Factors Affecting Conidial Exudation and Survival, and Ascospore Germination of *Leucostoma cincta*

Radwan M. Barakat, D. A. Johnson, and **G. G. Grove,** Department of Plant Pathology, Washington State University, Pullman 99164

ABSTRACT

Barakat, R. M., Johnson, D. A., and Grove, G. G. 1995. Factors affecting conidial exudation and survival, and ascospore germination of *Leucostoma cincta*. Plant Dis. 79:1245-1248.

Conidia of *Leucostoma cincta* from sweet cherry were exuded from pycnidia at 2 to 28°C with an optimum exudation at 20°C. Free water was needed for exudation at incubation periods of 6 to 24 h at 20°C. Conidia were exuded at 100% relative humidity during 48 and 72 h of incubation. A large proportion of conidia in dried cirrhi survived summer conditions in the field. Ascospores germinated at 10 to 28°C with an optimum at 28°C during incubation times of 12 to 24 h. Conidia germinated at 15 to 28°C during an incubation period of 24 h. Maximum germination of conidia occurred at 28°C during 24 h of incubation. Conidia germinated at 5 to 20°C when incubation periods were extended to 48 and 72 h. Inoculations of sweet cherry trees with ascospores of *L. cincta* resulted in infection and development of stem cankers.

Leucostoma canker (perennial canker, Cytospora canker, and Valsa canker) causes a dieback disease of sweet cherry (*Prunus avium* L.) in Washington State (15) and of *Prunus* spp. throughout the world (1,3,8,10,23,24). The disease is caused by one of two fungi, *Leucostoma cincta* (Fr.:Fr.) Höhn. (anamorph = *Cytospora cincta* Sacc. and *L. persoonii* Höhn. (anamorph = *C. leucostoma* Sacc.). *L. cincta* predominates in central Washington (15).

Leucostoma canker of sweet cherry in Washington State is characterized by dieback of tree branches, bark cankers, gummosis, and tree decline. Many perennial cankers of stone fruit trees originate at pruning wounds (9,23). Infections at pruning cuts are potentially devastating because they often are located at points critical to the architecture of the tree (3). Other avenues of infection are at points of injury to bark caused by insects, machinery, and cold temperatures (3,23). Healthy, intact bark of stone fruit trees is not invaded by *Leucostoma* spp. (3,9,17).

Conidia are exuded in a gelatinous cirrhus during wet weather from pycnidia on infected branches and can be disseminated by splashing and windblown rain or sprinkler irrigation (15). During brief rain showers in the semiarid environment of central Washington, conidia are exuded, and then, as the atmospheric humidity decreases, they dry en masse near the ostiole of the pycnidia. Viable conidia in dried cirrhi may be effectively disseminated with later rains and play an important role in the epidemiology of Leucostoma canker in Washington State and other areas of stone fruit production.

The teleomorph of *Leucostoma* spp. is not commonly observed on *P. avium* in central Washington, and little is known about the role of ascospores in the epidemiology of Leucostoma canker. Ascostromata of *L. cincta* were collected on dead branches of *P. avium* in an orchard near Prosser, Washington, in 1990. This is the first reported occurrence of *L. cincta* on *P. avium* in Washington and presented the opportunity to investigate the pathogenicity of ascospores of *L. cincta* on *P. avium*. The purposes of this study were to quantify the effects of temperature and humidity on the exudation of conidia from pycnidia, determine the viability of conidia in dried cirrhi, test ascospores for pathogenicity on sweet cherry, and determine the effect of temperature on germination of ascospores.

MATERIALS AND METHODS

The teleomorph and anamorph of *L. cincta* used in this study were identified using the criteria described by Kern and others (7,12,20,22). Characteristics considered were the shape and size of the perithecial and pycnidial stromata, stromatal disk, the presence or absence of a pycnidium in the perithecial stromata, and size and shape of conidia, asci, and ascospores.

Dead cherry branches with pycnidia of *L. cincta* were collected from living trees of the sweet cherry cultivar Bing in an orchard near Prosser. Pycnidia were removed from the branches with a razor blade. Before each experiment, a sample of 50 pycnidia was tested for the presence of conidia by placing pycnidia on moistened filter paper in glass petri dishes, wetting each pycnidium with about 0.01 ml of distilled water, and incubating at 21°C for 24 h. Pycnidia were only used when the sample for the collection had greater than 90% of the pycnidia exuding conidia within 24 h.

