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DEVELOPMENT OF ANTIBODIES FOR DETECTION OF THE COTTON APHID FUNGUS, *NEOZYGITES FRESENII*

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Abstract

The cotton aphid, *Aphis gossypii* Glover, is a serious pest throughout the Cotton Belt and is difficult to control by insecticides. *Neozygites fresenii* (Nowakowski), an obligatory fungus infects cotton aphid populations in the Mid-South. Cotton aphid populations usually begin to decline when the fungal infected aphid population reaches 15% and above. The timely identification of such fungal epizootics would aid the growers in making pesticide application decisions. Agdia, Inc. has developed a rapid and simple to use antigen coated plate enzyme-linked immunosorbant assay (ACP-ELISA) for the detection of fungal-infected cotton aphid.

Introduction

The cotton aphid, *Aphis gossypii* Glover, is an important pest on cotton. In addition to the feeding damage, it deposits excretions known as "honey-dew" onto cotton fibers. This "sticky cotton" causes major problems in textile mills resulting in a lower price to producers (Reed et al. 1999).

Cotton aphids are very difficult to control with currently labeled foliar insecticide treatments and many aphid populations are resistant to insecticides. Due to their parthenogenetic reproduction, resistant populations can reach very high levels within a growing season. Cotton aphid populations in the Mid-South are subject to epizootics of a naturally occurring fungus, *Neozygites fresenii* (Nowakowski) which consistently appears during mid July. The fungus causes drastic declines in aphid populations, and frequently provides effective population suppression for the remainder of the season (Steinkraus et al. 1992). Research indicates that when at least 15% of the aphids in a field are infected a rapid aphid decline can be expected (Steinkraus and Boys 1997). Coupling this information with accurate sampling of aphids and a fast, easy way to diagnose the prevalence of fungus in aphid populations increases the possibility of reducing insecticide treatments when epizootics can be detected early.

Currently, the University of Arkansas operates an extension-based sampling program for determining fungal prevalence in individual fields throughout the Mid South by sampling the aphids from cotton fields (Steinkraus, 1998). The sampling procedure usually involves examination of a sub-sample of 50 aphids from a sample of 500 aphids collected per field. The infected aphids are currently identified by microscopic observation, which is time consuming and requires special technical skills and equipment. However, use of an immunoassay utilizing the antibodies specific for *N. fresenii* would allow rapid sample processing and may not require special skills. Agdia, Inc. in collaboration with the University of Arkansas developed an ACP-ELISA for detection *N. fresenii* infected aphids in individual and composite aphid samples.

Materials and Methods

Preparation of immunogens: An epizootic of *N. fresenii* in a commerical cotton field in Portland, Arkansas was identified and the leaves with aphids were harvested, and placed on wire racks in ice chests containing either silica gel or rock salt and brought to University of Arkansas. Each aphid was examined under a dissecting microscope for the presence of *N. fresenii* and infected aphids in the late hyphal body stage were collected and sent to Agdia for production of monoclonal antibodies. Healthy cotton aphids were reared in a University of Arkansas green house. Infected aphids were dissected in PBS pH 7.4 and the fungal hyphae and conidia were collected with the aid of a pipette and resuspended in PBS. The prepared antigen solution was divided into 250ul aliquots and frozen until further use.

Production of monoclonal antibodies: Five Balb/c mice (10 week old) were injected three times at monthly intervals with 50ul of antigen. Test bleeds from the mice showed presence of antibodies to both aphid fungus and aphid. The test bleeds were cross-absorbed with healthy aphids and immunoblots were performed to select the mice for generation of monoclonal antibodies. The mouse received 100ul of antigen as a final booster and the hybridoma fusion was performed on the fourth day. Hybridoma cell lines were screened with both infected and healthy aphids using antigen-coated-plate indirect ELISA (ACP-ELISA).

