

Evaluation of a Lateral Flow Device for the Early and Reliable Field Detection of *Phakopsora pachyrhizi*

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INTRODUCTION

Asian Soybean Rust (ASBR), caused by the fungus *Phakopsora pachyrhizi*, may result in soybean yield losses of up to 80%. Accurate and timely diagnosis of ASBR is the key to determining whether a response will be attempted, and if so, the extent, direction and magnitude of that response. To date, a reliable way to diagnose ASBR in the field is the identification of the small (2-5mm) tan to dark brown-spore lesions that occur days later after infection. Attempts to identify the disease before sporulation takes place are difficult since symptoms could be confused with other common soybean diseases.

A Lateral Flow Device (LFD) test was developed to assist in the diagnosis of this disease in the field prior to the development of mature pustules.

The performance of the test was assessed against diseased soybean leaves inoculated with spores of ASBR. Leaves of the V1-V2 stage were inoculated with two levels of spores (high and low) for 11 consecutive days. Inoculated leaves/plants on a given day were considered a set. Each set, after inoculation, was transferred to a growth chamber to allow the infection process to continue under a controlled environment for the duration of the experiment.

On the 11th day, only non-symptomatic leaves and leaves showing chlorotic lesions were assessed with the LFD by two sampling procedures, Mesh Bag Extraction and Snap Cap Punch. These leaves corresponded to those sets inoculated on days 5-11. The levels of visual infection observed on the tested sets were in the order 0.2-0.5% (low level of spores) and 10-15% (high level spores). The LFD was able to detect the presence of ASBR on chlorotic lesions and to some extent on non-symptomatic tissue.

MATERIALS

1. Lateral flow device LFD (QuickStix™ Strip for Soybean Rust, manufactured by EnviroLogix Inc.).
2. Dew chamber at 20°C @ 100% RH.
3. Growth chamber at 22°C and 12-hour light photo period.
4. 44 healthy soybean plants at V1-V2 stage (hereinafter the "Healthy Plants") presenting at least one trifoliate expanded leaf (~10-15 days old).
5. Fresh inoculum supplied by 20 soybean plants at the V1-V2 stage ("Source Plants"). Inoculum was collected on a daily basis.
6. Vials to collect and quantify the spores from the infected leaves.
7. Hand-held spray devices, used for: (a) spraying spores onto healthy soybean leaves; (b) removing excess spores from the inoculated leaves.
8. Microscope and hemacytometer to count spores.
9. Level of spores: High (HD) = 5×10^5 spores/ml. Low (LD) = 1×10^5 spores/ml.
10. LFD device reader (QuickStix Reader), which measures relative light absorbance of Test Line compared to Control Line and gives a reading of "mean reflectance value".

METHODS

Spore Collection and Quantification

Spores were collected from heavily infected soybean leaves. Leaves were held over a funnel placed in a centrifuge vial. The leaves were sprayed with clean fresh water to wash loose ASBR spores into the vial.

The vial with the water-spore suspension was centrifuged at 2000-3000 G for 5 minutes. Spores were collected at the bottom. The excess water was removed and 0.02% inoculation buffer (water-Tween-20) was added to re-suspend the spores.

The number of spores was counted under the microscope with a hemacytometer. Buffer was added to adjust the level of spores to 5×10^5 spores/ml and 1×10^5 spores/ml.



Inoculation

The undersides of the leaves were sprayed with the spore suspension ensuring that the leaves were fully covered. Plants were labeled according to the inoculation level and the inoculation day.

Plants, after inoculation, were transferred to a dew chamber for 18 hours, after which they were sprayed with RoH_2O to remove any remaining un-germinated spores. The plants were transferred to a growth chamber where they remained until day 11 when they were all assessed.

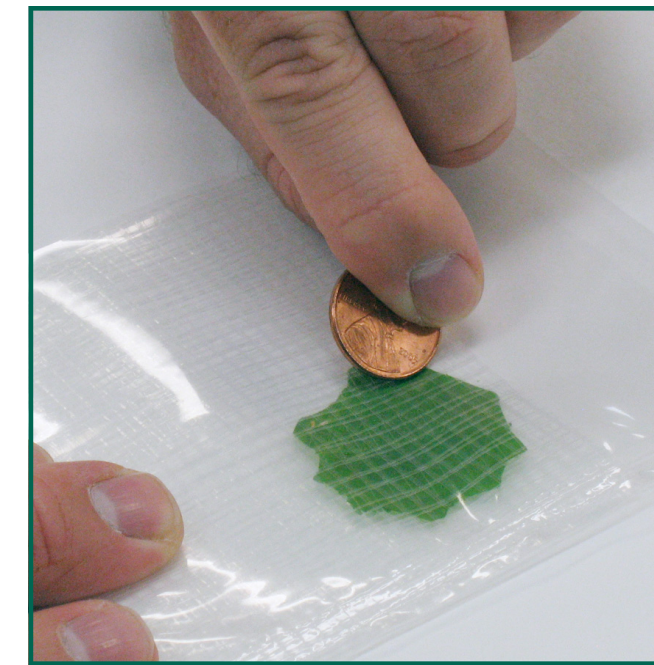
LFD Testing

All the LFD tests were performed on the 11th day. Only those leaves/plants sets presenting non-visible symptoms and chlorotic lesions were assessed (sets inoculated on days 5-11).

