands between them in either of the used methods, while *P. fluorescens* antigens gave only one common precipitin antigen when electrophoresed against antibodies of the pathogen using both methods. This clearly indicated that *T. viride* was able to recognize to the pathogenic fungus *F. semitectum*, which showed more common antigens.

Keywords: biocontrol, *Fusarium*, *Pseudomonas*, serological techniques, *Trichoderma*

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important crops cultivated successfully in Egypt and worldwide. The importance of this crop is as a basic staple food for humans, in addition to its straw, which serves as important fodder for animals. This crop is subjected to infection by several diseases such as rusts, loose smut, powdery mildew, root rot, and wilt. These diseases cause tremendous loss by *Fusarium* sp. in different areas of the world (El-Nashar *et al.* 2000; Andres-Ares *et al*. 2004; Strausbaugh *et al.* 2004; El-Shamy 2006; Muthomi *et al*. 2008).

Fusarium semitectum is a widespread species often isolated from plants with complex disease and also known to be toxigenic (Zaccardelli *et al*. 2006; Pratt and Tewolde 2009). The control of this pathogen depends mainly on chemical fungicides that pollute the environment, disturb the ecological balances for all living microorganisms, and cause harmful effect for beneficial microorganisms (Hooda and Grover 1983). Biological control is increasingly becoming an important component of plant disease management and offers solutions to many of the persistent problems in agriculture (Cook and Baker 1983). The biological control of soil-borne plant pathogens by antagonists i.e. *Trichoderma* spp., *Gliocladium* spp., *Bacillus subtilis* and *P. fluorescens* is likely to be the best alternative to conventional chemical control methods.

Immunological techniques for the detection and identification of particular microorganisms are of great value because of the specificity of the reaction between the antigens of the organisms and the corresponding antibodies (Abs), which are produced in the serum of the animals inoculated with the organism (Ouchterlony and Nilsson 1978). Serological differences were frequently found between *formae* and races of fungi (Iannelli *et al*. 1982; Ala El-Dein and El-Kady 1985). The common features of these attempts were a few common antigens for comparison either between microorganisms or between pathogens and plants (El-Kazza *et al*. 1994, 1997; Meyer *et al*. 2000; Eibel *et al*. 2005; El-Shamy 2006).

The main objective of this study is to evaluate the potential use of two serological techniques, namely crossed-immunoelectrophoresis (CIE) and double diffusion reaction (DDR) to illustrate the relationship between two biocontrol agents i.e. *T. viride*, *P. fluorescens* and the pathogenic fungus, *F. semitectum*, causing rot and wilt diseases.

MATERIALS AND METHODS

Experimental design

This experiment consisted of three treatments where the antigen obtained from *F. semitictum*, *T. viride*, or *P. fluorescens* were injected in male Boscat rabbits (3 kg each; Faculty of Agriculture, Benha University, Benha, Egypt) and their antisera were obtained. The antigen-antiserum interaction was examined by CIE and DDR.

Crossed-immunoelectrophoresis

1. Preparation of antigens

The fresh biomass of either the pathogenic fungus or the biocontrol agents used were ground in a mortar with 20 g glass beads under $N_{2(l)}$ and diluted with HCl-Tris buffer (0.05 M) at pH 7.2 and kept overnight at 5°C in a refrigerator. The extractions were centrifuged at 10,000 rpm for 20 min. The supernatant of each was collected and protein content was adjusted to 20 mg/ml before injection into male Boscat rabbits according to Lowry *et al*. (1951).

2. Immunization and production of antisera

Antigens of either the bio-control agents or the pathogenic fungus were mixed with incomplete Freund adjuvant (1: 1 ratio). The mixed antigens-incomplete adjuvant was administered intramuscularly and subcutaneously at male Boscat rabbits. Each rabbit received a course of 10 injections (two per week); the volume of the