Effect of temperature on conidial discharge. Pycnidia were removed from the branches and placed on moistened filter paper in petri dishes at a concentration of 20 pycnidia per petri dish. Pycnidia were wetted with 0.01 ml of sterile distilled water per pycnidium and incubated at either 2, 5, 10, 20, or 28°C for 6 h. Treatments were arranged in a randomized complete block design with three replicates. Temperature of incubators and of the distilled water used in petri dishes in each incubator was previously adjusted to that of the desired treatment.

Pycnidia were observed with a stereomicroscope at 30x magnification for exuded conidia. The percentage of pycnidia with exuded conidia was calculated for each petri dish. The experiment was done twice. Regression was used to analyze data with percentage of discharge as the dependent variable and temperature as the independent variable. Data from the two experiments were combined for analysis because they were not significantly different (4,5).

Effect of moisture on conidial discharge. Pycnidia were removed from branches and placed on wax paper in petri dishes at a concentration of 20 pycnidia per petri dish. Dishes were placed inside four 0.5-liter glass jars containing 100 ml of various solutions of anhydrous glycerol and sterile distilled water mixed at proportions calculated to result in relative humidities of 80, 90, 95, and 100% (6,11,16,18) at 20°C. Jars were covered with plastic petri dish tops, each coated on the inside with high-vacuum silicone grease. Tops were secured with large rubber bands. Pycnidia were kept 1 to 2 cm above the surface of humidifying solutions by placing the wax paper on wire screens. Each incubation jar was sealed in a polyethylene bag and placed in a cylindrical petri dish sterilization can. The four cans were placed in a controlled-temperature circulating water bath (Tempete TE-8A, Techne, Duxfor, England) and incubated at 20 ± 0.2°C for 6, 12, 24, 48, or 72 h. Temperature and relative humidity within each incubation chamber were continuously

Corresponding author: D. A. Johnson
E-mail: djohnsn@wsuvm1.csc.wsu.edu

Accepted for publication 27 September 1995.

© 1995 The American Phytopathological Society

monitored with sensors (Vaisala HMP-123Y sensors, Vaisala Inc., Warburn, MA) inserted into the chambers through a small rubber grommet that lined a circular opening in each petri dish top. At the conclusion of the five respective incubation periods, pycnidia were removed from the four containers and observed as described above for exuded conidia. The percentage of pycnidia with discharged conidia was calculated for each petri dish. The experiment was done twice, and data were combined for analysis.

Viability of conidia in dried conidial cirrhi. Pycnidia were removed from branches and 15 pycnidia were placed on moistened filter paper in each of five petri dishes. Pycnidia were wetted with water and incubated at 20°C for 6 h to induce release of conidia. Petri dish covers were then removed, and pycnidia, conidial exudation, and filter paper were air-dried. Pycnidia were incubated at 20°C in a 15-h photoperiod at approximately 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 0, 2, 5, 10, and 20 days.

After incubation for the various time periods, pycnidia and conidial exudation were suspended in sterile distilled water. Pycnidia were removed by filtering the suspension through four layers of cheesecloth. Concentration of the conidial suspension was determined with a hemacytometer and adjusted to 7.5×10^7 conidia per ml. Two adjacent wounds on branches of 6-year-old sweet cherry trees cv. Rainier were made to xylem depth with a modified staple gun (15) and then either inoculated with 0.25 ml of the conidial suspension or sterile distilled water. Five sets of wounds on separate branches with similar stem circumferences were inoculated for each time duration that conidia had been released from pycnidia. After inoculation, wounds were wrapped with Parafilm for 10 days. Canker length and percentage of the stem circumference colonized were determined 2, 4, and 8 weeks after inoculation. Tissue samples from cankers were then plated on malt agar to verify the presence of *L. cincta*.

Dead cherry branches with pycnidia of *L. cincta* from living Bing sweet cherry trees were cut into 12 segments 25 cm in length. Branch segments were rinsed in cold tap water for 5 min, placed in plastic bags on a moistened paper towel, and incubated for 10 h at 21°C. Branch segments were removed from the plastic bags, and pycnidia with conidial cirrhi were labeled. Branch segments were placed outdoors on 17 May 1991 under an overhead shelter 2.5 m above the samples to prevent contact with rain. Branch segments were exposed to direct sunlight for about 4 h on cloudless days. Air temperature at a height of 1.6 m and rainfall about 100 m from the overhead shelter were measured with a probe thermistor (model XN217, Campbell Scientific, Logan, UT) and a tipping rain gauge (Qualimetriz, Oakland, CA), respectively.

