Short communication

# Colony Establishment and Maintenance of the Eriophyid Wheat Curl Mite *Aceria tosichella* for Controlled Transmission Studies on a New Virus-like Pathogen

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**Abstract**

High Plains Disease (HPD) is of serious economic concern for wheat and corn production but little is known about the virus-like causal agent. In the field HPD is often associated with *Wheat streak mosaic virus* (WSMV) and both pathogens are transmitted by the same eriophyid wheat curl mite, *Aceria tosichella* Keifer. The objective of this study was to develop methods for establishing and maintaining HPD-transmitting wheat curl mite colonies for their use in studies on HPD. Towards this goal, mite colonies from a mixed infection source were separated into colonies either i) not viruliferous, ii) only transmitting WSMV, or iii) only transmitting HPD. Maintenance of these colonies required strictly separated incubator facilities and adaptation of mite-suitable transfer techniques to permit frequent passages of mites to healthy plants. The established colonies provided reliable sources of infective material to study the progression of HPD and/or WSMV in plants using sensitive immuno-detection assays. In conclusion, we have developed reliable methods with a poorly studied arthropod vector to study the biology and properties of a new virus-like disease.

*Keywords*: HPD, Eriophyid mites, Colony Establishment and Maintenance, Transmission, Immuno-detection.

High Plains Disease (HPD) is a recently recognized disease affecting wheat, corn, barley and other plant species in areas spanning the Great Plains to the Pacific Northwest of the USA. The exact nature of the HPD-pathogen remains somewhat elusive, although all evidence to date strongly suggests a viral agent (Ahn et al., 1995; Jensen, 1994; Mirabile et al., 2000) that causes a disease resembling wheat spot mosaic described in the 1950 s (Slykhuis, 1956).

In the field HPD is often present as a co-infection with other viruses (Jensen et al., 1996; Mahmood et al., 1998; Marcon et al., 1997; Scholthof and Scholthof, 1995). Consequently, research on the causal agent of HPD has been thwarted in part due to the difficulty in maintaining a reliable source of singly infected plants. Laboratory studies are hampered because the pathogen is not mechanically transmissible by rub-inoculation and it is poorly seed transmitted (Forster et al., 2001). Vascular puncture methods for transmission have been developed for corn seeds (Forster et al., 2001; Seifers et al., 2002), but these methods have not yet proven successful for HPD transmission to wheat (unpublished observations). HPD is vector-transmitted by the eriophyid wheat curl mite, *Aceria toschiella* Keifer, but very little is known about these mites except that they also transmit *Wheat streak mosaic virus* (WSMV) which is often found in conjunction with HPD in the field. Vector transmission can vary within the species according to recent results that mites from different geographical sources transmit HPD with different efficiencies (Seifers et al., 2002).

Development of a reliable experimental tri-component HPD/vector/host system is important to permit controlled and biologically relevant studies under laboratory

conditions, especially since continuous mechanical passages of vector transmitted viruses can select variants defective for vector transmission. Therefore, despite the challenges associated with developing a system for the poorly studied wheat curl mite vector, the objective of this study was to design methods for the establishment and maintenance of mite colonies that reproducibly transmit HPD. Here we describe the establishment of viruliferous and non-viruliferous colonies using a source of mites that initially was doubly infected with HPD and WSMV. Maintenance of the colonies required strictly separated incubator conditions and their frequent passaging to healthy plants. The colonies provided a reliable source to infect plants under laboratory conditions to permit precise monitoring of HPD progression through plants using standard immunodetection analyses.

**Establishment**

*Aceria tosichella* eriophyid mites (Fig. 1) collected from the field were kindly provided by D. Seifers (Kansas State University, Hays). These original mites not only transmitted the HPD pathogen, but they were also viruliferous for WSMV. To establish a colony that would only transmit the HPD pathogen, infective mites were transferred onto yellow foxtail [*Setaria glauca* (L.) Beauv.] which is a host for the mites and HPD, but not for WSMV (Seifers et al., 1998). Since the HPD pathogen and WSMV are not transovarially passaged to offspring (Seifers et al., 2002; Slykhuis, 1955; Wijkamp et al., 1996), all the newly hatched mites could be expected to be pathogen free. These young mites acquired HPD from their HPD-infected yellow foxtail but did not acquire WSMV because this virus did not infect the foxtail plants. Because of the relatively short life span

of the mites (7-10 days at 24-27 C), the original WSMV-transmitting vectors expired fairly rapidly. Consequently, the population soon consisted solely of progeny HPD-transmitting mites that were then transferred to Siouxland wheat. On this host it was often difficult to visually discern the spotting symptoms caused by mite infestation from the mild symptoms caused by HPD under laboratory conditions. Therefore, the absence of WSMV and presence of HPD on wheat was verified with routine immuno-detection experiments for WSMV CP (data not shown) and a HPD-specific 32 kDa protein (described below).

