Viability of *Puccinia horiana* Teliospores Under Various Environmental Conditions

Morris R. Bonde, USDA-ARS, Foreign Disease-Weed Science Research Unit (FDWSRU), Fort Detrick, Frederick, MD 21702; Cristi L. Palmer, The IR-4 Project, Rutgers University, Princeton, NJ 08520; Douglas G. Luster and Susan E. Nester, USDA-ARS-FDWSRU, Fort Detrick, Frederick, MD 21702; Jason M. Revell, The IR-4 Project, Rutgers University, Princeton, NJ 08520 and USDA-ARS-FDWSRU, Fort Detrick, Frederick, MD 21702

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### ABSTRACT

*Buccinia horiana* Henn. is a quarantine-significant fungal pathogen and causal agent of chrysanthemum white rust (CWR). The pathogen and disease were first discovered in the United States in 1977 and quickly eradicated. During the early 1990s, CWR reemerged in several instances, but in each instance was declared eradicated. However, since approximately 2004 CWR has reappeared at an accelerated frequency. This has suggested that either *P. horiana* is entering the country more frequently from foreign locations or that *P. horiana* is now established in the field, implying that spores are capable of surviving winter conditions in plant debris or soil. As a result of the possibility that the pathogen has become established in the United States, we initiated several lines of research. The objectives of the study reported here were: (i) develop a better and more sensitive method to measure teliospore longevity; and (ii) determine if the pathogen is able to survive northeastern winters as viable teliospores. Results from the study showed that teliospores survived in the greenhouse a maximum of 28 days in dry soil and 7 days in moist soil. In a growth chamber simulating winter temperature conditions in the northeastern United States, teliospores survived a maximum of 35 days. It was concluded that *P. horiana* teliospores are not able to survive through typical northeastern U.S. winters.

### INTRODUCTION

Indigenous to Asia (12), *Puccinia horiana* Henn., causal agent of Chrysanthemum white rust (CWR), is a fungal pathogen of quarantine significance in the United States. The pathogen and disease were first discovered in the United States in 1977 and apparently were quickly eradicated (15). Over the next 25 years, CWR reappeared on several occasions, but was again reportedly eradicated (5,6,7,8,9,11). However, beginning in about 2004, CWR began to be discovered more frequently and at an increasing frequency (4,13,14). In 2011, Kim et al. (13) and later O’Keefe and Davis (14) independently reported evidence that *P. horiana* might have overwintered in Pennsylvania. There are several possible ways which *P. horiana* might overwinter. One possibility is that teliospores, on or in soil, are able to survive winter conditions. A second possibility is that the pathogen is able to survive in systemically infected plants during the winter, at least under moderate environmental conditions. This manuscript examines the first possibility.

*Puccinia horiana* is an autoecious microcyclic rust fungus, and therefore completes its life cycle on a single host species. The pathogen produces two spore types. Teliospores embedded in infected chrysanthemum leaves germinate to produce basidiospores, the infectious propagules that reinfect chrysanthemum plants. Teliospores have been shown to survive a maximum of 8 weeks at 50% relative humidity, and for shorter durations at higher humidities (10). When buried in compost, they have been reported to survive a maximum of 2 weeks (10).

However, the single report describing teliospore survival relied on methodology not conducive for conclusive determination of teliospore viability at very low levels, which is required for regulatory purposes (10).

The objectives of the study reported here were to develop a more sensitive method to detect viable teliospores in soil, and use that technique to determine if teliospores are able to survive for a duration that allows the pathogen to survive northeastern U.S. winters. Establishing that *P. horiana* can overwinter and become established in the United States would influence regulatory decisions and cause producers to revisit their disease management and control strategies for the pathogen.

### EFFECTS OF SPECIFIC ENVIRONMENTAL CONDITIONS ON TELIOSPORE LONGEVITY

Leaves bearing CWR pustules were cut into two nearly equal halves and one half leaf of each pair was mounted to the inside surface of a Petri dish lid by means of a thin coat of petroleum jelly between the lid and upper leaf surface. The half leaves were soaked for 5 min in Tween 20 (Sigma-Aldrich, St. Louis, MO) water (1 drop per 100 ml RO water) to increase sporulation capability, and placed over 1% water agar in a petri-dish bottom. The closed dishes were incubated in darkness in a plastic box at 17°C. Water agar was employed to prevent basidiospores produced by the pustules from adhering to the lower surface of the dish. Basidiospores from sporulating leaves fell onto a semi-solid medium, rather than into a liquid medium, prior to counting, thus the deposited teliospores were in a nearly single plane for observation. However, when deposition was especially heavy, basidiospores became vertically stacked within the semi-solid agar medium. Theoretically we were able to detect a single released basidiospore using our technique, whereas in prior,
preliminary trials using a liquid medium and counting with a hemocytometer we estimated that at least $7 \times 10^3$ basidiospores would be required for detection. However, our method using semi-solid medium was not quantitative, because the basidiospores in areas of higher density were piled vertically and more difficult to enumerate. An infected, nonsporulating chrysanthemum leaf is shown in Fig. 1, and the experimental setup illustrating basidiospores on the surface of water agar is depicted in Fig. 2.