After 0, 2, 5, 10, 20, and 30 days, discharged conidia from two branch segments were collected with a scalpel and suspended in sterilized distilled water. The concentration of the conidial suspension was determined with a hemacytometer and adjusted to 2.5×10^7 conidia per ml. About 0.25 ml of the suspension was transferred to Difco malt agar (MA) in four petri dishes and incubated at 28°C for 24 h. Conidia were then observed with a microscope for germination. Number of conidia observed per petri dish was 500; conidia were considered germinated when the germ tube length equaled the length of the conidium.

Germination of ascospores and conidia. Sweet cherry branches of the cultivar Bing that contained perithecial stromata of *L. cincta* were collected in July and August 1990 from an orchard near Prosser. The bark was peeled off, and the perithecial stromata were rinsed with tap water. Perithecia were removed with a razor blade, placed in 5 ml of sterile distilled water, and shaken for 10 min. The resultant spore suspension was adjusted to 1.26×10^6 ascospores per ml after filtering through four layers of cheesecloth. Approximately 0.25 ml of the ascospore suspension was transferred to MA in petri dishes. Two dishes of ascospores were incubated at 5, 10, 15, 20, and 28°C for 12, 18, and 24 h. The percentage of germination of ascospores was calculated after observing 100 spores per treatment. This experiment was repeated three times.

Pycnidia from sweet cherry branches were placed on moistened filter paper in petri dishes for 24 h. Conidia that exuded from pycnidia were lifted off with a scalpel and suspended in sterile distilled water at a concentration of 6.25×10^6 conidia per ml. Approximately 0.25 ml of the conidial suspension was transferred to malt agar in petri dishes and incubated at 5, 10, 15, 20, and 28°C for 12, 18, and 24 h. The percentage of germination of conidia was calculated after observing 500 conidia per treatment. The experiment was repeated three times. One experiment was done concurrently with one ascospore germination test. Conidia were further tested, using the above techniques, for germination at 5, 10, 15, and 20°C during 48 and 72 h of incubation.

Pathogenicity of ascospores. Ascospores that were extracted from perithecia as described above were suspended in sterile distilled water and adjusted to a concentration of 1×10^7 ascospores per ml of water. Wounds made with a modified staple gun as previously described (15) on three branches of three 6-year-old sweet cherry trees (cv. Rainier) were inoculated in the same manner as the conidial inoculations. Sterile distilled water was used as a noninoculated control. Disease symptoms were observed 2, 4, and 8 weeks after inoculation. Bark and wood tissues from the margin of cankers were plated on MA to reisolate *L. cincta*.

Analysis of variance and regression were used to analyze data. Data expressed as percentages were transformed using the arcsine transformation to stabilize variances (14).

RESULTS

Effect of temperature on exudation of conidia. The percentage of pycnidia that discharged conidia increased significantly ($P = 0.001$) as the temperature increased from 2 to 20°C and then decreased as the temperature increased to 28°C. The mean percentage of pycnidia that exuded conidia was 24, 36, 41, 93, and 47 at 2, 5, 10, 20, and 28°C, respectively. The cubic equation $\hat{y} = 35.18 - 5.48x + 0.914x^2 - 0.03x^3$ described the data with a coefficient of determination of 0.93.

Effect of moisture on discharge of conidia. Conidia were not released from pycnidia at relative humidity (RH) less than 100% during incubation periods of 6 to 72 h at 20°C. Conidia were released from 80 to 83% of pycnidia when free water was present during 6, 12, and 24 h of incubation. Pycnidia failed to extrude conidia during 6, 12, and 24 h of incubation at 100% RH, but 30 and 46% of the pycnidia discharged conidia after 48 and 72 h of incubation, respectively, at 100% RH.

Viability of conidia. Inoculations with conidia from cirrhi that had been dried for up to 20 days after extrusion from pycnidia all resulted in infection. *L. cincta* was reisolated from cankers of all inoculated wounds 9 weeks after inoculation. *L. cincta* was not isolated from wounds on branches used as noninoculated controls. Canker length and percentage of stem circumference colonized from the inoculated treatments were all significantly greater ($P = 0.05$) than wounds on the noninoculated control. Canker length and percentage of stem circumference colonized did not differ significantly among the 0-, 2-, 5-, 10-, and 20-day treatments.