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Research Note

antigens was 0.5 ml for 5 injections and 1.00 ml for the last injections. The rabbits were bled by cutting the lateral veins of the ear 7, 9 and 11 days after the last injection. The blood was received in a sterilized test tube. The blood was left to clot at room temperature overnight. The clot was loosened from the wall of the tube with a fine glass rod, and then the tube of blood was kept at 4°C overnight to allow the clot to retract and express the straw-colored serum. The antisera were obtained and centrifuged at 10,000 rpm for 20 min to remove any precipitates and kept at 5°C. Sodium azide (0.02%) was added to prevent any contamination. The immunoglobulin was concentrated by ammonium sulphate (37% w/v). CIE was performed according to Axelsen *et al.* (1973) where 1% agarose gel (Litex, Glostrup, Denmark) was mixed with barbital buffer (pH 8.6; ionic strength 0.02). The first-dimension electrophoresis of the fungus and bio-control agents was performed at 12°C, applying 10V per cm for 60 min. The second dimension electrophorosis was run at 12°C, applying 3V per cm for 18 h through a gel containing 10 µL of rabbit Abs per cm^2. Dimensions of the plates were 10 × 10 cm in the first and 6 × 10 cm in the second. The non-precipitated proteins were removed by washing the gel with 0.1 µL NaCl for 24 h and distilled water for 1 h. After drying, the plates were stained for 10 min in a solution of 0.5% Comassie brilliant blue R-250 (Sigma, St. Louis, USA) in ethanol: glacial acetic: water (45: 10: 45). Excess dye was removed by repeated washing in destaining solution (ethanol: glacial acetic acid: water, 45: 10: 45).

Double diffusion test

DDR was carried out according to Ouchterlony and Nilsson (1978) with some modifications. Melted 2% ion-agar (Sigma, St. Louis, USA) in saline and supplemented with merthiolate (1: 10.000), was poured on to 5 × 5 cm glass slides (Al-Gomhouria Co. for Chemicals, Zagazig, Egypt) to obtain a layer of agarose 1-2 mm thick. Two wells were punched (5 mm in diameter for each and 15 mm distance between wells). One of the two wells was filled with antiserum of the pathogenic fungus, while the other one was filled with the antigen of each bio-agent individually. The glass slides were incubated at 30-32°C for 48-72 h and kept in humid conditions (85% relative humidity). The developing precipitin bands were visually examined to know the homologus and heterologous nature of antigens; the agarose slides were pressed by a heavy weight for 15 min, washed in saline solution (8.5 g NaCl/L distilled water), dried by a hair dryer (40°C), stained using Coomassie Brilliant blue R-250 (Sigma, St. Louis, USA) then destained in solution consisting of 96% ethanol: glacial acetic acid : water (45: 10: 45) and dried by a hair dryer. Precipitin bands were recorded and hand drawn (Hussein 1992).

RESULTS AND DISCUSSION

Table 1 and **Fig. 1** reveal homologous and heterologous reactions using the DDR test. In homologous reactions, antigens of *F. semitectum*, *T. viride* and *P. fluorescens* showed 2, 2 and 3 bands when electrophorized against their corresponding Abs. In heterologous reactions, antigens of *T. viride* gave two common bands when electrophorized against *F. semitectum* Abs, while one common band was detected when antigens of *P. fluorescens* electrophorized against *F. semitectum* Abs. Homologous reactions were carried out for each of *F. semitectum*, *T. viride*, and *P. fluorescens* to detect their antigenic structure, while heterologous reactions were performed to detect the common antigens between them.

Table 2 and **Fig. 2** reveal homologous and heterologous reactions using CIE. In homologous reactions, 7, 5 and 4 precipitin bands were detected when antigens of *F. semitectum*, *T. viride* and *P. fluorescens* were electrophoresed against their corresponding Abs, respectively. In heterologous reactions, two common bands were detected when antigens of *T. viride* were electrophoresed against *F. semitectum* Abs, while one common band was detected between antigens of *P. fluorescens* and *F. semitectum* Abs.

In several instances, it has been found that the relationship between organisms controlled by antigenic substances is termed a common antigen. The greater antigenic similarity between pathogens and their hosts or between any two organisms means that they completely recognize them, according to the definition of gene-for-gene concept (Flor 1971). *T. viride* showed more common antigen with *F. semitectum* (two bands), while *P. fluorescens* gave only one precipitin band. These results are agreement with the results of antagonism of *T. viride* and *P. fluorescens* against *F. semitectum in vitro* and *in vivo*. Homologus CIE was thus used to demonstrate the cross-reaction between *F. smitectium* and biological control, serological homogeneity within each group and an antigenic relatedness between the two (*Fusarium* and bio-control agents).