In a recent study, infestations by aviruliferous *Aceria tosichella* on Ike wheat were found to cause sufficiently severe symptoms to induce grain yield losses of 1-15% (Harvey et al., 2000). This further illustrated the necessity for maintaining nonviruliferous mites as a negative control in our studies on HPD. For this purpose, mite eggs were transferred from the HPD-transmitting colony to healthy Siouxland wheat plants (Seifers et al., 2002). These transfers were performed using a specially adapted tool created from an ethanol-sterilized eyelash taped to a toothpick (Fig. 2). Because newly hatched mites from the toothpick transferred eggs were free of virus, this procedure yielded a nonviruliferous colony.

To obtain a WSMV-transmitting mite colony, healthy wheat plants were first infected by a traditional rub-inoculation technique with WSMV-infected tissue. Subsequently, mites from the above described healthy nonviruliferous colony were transferred (Fig. 2) to these infected plants to establish a WSMV-transmitting mite colony.

Although the original HPD-source mites transmitted more than one pathogen, the efforts described in this section resulted in the establishment of three separate wheat curl

mite colonies that were: 1) non-viruliferous, 2) WSMV-transmitting, or 3) HPD-transmitting.

**Maintenance**

Wheat curl mite colonies were kept on densely planted Siouxland wheat grown in autoclaved Bacto-mix soil (Fig. 3). Transfer of mites from highly infested older plants (~3-4 weeks) to younger plants (~1 week) was quickly accomplished by gently rubbing upper portions of the plants together which would cause a sufficient number of mites to be transferred. Such periodic transfers were generally sufficient to maintain a continuous supply of the separate colonies on wheat plants.

The eriophyids provided a complex problem for containment due their minute size (as small as 33x80 m for the first nymphs) ( Slykhuis, 1955) (Fig. 1). Although the mites are apterous and are not known to crawl from plant to plant (del Rosario and Sill, 1964), their ability to become swept up and carried by the slightest breeze, as is their method of dispersal in nature, made containment a challenge. Initially we attempted housing all the mite colonies in a single area utilizing cages (Slykhuis, 1956), however due to the necessarily small exclusion limits for the cages, moisture and fungal contamination was a problem, quickly overcoming and eradicating the colonies. We determined that instead it was best to keep the colonies separate in their own individual growth chambers (16 hr light, 25°C) or alternatively, to maintain the colonies at room temperature on window sills receiving a mixture of natural and fluorescent light. Since mites can sustain low temperatures (del Rosario and Sill, 1964), reserve back-up colonies were also established. This was achieved by placing a well watered, whole colony potted plant in a sealed

plastic bag at 5°C. Although egg viability was compromised, these colonies remained viable for several months.

In addition to avoiding cross-contamination between the separate colonies, it was also imperative to keep the colonies free from foreign arthropod invasion and microbial contamination. Foreign arthropod species such as the predaceous mite *Tylodromus cucumeris* were detrimental to the mite colony not only by acting as a predator (Slykhuis, 1956), but also by competing with the mites for the same habitat niche. Similar problems occurred when several species of aphids invaded the eriophyid colonies. Contamination was best controlled by avoidance practices, not allowing colonies to come into contact with plants brought in from the greenhouse or general use growth chambers, or with plants growing in non-autoclaved soil. Despite these measures, routine inspections were necessary to ensure the purity of the colonies. For this, the colonies were viewed macroscopically to determine if proper infestation symptoms were present with longitudinal leaf curling and bowed out leaves (Fig.1B) as is typical upon infestation (Slykhuis, 1965). Although this was a good indicator for whether plants were infested, it did not verify the health of the colony since these symptoms remained long after a colony had perished. Colonies were inspected weekly using a stereoscope (Fig. 2A) to determine if the mites were present and appeared healthy (slightly translucent) and also to monitor for signs of microbial or foreign arthropod invasion, i.e. foreign feces, exoskeletons, eggs, symptomatic mites, etc. If contamination had occurred, healthy mites were transferred to several young healthy plants using the hair-toothpick, and the growth chambers were sterilized. As many mites as possible (50+) were transferred to each new plant to minimize the occurrence of genetic bottlenecks. This was also based on a study

that single eriophyid mites become less effective at transmitting the HPD pathogen over time, and eventually 10 or more mites were required to invoke the same symptom severity as observed previously with one mite from the original colony (Seifers et al., 1997). Alternatively, we replaced contaminated colonies with reserve colonies placed in storage as described in a previous section.