Leaves were separated and labeled according to inoculation level and day of inoculation. Leaves were tested using the following protocols: Mesh Bag Extraction and Snap Cap Punch. Healthy tissue was included as a Negative Control in both protocols.

Mesh Bag Extraction Protocol

Sections of leaf tissue of approximately 1½" x 1½" were selected and placed in a mesh bag. The leaf sample was macerated by rubbing with a pestle or other hard object (coin) against a hard surface to force the mesh through the tissue. Ten ml of Extraction Buffer was added directly into the mesh bag and massaged to mix buffer and leaf tissue. Five hundred µL of extract was transferred from the mesh bag to a reaction vial. A QuickStix Strip for Soybean Rust was inserted in the vial, and after 10 minutes was removed and evaluated.



Snap Cap Punch Protocol

Sections of leaf tissue were sandwiched between the cap and body of the Disposable Extractor Tube (as pictured). Two sections of tissue per tube were used for this type of assay. The tissue was ground for 20-30 seconds by inserting a pestle into the tube and grinding it by rotating the pestle against the insides of the tube with twisting motions. Five hundred µL of Extraction Buffer was added to the tube. A QuickStix Strip for Soybean Rust was inserted in the vial, and after 10 minutes was removed and evaluated.

Interpreting the Results

The LFDs were evaluated visually according to Figure 1. If the sample extract contained Soybean Rust spores, a line (Test Line) developed on the membrane strip between the Control Line and the protective arrow tape. The results were interpreted as positive for *Phakopsora spp.* If no Test Line was observed after 10 minutes, the results were interpreted as negative for the sample tested.

Development of the Control Line within 10 minutes indicates that the strip has functioned properly.

The LFDs were also evaluated using a QuickStix Reader. The strip reader uses an LED and photodiode to measure reflected light. This reflected light will be proportional to the Test Line intensity.

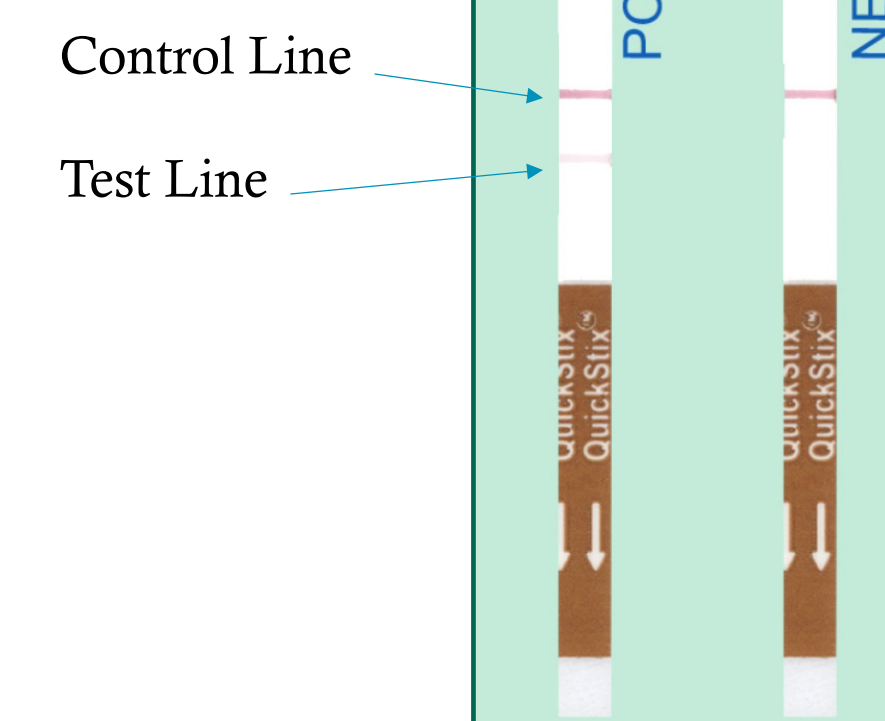


Figure 1

RESULTS

Sensitivity and Specificity of the Test

The antibodies used for the development of the LFD test were evaluated for sensitivity and specificity. Different spore concentrations of ASBR pathogen were evaluated in both ELISA plates and LFD formats. Both formats demonstrated the antibodies to have a good detection level, with the ELISA plate detecting ~200 spores and the LFD ~3000 spores (a new version of the LFD detects ~1500 spores/ml).