Development and optimization of ELISA: An indirect ACP-ELISA was developed and optimized for detection of 1 infected aphid plus 4 negative aphids. This would allow testing composite samples of 5 aphids. The assay can be done in 5 hours including the 2-hour sample incubation. Alternatively, samples can be incubated overnight. For diagnosis of individual infected aphids, a shorter protocol was developed and optimized. Using this protocol the test can be completed in 1.5 hours (Figures 1 & 2).

Results

Three monoclonal cell lines were selected based on their specific reactivity to the infected aphids and are subsequently cloned. These cell lines were further sub-cloned to homogeneity, isotyped and designated as AGD 206, AGD 207 and AGD 208. The cell lines were scaled-up and the supernatant was harvested. The supernatants were centrifuged to remove the cell debris, filter sterilized and used for the development of ELISA. Experiments were conducted to identify and optimize the antibody dilution buffer and the optimum dilution of the supernatants was found to be at 1:500. The principle of antigen extraction and ELISA is shown in Figure 1. Mixing of the three monoclonal antibodies does not have any synergetic affect on the test signal (Table 1).

The test can differentiate a *N. fresenii* infected aphid from healthy aphid. In a blind test the test identified all the infected aphids from healthy (Table 2). With the help of a motorized pestle and mortar, one can process 2 sub samples of 50 each in about 3 hours. The test can also be used to process composite samples of five aphids by increasing the antigen and antibody incubation time from 30 minutes to two hours. The test also accommodates an overnight incubation protocol and did not cross react with any saprophytic fungi tested (Table 3).

Acknowledgement

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Table 1. Absorbance values at 450 nm for individual monoclonal antibodies.

	Mix	AGD 206	AGD 207	AGD 208
2 Infected Aphids/ml	0.347	0.368	0.359	0.293
10 Infected Aphids /ml	0.610	0.685	0.611	0.523
10 Healthy Aphids/ml	0.027	0.027	0.023	0.018

Table 2. Absorbance values at 450 nm for individual aphids in a blind test.

Aphid	Code	OD value at 450 nm
A	Positive	0.468
C	Positive	0.481
I	Negative	0.040
O	Negative	0.027
P	Negative	0.052
R	Positive	0.252
V	Positive	0.612
X	Negative	0.019

Table 3. Absorbance values of composite samples at two different protocols.

	2hr/2hr/1hr protocol	Overnight/2hr/1hr protocol
2 infected aphids in 8 healthy aphids/ml	0.515	0.533
2 infected aphids in 8 healthy aphids/ml	0.534	0.516
2 infected aphids in 8 healthy aphids/ml	0.704	0.714
10 healthy aphids/ml	0.034	0.045
10 healthy aphids/ml	0.035	0.051
10 healthy aphids/ml	0.038	0.062
Buffer	0.032	0.065
Penicillin spp. Aspergillus spp.	N/A	0.041

