

Table 2. Summary of LFD Testing Results

High Inoculation Level = 500,000 spores/mL			
# of Days after Inoculation	Visual Infection Level	Results: Mesh Bag Extraction	Results: Snap Cap Extraction
4	No Lesions	Positive	Positive
5	0 → 35% Chlorotic Lesions (no pustules)	Positive across the symptom range	Positive
6	Chlorotic Lesions - 40-50% (no pustules)	Positive	Positive
Low Inoculation Level – 100,000 spores/mL			
4	No Lesions	50% of Samples tested ran positive	Positive
5	No Lesions	Positive	Positive
6	Chlorotic Lesions – 40-50% (no pustules)	Positive	Positive

Table 3. Comparison of Test Line Mean Reflectance Values (MRV) between the Generation #1 Format (Paby) and the new Generation #2 Format (Maby)

High Inoculation Level = 500,000 spores/mL			
# of Days after Inoculation	Visual Infection (Chlorotic Lesions)	MRV Generation #1	MRV Generation #2
4	No Lesions	142.74	581.71
5	25-35%	103.23*	817.5
6	40-50%	151.05	822.19
Low Inoculation Level – 100,000 spores/mL			
4	No Lesions	25.74	92.55
5	No Lesions	38.26	211.11
6	40-50%	165.50	827.30

* With the Generation #1 device format this sample was showing a hook effect. No hook effect was observed with the Generation #2 format.

SUMMARY

- The new Generation #2 LFD, incorporating the use of a Maby, gave significantly higher positive signal intensities across the detection range. A second advantage with the new format is the absence of any hook effect (as seen with the Generation #1 format) when running strongly positive samples.
- No cross reactivity was found with other common soybean diseases.
- The LFD device is capable of detecting ASBR before the formation of pustules. The higher the level of inoculation, the greater the chances of asymptomatic detection.

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A Monoclonal-based 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 (2-5 mm) tan to dark brown-sporulating lesions that occur days after infection. Attempts to identify the disease before sporulation are difficult, since symptoms could be confused with other common soybean diseases.

A polyclonal antibody (Paby) -based lateral flow device (LFD) developed by EnviroLogix in 2006 assisted in the diagnosis of this disease in the field prior to development of mature pustules. EnviroLogix has now developed an improved second generation LFD that incorporates a monoclonal antibody (Maby). **The major improvement with this new LFD is significantly increased positive signal intensities across the detection range (early non-characteristic visual symptoms through mature ASBR symptoms).**

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). 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. Leaf samples were tested on Days 4, 5 and 6 post-inoculation. This range incorporates visual symptoms from asymptomatic to heavy chlorotic lesions (covering up to 40-50% of the sample). There were no pustules present on any of these samples.

Starting with the earliest visual symptoms (lighter green spotting on the leaf before yellowing) through the most advanced symptoms tested (40-50% chlorotic lesions), the new LFD ran positive with **significantly higher signal intensities** than the previous 2006 generation. 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 and 100% RH.
3. Growth chamber at 22°C and 12-hour light photo period.
4. Healthy soybean plants at V1-V2 stage (hereinafter the “Healthy Tissue”) presenting at least one trifoliolate expanded leaf (~10-15 days old).
5. Fresh inoculum supplied by soybean plants at the V1-V2 stage (“Source Plants”). Spores were collected immediately before the inoculation.
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 = 5×10^5 spores/mL. Low = 1×10^5 spores/mL.
10. LFD device reader (QuickStix Reader), reading signal intensities of the Test and Control Lines by measuring changes in reflectance relative to device background.

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 five 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.

Spores were counted under the microscope with a hemacytometer. Buffer was added to adjust the spore counts to the High (5×10^5 spores/mL) and Low (1×10^5 spores/mL) levels.

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 RO-H₂O to remove any remaining un-germinated spores. The plants were transferred to a growth chamber where they remained until the desired post inoculation date (Days 4, 5 and 6), at which time they were harvested, refrigerated and tested within 24 hours.

To evaluate the washing procedure, a Day 0 leaf set was inoculated at both High and Low levels, and allowed to dry for one hour. Following the same wash procedure, both levels were evaluated and found to run negative, demonstrating that any positive signal in the study is the result of an active infection and not due to residual inoculum.

LFD Testing

Only those leaf sets presenting no visible symptoms and chlorotic lesions were assessed with the LFD (Days 4, 5 and 6).

Leaves were tested using the following extraction 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 approximately 1” in diameter (roughly the size of a quarter) 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. Five mL of Extraction Buffer was added directly into the mesh bag and massaged to mix buffer and leaf tissue. Five hundred μ L 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 μ L of Extraction Buffer was added to the tube followed by additional grinding for 20-30 seconds. 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 antigen, a 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.

RESULTS

Sensitivity and Specificity of the Test

It is important to note that the antigen detected by the LFD is also present in the absence of spores; this is why the device is able to detect ASBR at the start of the earliest visual symptoms, well before any spore formation is present.

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 the ELISA plate and LFD formats. The antibodies were shown to have a good detection level in both formats, with the ELISA plate detecting ~200 spores/mL and the LFD between 500 and 1000 spores/mL.

Additionally, the cross reactivity of the LFD was assessed against four common soybean diseases that could be confused with symptoms encountered in the field. No cross reactivity was found to *Cercospora sojini* (Frog Leaf Spot), *Cercospora kikuchii* (Seed Stain and Leaf Blight), *Septoria glycines* (Brown Spot), or *Xanthomonas axonopodis pv. Glycines* (Bacterial Pustule).

Visual Assessment

The samples were visually rated for percentage of leaf area infected, using a common scale for ASBR. Visual symptoms began to appear on the Day 5 post-inoculation leaf sets. Symptoms ranged from light green spotting (on leaves inoculated with the Low 100K spores/mL) to chlorotic lesions covering 25-35% of the leaf (on leaves inoculated with the High 500K spores/mL).

LFD Results

Mesh Bag: At the Low inoculum level, the Day 4 samples showed inconsistent positive/negative results between different samples. At the High inoculum level, the samples ran consistently positive. Once visual symptoms started to appear on the Day 5 samples, both inoculation levels ran consistently positive. As soon as visual symptoms appeared, the Generation #2 LFDs showed significantly stronger positive signals compared to the Generation #1 format.

Snap Cap: With the Day 4 samples at both inoculation levels, consistent positive signals were detected. The snap cap samples from the Day 5 and 6 leaf sets also ran consistently positive.

Summary

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

No hook effect was observed with the Generation #2 format.