Table 1 Number of precipitin bands detected in homologous and heterologous reactions of *F. semitectum T. viride* and *P. fluorescens* using double diffusion reaction.

	No. of precipitation peaks detected among the isolate		
	F. semitectum	***T. viride***	***P. fluorescens***
Fusarium semitectum	2	2	1
Trichoderma viride	2	2	-
Pseudomonas fluorescens	1	-	3

Table 2 Number of precipitin bands detected in homologous and heterologous reactions of *F. semitectum T. viride* and *P. fluorescens* using crossed–immunoelectrophoresis technique.

	No. of precipitation peaks detected among the isolate		
	F. semitectum	***T. viride***	***P. fluorescens***
Fusarium semitectum	7	2	1
Trichoderma viride	2	5	-
Pseudomonas fluorescens	1	0	4

Table 3 Comparison between Double diffusion (DDR) technique and Crossed immuno-electrophoresis (CIE) technique.

	Homologous reactions	
	CIE technique	**DDR technique**
Fusarium semitectum	7	2
Trichoderma viride	5	2
Pseudomonas fluorescens	4	3

Table 3 compares CIE and DDR in detecting the antigenic structure of *F. semitectum*, *T. viride* and *P. fluorescens*. CIE showed in the most homologous reactions of *F. semitectum* (7 bands), *T. viride* (5 bands) and *P. fluorescens* (4 bands), while few precipitin bands were detected in the DDR test (2, 2, and 3 bands, respectively).

CIE was more sensitive than DDR in terms of precipitin band detection. It evidenced that electric migration is considered more active than normal diffusion. Similar results were obtained by Centurion and Kimati (1992) who used the Ouchterlony gel agar double-diffusion method for serological characterization of *F. moniliform* and *F. moniliform* var. *subglutinans* isolates. They found that this method appeared to be ineffective for serological characterization of these isolates. These common antigens may be considered as a specific antigen and may be used to define the serological homogeneity of the pathogen and bio-control agent. Serological classification on the basis of quantitative similarity may be supported by CIE. Both *T. viride* and *P. fluorescens* had an antigenic determinant in common with *F. semitectum*. Collectively, these studies suggest that pathogen and biological control have several common antigenic determinates. The information derived from such serological studies is considered of value in taxonomy (Jones and Krieg 1984) to define the several relationships between pathogen and their biological agent(s), for taxonomic purpo-

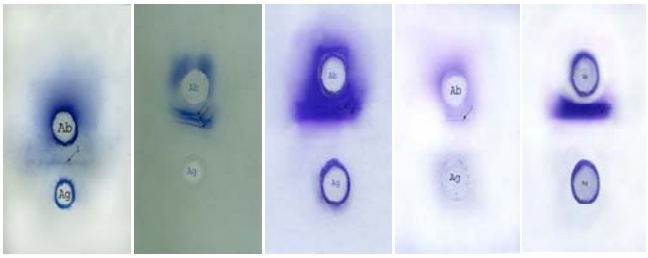

Fig. 1 Precipitation bands showing the antiserum reaction with its homologous and heterologous antigen using Ouchterlony DDR test. *F. semitectum* (F), *P. fluorescens* (P), *T. viride* (T), FP, and, FT, respectively.

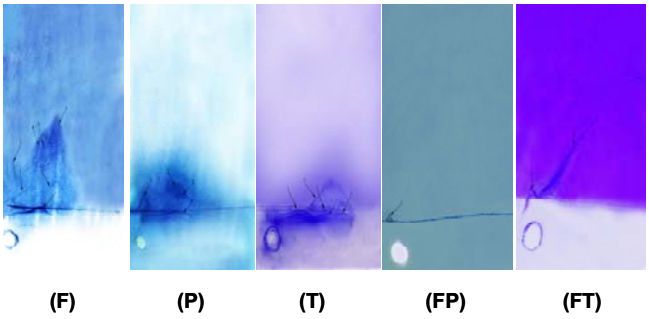

Fig. 2 Precipitation bands showing the antiserum reaction with its homologous and heterologous antigen using the CIE technique. *F. semitectum* (F), *P. fluorescens* (P), *T. viride* (T), FP, and, FT, respectively.

ses (Wroblewaki *et al*. 1977) or to obtain a precipitation pattern, which as far as possible reflects the complete protein composition.

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