After 2 days of incubation, the agar surface below the half leaves in each dish was examined microscopically to determine relative amounts of basidiospore deposition. Depositions were rated as “heavy” (rating = 3), indicating that the basidiospores were several layers deep under most pustules and appeared white to gray and opaque to translucent, to the unaided eye; “moderate” (rating = 2), denoting that the basidiospore drop was generally one layer thick under a majority of the pustules and appeared pale gray and translucent to the unaided eye; “light” (rating = 1), denoting that the basidiospore drop was diffuse, a single layer, and/or deposited from a few pustules, appearing faintly cloudy or not visible to the unaided eye; and “none” (rating = 0), denoting basidiospores were not detected at 100× magnification. Paprika powder sprinkled lightly onto the agar surface aided in focusing rapidly to the correct plane of vision. These particles, present only in some fields, were easily differentiated from basidiospores because of their color. These samples served as controls and the measured sporulation served as the basis for subsequent measurements of sporulation.

The second half of each half-leaf pair was placed in a mesh bag made of 100% polyamide with a 25-μm pore size (Sefar Inc., Depew, NY) containing 2.5 g Sunshine Mix soil (SunGro Horticulture Ltd, Agawam, MA) dampened with 25 ml of water, or without soil, and the mesh bags sealed with laboratory tape. The mesh bags with leaf pieces in soil were buried in 300 g soil in a 0.9-liter plastic bag, four mesh bags per plastic bag. The mesh bags containing half leaves without soil were placed in a 600 ml beaker.

One third of the samples were placed in a -20°C commercial freezer to simulate freezing conditions, one third in a commercial cold room to simulate cold but nonfreezing conditions, and one third on a bench in a greenhouse to simulate early fall temperate conditions. Four mesh bags for each treatment were collected weekly, half leaves removed from the bags and gently rinsed to remove soil from the leaves. This did not remove any spores because they had not been formed. Temperatures were recorded during the experiments using a Hobo H8 Pro Serieslogger (Onset Computer Corporation, Pocasset, MA) with an accuracy of ±3.0%, based on information from the manufacturer. The half leaves were mounted to the inside surface of water agar dishes and tested as above to determine sporulation capacity as described above.

Statistical analyses. Sporulation on each leaf-half (rated as “none” = 0; “light” = 1; “moderate” = 2; or “heavy” = 3, as described above) was used to generate a ratio of sporulation on the test half leaf/sporulation on the corresponding control “zero-time” half leaf. A mean ratio based on numerical sporulation ratings was computed for each location, treatment, day of observation, and repetition of the experiment by averaging over the number of samples tested. The mean ratios were analyzed by first testing the data for normality with the UNIVARIATE procedure of SAS (SAS Institute Inc., Cary, NC) and noting the significance of the $P$-values of the test statistics. Significant ($P \leq 0.05$) test statistics indicated that the hypothesis that the data were normally distributed should be rejected. Next the data were tested for fit to a Poisson distribution with the GENMOD procedure of SAS and the model: ratio = location, a Poisson distribution, and a log link function. A $P$-value for fit to the Poisson distribution was generated from the chi-square deviance value from the GENMOD output, associated degrees of freedom, and the probchi function of SAS: $P$-value = 1 - probchi(chisq, df). Lack of a significant $P$-value was the criterion to indicate that the data fit a Poisson distribution. The data were then analyzed with the GLIMMIX procedure of SAS, a log link function, and Poisson distribution. Repetitions of the experiment were included in the analyses as random effects while all other effects were analyzed as fixed effects. Four different models were run: ratio = loc tmt loc*tmt days(loc*tmt); ratio = loc tmt loc*tmt; ratio = loc days(loc); and ratio = loc, where “loc” = location (greenhouse, cold room, or freezer); “tmt” = buried or placed in beaker; and “days” = day of observation. Least squares means and t-tests of the least squares means against zero and against the other effects in the analyses were generated in each analysis.

The mean ratio data were determined to be not normally distributed, but they were Poisson distributed ($P$-value for deviance chi square = 1). None of the F-values for any of the effects were significant in any of the models except for the model ratio = loc. This model had the best fit of any of the models (<2

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**FIGURE 1**
Infected chrysanthemum leaf with nonsporulating lesions of chrysanthemum white rust.

**FIGURE 2**
Infected chrysanthemum leaf mounted to the lid of a petri dish to induce and collect basidiospores. Note the copious amounts of basidiospores on the surface of the agar.
Res Log Pseudo-Likelihood = 390.82), and the F-value for location was significant at $P = 0.0747$. The least squares means for each location were each significantly different than zero in this model. The back transformed (anti-log) means were: greenhouse = 0.1267 ± 0.0777; cold room = 0.3973 ± 0.1323; freezer = 0.5013 ± 0.1543. The only significant difference among locations was for the freezer versus the greenhouse, $P > |t| = 0.0253$.