Mean maximum, mean minimum, and mean temperatures for the 30-day period that pycnidia with exuded spores on branch segments were exposed under the overhead shelter were 23.0, 7.3, and 15.1°C, respectively. A maximum temperature of 32.6°C occurred on day 25. Rainfall occurred on 4 days and totaled 6.61 mm. Additional sporulation was not observed on any of the pycnidia during the 30-day period. Percentage of germination of conidia from cirrhi that had been dried for up to 30 days after extrusion from pycnidia ranged from 94% for the 0-day-old conidia to 97% for the 30-day-old conidia.

Germination of ascospores and conidia. Ascospores germinated at 10, 15, 20, and 28°C during the 12, 18, and 24 h of incubation. Maximum germination occurred

at 28°C (Table 1). Conidia germinated at 15°C during 24 h of incubation and at 20°C during 18 and 24 h of incubation. Maximum germination occurred at 28°C (Table 2). Conidia germinated at 5, 10, 15, and 20°C when incubation periods were extended to 48 and 72 h (Table 2).

Infection by ascospores. Inoculations with ascospores resulted in cankers and gumming typical of those caused by *L. cincta*. *L. cincta* was reisolated from canker margins 2 and 3 months after inoculation.

Table 1. Percent germination of ascospores of *Leucostoma cincta* on malt agar at five temperatures and three times of incubation

Incubation time (h)	5°C	10°C	15°C	20°C	28°C
12	0[a]	0.3	4	8	74
18	0	3	12	62	90
24	0	10	60	81	96

[a] Data are mean values of three replicates. LSD (0.05) = 6.9.

Table 2. Percent germination of conidia of *Leucostoma cincta* on malt agar at various temperatures and times of incubation

Incubation time (h)	5°C	10°C	15°C	20°C	28°C
12	0[a]	0	0	0	0.1
18	0	0	0	5	91
24	0	0	0.7	95	99
48	0.1	25	48	100	...
72	5	60	100	100	...

[a] Data are mean values of three replicates. LSD (0.05) = 4.0.

DISCUSSION

Previous work based on the occurrence of the anamorph indicated that *L. cincta* predominated in sweet cherry orchards in south central Washington (15). The collection of the teleomorph of *L. cincta* in this study confirms the identification of the species on sweet cherry and raises questions about the relative importance of the sexual state in the epidemiology of the disease in south central Washington. Conidia and ascospores may both serve as primary inoculum for *L. cincta* (22). The teleomorph, as observed in this and a previous study (2), does not occur in sweet cherry orchards that are pruned on a yearly basis because ascostromata usually do not form until infected branches have been dead for 2 to 3 years. Because conidia are more common than ascospores (2), they are probably a more frequent source of infection. However, the sexual state may play an important role in increasing genetic variability within south central Washington. Ascospores may also disseminate the fungus greater distances than do conidia since they are forcibly discharged and become airborne (2). Ascospores were found to be infective on prune and plum trees in California (2) and on sweet cherry in this study.

Conidia of *L. cincta* were released from pycnidia on sweet cherry over a wide temperature range. Conidia were released at a low temperature of 2°C, indicating that conidia can be released during relatively warm periods during the winter. In other studies, conidia of *L. persoonii* were trapped in plum and prune orchards in California during rains occurring in all seasons (2), and in a peach orchard in Colorado throughout the year, with the highest counts occurring during the summer (13).

Conidia were most rapidly released from pycnidia in the presence of free water. Conidia of *L. persoonii* and *L. cincta* are disseminated mainly by splashing and windblown water (2,13,24), and conidia are only released when they can possibly be splash dispersed. Incubation of pycnidia at 20°C with free water showed that conidia were released in less than 6 h, whereas at 100% RH they required more than 48 h to sporulate. Schulz and Schmidle (19) showed significant correlations between conidia captured in spore traps and the duration of leaf wetness. No evidence was found for any non-waterborne release of conidia in prune and plum orchards in California (2).

Conidia of *Leucostoma* spp. separated from the gelatinous matrix are short-lived (17,21). Tekauz (21) showed that individual conidia of *L. cincta* died within 6 h when released from their gelatinous matrix, wetted, and then allowed to dry. In this study, conidia in dried cirrhi survived for 30 days under orchard conditions without a significant reduction in germination. Conidia in dried cirrhi incubated at 20°C for 20 days were just as infective as conidia that were recently exuded from pycnidia. These results suggest that conidia in dried cirrhi remain viable for extended periods of time. This is important in the epidemiology of Leucostoma canker in Washington. After brief rain showers, conidial cirrhi frequently dry on infected branches and are not disseminated until additional rainfall, which may not occur for several weeks. The wide temperature range in which conidia were exuded from pycnidia and the longevity of conidia in dried cirrhi accounts for the presence of viable conidia in orchards throughout the year (2,13,19).