In summary, the maintenance of wheat curl mite colonies was and remains a labor-intensive process. However, by diligent passaging and inspection, and implementing strictly separated incubator facilities the three separate wheat curl mite colonies were reproducibly maintained on wheat. In fact, the HPD-transmitting colony has now been sustained for five years in our laboratory.

**Vector Transmission Experiments**

As it is not possible to transmit HPD by rub-inoculation, the mite colonies are instrumental as an inoculum source for studies on the biology of HPD. To standardize the inoculum procedure for different experiments, 1 cm of highly infested leaf tissue (Fig. 1B) from the colony infected plants was placed with forceps in the whorl of healthy wheat (or corn) plants at the two-leaf stage. To study progression of HPD infection through plants, it was necessary to eliminate the mites after they had transmitted the disease to avoid continuous reintroduction of pathogen into healthy tissue by mite feeding. Previous studies have shown that foliar applications with Carbofuran-based aracicides were either non-effective (Fritts et al., 1999) or effective for control (Seifers et al., 2002). Perhaps the effectiveness of the foliar application is influenced by the extent of mite-induced leaf curling (Fig. 2B) that creates a protective cocoon. Because of the

mixed results with the Carbofuran treatments, we opted for an alternative to ensure maximum control. For this purpose we allowed the mites to feed and transmit for three days, prior to an application of the systemic aldicarb aracicide Temik (4 granules per 14 cm diameter pot were thoroughly watered in as per instructions of the supplier; Aventis, NC). Periodic inspections verified that this treatment effectively destroyed all the mites (data not shown).

The established mite colonies in combination with the precisely timed application of Temik were used in an experimental capacity to monitor HPD infection. For example, viruliferous mites were transferred onto young healthy wheat plants; 3 days later mites were eliminated with Temik, and at 28 days post-infestation leaves and roots were harvested for routine immuno-blot detection assays (Scholthof et al., 1994). For this purpose we used rabbit antiserum specific for a HPD-specific 32 kDa protein (Fig. 4) that is specifically associated with HPD infection (Jensen et al., 1996). These tests also revealed the presence of a non-specific host-encoded protein that interacts with the antiserum (Fig. 4, right panel), suggesting that traditional ELISA-mediated detection could potentially result in false positive readings due to the reaction of the non-specific protein. Nevertheless, these results and related time-course experiments (data not shown) revealed a classical virus movement pattern (Samuel, 1934) with the HPD infection progressing from inoculated leaves down to the roots prior to infection of non-inoculated upper leaves. This movement pattern appeared to be influenced in a host-specific manner by the presence of WSMV and this feature is currently actively pursued in the laboratory through transmission studies that implement our established colonies. The results

obtained thus far on the movement pattern of HPD and its interaction with WSMV provide biological support for the viral origin of HPD.

In conclusion, we have developed a reliable experimental system involving a poorly described mite vector to study a new virus-like pathogen of wheat and corn under laboratory conditions. This system will allow biological studies on HPD and provides a reliable source of material to characterize the causal agent at the molecular level.

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## Figure legends

**FIG. 1.** The wheat curl mite vector, *Aceria tosichella* Keifer. (A) Scanning electron micrograph of the ~30x230 μm mites (B) Mites on a section of a wheat leaf exhibiting infestation characteristics such as longitudinal leaf curling due to mite feeding.

**FIG. 2.** Tools for mite colony inspection and transfer (A) Mite colonies were inspected using a stereoscope to observe foreign microbial and arthropod invaders. (B) Eriophyid mite transfer tool made of an eyelash taped to a toothpick.

**FIG. 3.** Wheat curl mite-infested wheat plant showing longitudinal leaf curling and bowed out leaves due to mite feeding.

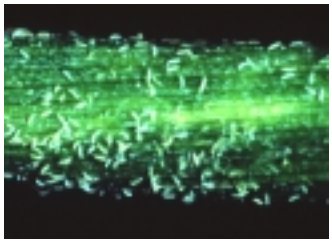
**FIG. 4.** Immunoblot detection of HPD infections. Left panel: wheat leaf (L) and root (R) samples harvested 28 days post-infestation with HPD-transmitting mites. The HPD-associated 32 kDa protein (arrow) was detected with alkaline phosphatase-conjugated secondary antibodies. Right panel: wheat tissue from plants infested with non-viruliferous mites (HM) versus HPD-infected plants (HPD). This test serves to illustrate

that the second reactive protein (marked with asterisk) that migrates above the 32 kDa protein in both samples is not specific for HPD.

Skare et al., FIG. 1

**A**

**B**



Skare et al., FIG. 2

**A**

**B**

Skare et al., FIG. 3

Skare et al., FIG. 4