Additionally, the cross reactivity of the LFD was assessed against the six most common soybean diseases that could be confused at some point with symptoms encountered in the field (Table 1). No cross reactivity was found to any of them.

Table 1. Cross Reactivity

Organism	Sample Type	Reactivity
<i>Cercospora sojini</i> (Frog Leaf Spot)	Culture	None
<i>Cercospora kikuchii</i> (Seed Stain and Leaf Blight)	Culture	None
<i>Septoria glycines</i> (Brown Spot)	Culture/field samples	None
<i>Peronospora manshurica</i> (Downy Mildew)	Field samples	None
<i>Pseudomonas savastanoi</i> pv. <i>Glycinea</i> (Bacterial Pustule)	Field samples	None
<i>Xanthomonas axonopodis</i> pv. <i>Glycines</i> (Bacterial Pustule)	Culture	None

Visual Assessment

On day 11, all plant sets were visually rated for percentage of leaf infected area (LIA) using a common scale for ASBR. Symptoms began to appear on plant sets inoculated on days 1-6, but not on those inoculated on days 7-11. Lower level of infection (lesions) were present on leaves from sets inoculated on days 5-6. Those inoculated with low levels of spore presented approximately 0.2%-0.5% LIA, and those sets inoculated with high levels presented about 10%-15% LIA.

LFD Results

Mesh Bag: At low levels of inoculation, leaves inoculated on days 7-11 were negative, but strong signals were obtained from leaves inoculated on days 5 and 6. At high levels, leaves inoculated on day 11 were negative and leaves inoculated on days 5-10 were positive (Table 2).

Snap Cap: At low levels of inoculation, leaves inoculated on days 8-11 were negative and those from days 5-7 were positive (Table 2).

Summary:

Both methods detected the presence of the ASBR pathogen on symptomatic tissue, and to some extent on non-symptomatic tissue.

At a high concentration of inoculum, the target protein may affect the results by reducing both the reading values and the strength of signal at the test line. This is clearly reflected on the results obtained from leaves inoculated with high levels of spores on days 5 and 6 (Table 3). At higher levels, the mean reflectance values seem to be lower than those of the low level of inoculation. This reduction is called the hook effect. In order to obtain the maximum signal, samples were diluted 1:16 with additional buffer.

Table 2. Summary of LFD Testing Results

High Level of Inoculation			
Inoculation day	Visual Infection	Mesh Bag—LFD Results	Snap Cap—LFD Results
1	Pustule	Not tested	Not tested
2	Pustule	Not tested	Not tested
3	Pustule	Not tested	Not tested
4	Pustule	Not tested	Not tested
5	Chlorotic Lesion	positive	positive
6	Chlorotic Lesion	positive	positive
7	Chlorotic Lesion	positive	positive
8	No lesion	positive	positive
9	No lesion	positive	positive
10	No lesion	positive	positive
11	No lesion	negative	negative
Low Level of Inoculation			
Inoculation day	Visual Infection	Mesh Bag—LFD Results	Snap Cap—LFD Results
1	Pustule	Not tested	Not tested
2	Pustule	Not tested	Not tested
3	Pustule	Not tested	Not tested
4	Pustule	Not tested	Not tested
5	Chlorotic Lesion	positive	positive
6	Chlorotic Lesion	positive	positive
7	Chlorotic Lesion	negative	positive
8	No lesion	negative	negative
9	No lesion	negative	negative
10	No lesion	negative*	negative
11	No lesion	negative	negative

Table 3. Comparison of Mean Reflectance Values (MRV) of Mesh Bag Extraction

Inoculation Day	5	6	7	8	9	10	11	Negative
Low Level MRV	80	101.7	Negative	Negative	Negative	Negative*	Negative	Negative
Symptom	Chlorotic 0.2-0.5%	Chlorotic 0.2-0.5%	No symptoms	No symptoms	No symptoms	No symptoms	No symptoms	No Symptoms
Inoculation Day	5	6	7	8	9	10	11	Negative
High Level MRV	^51.00	^42.40	66.23	30.00	76.65	40.94	*Negative	Negative
Symptom	Chlorotic 10-15%	Chlorotic 5-10%	No symptoms	No symptoms	No symptoms	No symptoms	No symptoms	No symptoms

*Light shadow on the stick due to over-inoculation or possibly poor washing.

^ Hook Effect. Maximum color at approximately 1:16 dilution.

SUMMARY OF RESULTS

The LFD device is capable of detecting ASBR before the formation of pustules. The higher the level of inoculation, the greater the chances of detection: as early as 24 hours after infection/deposition.

No cross reactivity was found with other common soybean diseases.

Similar results following the same protocols were obtained in August 2006 by a leading South American agricultural research and development institute.