A comparison of maximum longevity showed that the highest longevity was with teliospores, buried or not buried, maintained frozen in the freezer (Fig. 3). After 112 days, some of the teliospores were still viable, as indicated by an average sporulation rating of 0.30. Sporulation for teliospores, buried or not buried, in the cold room tended to be intermediate in longevity, surviving up to 77 days (Fig. 3). However, most striking was longevity of teliospores in the greenhouse. At this location, nonburied teliospores survived a maximum of 28 days, and those buried in soil failed to germinate at 7 days, the shortest duration tested, in all experiments (Fig. 3).

LONGEVITY UNDER SIMULATED WINTER TEMPERATURES

An environmental plant growth chamber was programmed to simulate diurnal temperature conditions during fall, winter, and spring in State College, Pennsylvania. The diurnal temperature pattern was based on diurnal temperatures recorded for State College over a 10-year period (National Climate Data Center, http://www.ncdc.noaa.gov). Inoculated plants were placed for 3 weeks in a greenhouse considered to simulate early fall temperature conditions, and then transferred to a growth chamber. The accelerated diurnal temperature highs and lows each gradually decreased during the fall and early winter, leveled off, and then gradually increased until reaching spring temperatures characteristic of the climate in State College. A set of 32 mesh bags containing half-leaf pairs in soil and a second set of 32 bags with half leaves without soil, as described above, were placed on a shelf in the growth chamber after having been in the greenhouse for 3 weeks. During the course of the experiment, four mesh bags with soil and four without soil were retrieved at a weekly interval, leaf pieces washed to remove soil, and the pieces treated as above to determine the capability of the teliospores to germinate. The experiment was conducted twice.

The highest longevity was with teliospores that had not been buried; their maximum longevity was 35 days in each of the experiments, which was approximately midwinter during the simulated overwintering. Teliospores that had been buried germinated at a maximum duration of 7 days, consistent with the previously outlined experiment. Because the teliospores were located in the greenhouse for 21 days prior to placement in the growth chamber, it was apparent that buried teliospores had lost viability prior to placement in the growth chamber. The higher germination of nonburied teliospores correlated with results of the above greenhouse experiment.

DISCUSSION

The sporulation ability of *P. horiana*-infected chrysanthemum leaves varied greatly among leaves. To compensate for this inherent difference, leaves were cut into halves. One half was used for the treatment, and the other half for the zero-time “control.” This permitted us to follow the decline in viability, measured by the ability of the teliospores to germinate, over time as the various treatments progressed. In spite of the high variability, it was shown by statistical analyses that teliospores survived best when frozen. Most important, however, under simulated fall, winter, and spring temperature conditions of the northeastern United States, teliospores survived at maximum only up to midwinter.

We assumed that if teliospores did not germinate under optimum conditions for germination, they were not viable. Although teliospores of some rust fungi apparently can become dormant (1), we have no evidence this occurred with *P. horiana*. However, this is an aspect of the pathogen that should be examined in the future.

Generally, it is believed that teliospores of rust fungi are important for overwintering and largely responsible for the formation of new physiologic races (2). However, Anikster (1) showed in a study with 27 rust species on a total of 59 plant hosts, each pathogen lost germinability within 1 year when outdoors. When teliospores were stored dry in paper bags at 2 to 4°C, germinability was high after 1 year, somewhat diminished by 2 years, and nonexistent by 3 years. Teliospores maintained dry at 5°C in sealed glass vials under partial vacuum remained viable for 14 years, and teliospores of the pathogens that cause stem rust of wheat, oats, and *Agropyron* spp. were viable after 8 years of storage at -18°C. It is apparent that teliospores of some rust species under certain conditions are capable of germination after many years (1,3). It should be noted that teliospores embedded in leaves, as with *P. horiana*, would remain hydrated for a longer period of time than free teliospores in soil that are shed from leaves.

In a previous study, we demonstrated that *P. horiana* teliospores remained viable in infected chrysanthemum leaves on plants only as long as the leaves remained living (4). The loss of viability may have resulted from desiccation of the leaves. In the current study, teliospores in the greenhouse survived a maximum of 28 days in dry soil and were not viable by 7 days in damp soil. Although not part of this study, in a separate study to determine

**FIGURE 3**

Relative sporulation ratings (as ratios) for leaf pieces that had been maintained dry, or in moist soil in the greenhouse at 22 to 26°C (top), cold room at 5°C (middle), or freezer at -20°C (bottom). Vertical bars represent the standard errors of the means, $n = 12$. 

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**PLANT HEALTH PROGRESS** ◆ Vol. 15, No. 1, 2014 ◆ Page 27
the effectiveness of soil steam sterilization to eliminate *P. horiana* teliospores, even without the steam treatment none of the teliospores survived past 3 days. This demonstrates the very short period of viability of *P. horiana* teliospores in soil. Although teliospores survived longer if immediately placed in a cold room, and even longer when frozen, neither of these treatments simulated natural conditions in the field where teliospores would be subjected to warm temperatures prior to the onset of freezing conditions. Thus under the natural progression of temperatures from autumn into winter, teliospores would become nonviable before being subjected to cold or freezing conditions. For that reason, it is very unlikely that *P. horiana* teliospores would survive through winters in the northeastern United States.

**DISCLAIMER**

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**LITERATURE CITED**