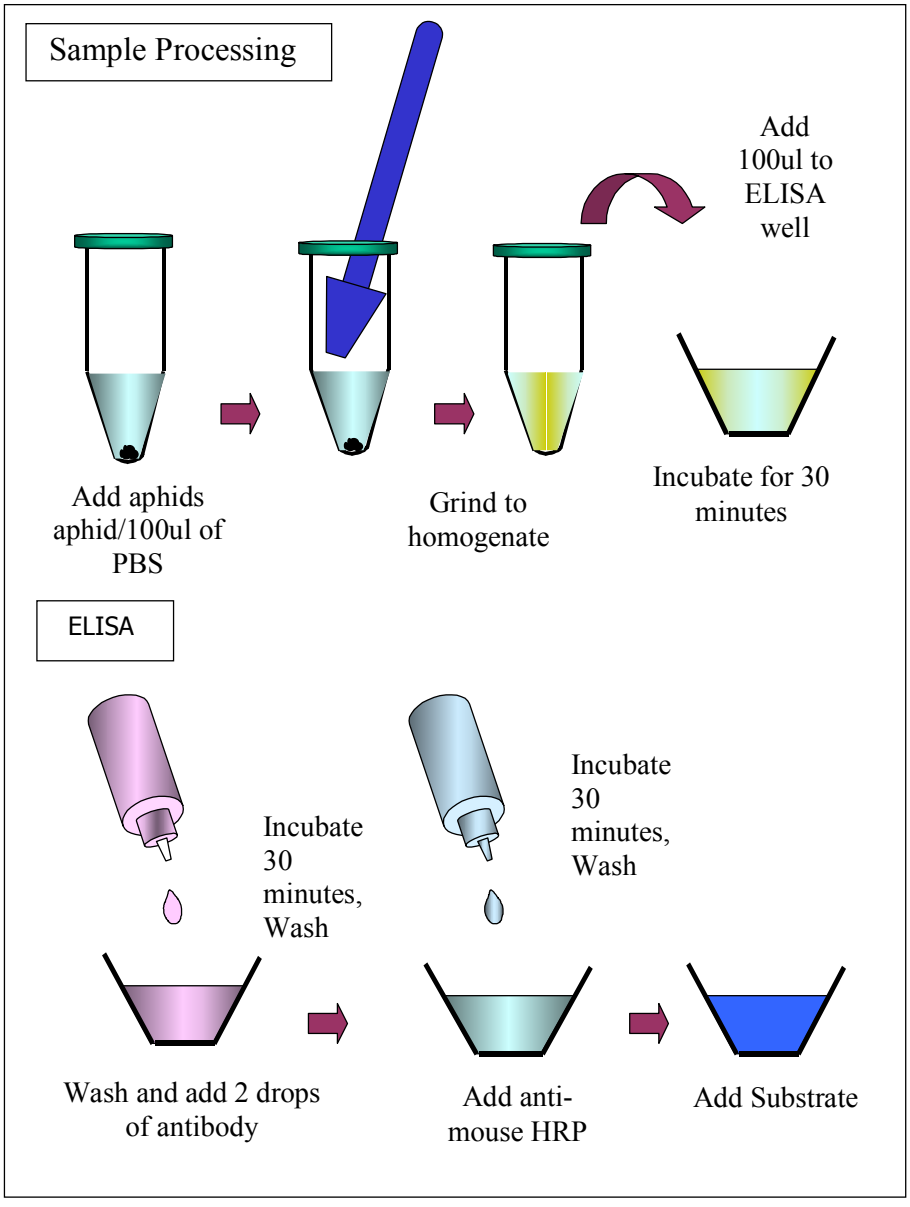

Figure 1. Schematic diagram of ACP-ELISA protocol.

1. Grind the aphids at a ratio of 100ul per aphid in phosphate buffered saline pH 7.4. The aphids can be tested in composites up to 5 aphids per sample. The best grinding method so far has been with small blue plastic pestles in microcentifuge tubes that match the shape of the pestle so it is a tight fit. If doing large numbers of samples an electrical tool that can attach to the blue pestles and turn them would be advantageous.
2. Pipette 100ul of aphid extract into each test well. Incubate the plate in a humid box either overnight at 4C or for 30 minutes at room temperature.
3. Wash the plate 8 times with PBST, let the plate soak for 1 minute on the last wash and tap dry on paper towel.
4. Squeeze 2 drops from bottle A into each test well and incubate in a humid box for 30 minutes at room temperature.
5. Wash the plate 4 times with PBST and tap dry on paper towel.
6. Squeeze 2 drops from bottle B into each test well and incubate in a humid box for 30 minutes at room temperature.
7. Wash the plate 8 times with PBST, let the plate soak for 1 minute on the last wash and tap dry on paper towel.
8. Add 2 drops per test well using the bottle of TMB substrate solution. Allow the plate to incubate for 10 to 15 minutes. It is not necessary to use a humid chamber at this point. Test wells that are negative will remain clear. Test wells that are positive will turn blue in color. A plain white piece of paper under the ELISA plate will aid in viewing the results.

Figure 2. Instructions for the ELISA test kit to detect the fungus *Neozygites fresenii* in the cotton aphid, *Aphis gossypii.*