Ascospores and conidia of *L. cincta* had the same optimum temperature of 28°C for germination. However, a higher percentage of ascospores germinated at 10 and 15°C during the shorter incubation periods of 12 to 24 h than did conidia. At the extended incubation periods of 48 and 72 h, conidia also germinated at the lower temperatures of 5 and 10°C. Germination of conidia was also shown at 10°C in a previous study (17). Both conidia and ascospores are potentially able to infect at relatively low temperatures if conditions remain sufficiently wet. Ascospores of *L. persoonii* tended to be most common in the spring (2) and may be able to more quickly infect the host than conidia at relatively low temperatures. Variation of virulence among isolates of the anamorph of *L. cincta* and *L. persoonii* has been found (8), and variation among isolates for time of incubation period needed for germination at low temperatures needs to be further investigated.

Removing infected branches and trees, avoiding bark injuries and winter damage, and maintaining tree vigor are recommended control practices for Leucostoma canker (24). The importance of sanitation practices in managing Leucostoma canker has not been demonstrated and published. Removing dead infected branches should lessen the effect of the sexual state as a component in the epidemiology of the disease. Pycnidia have a wide temperature range in exuding conidia, and pruning and destroying infected branches should reduce the numbers of pycnidia and the amount of dried conidial cirrhi in orchards.

ACKNOWLEDGMENTS

Plant Pathology New Series 0217, Project 0678, College of Agriculture and Home Economics, Research and Extension Center, Washington State University, Pullman, WA 99164-6430.

LITERATURE CITED

1. Bertrand, P. F., and English, H. 1976. Virulence and seasonal activity of *Cytospora leucostoma* and *Cytospora cincta* in French prune trees in California. Plant Dis. Rep. 60:106-110.
2. Bertrand, P. F., and English, H. 1976. Release and dispersal of conidia and ascospores of *Valsa leucostoma*. Phytopathology 66:987-991.
3. Biggs, A. R. 1989. Integrated approach to controlling Leucostoma canker of peach in Ontario. Plant Dis. 73:869-874.
4. Chakravarti, I. M., Laha, R. G., and Roy, J. 1967. Handbook of Methods of Applied Statistics. Vol. 1. Techniques of Computation, Descriptive Methods, and Statistical Inference. John Wiley & Sons, New York.
5. Garybill, F. A. 1976. Theory and Application of the Linear Model. Duxbury Press, North Scituate, MA.
6. Grove, G. G., and Boal, R. J. 1991. Factors affecting germination of conidia of *Podos-*

phaera clandestina on leaves and fruit of sweet cherry. Phytopathology 81:1513-1518.
7. Grove, W. B. 1923. The British species of *Cytospora*. Bull. Misc. Inf. R. Bot. Gard. No. 1:1-30.
8. Helton, A. W., and Konicek, D. E. 1961. Effects of selected *Cytospora* isolates from stone fruits on certain stone fruit varieties. Phytopathology 51:152-157.
9. Hildebrand, E. M. 1947. Perennial peach canker complex in New York, with methods of control. Cornell Univ. Agric. Exp. Stn. Memoir 276:45-61.
10. James, W. C., and Davidson, T. R. 1971. Survey of peach canker in the Niagara Peninsula during 1960-1970. Can. Plant Dis. Surv. 51:148-153.
11. Johnson, C. G. 1940. The maintenance of high atmospheric humidities for entomological work with glycerol-water mixtures. Ann. Appl. Biol. 27:295-297.
12. Kern, H. 1955. Taxonomic studies in the genus *Leucostoma*. Mich. Acad. Sci. Arts Lett. 40:9-22.
13. Luepschen, N. S., and Rohrbach, K. G. 1969. Cytospora canker of peach trees: Spore availability and wound susceptibility. Plant Dis. Rep. 53:869-872.
14. Neter, J., Wasserman, W., and Kutner, M. H. 1985. Applied Linear Statistical Models. 2nd ed. Richard D. Irwin, Homewood, IL.
15. Regner, K. M., Johnson, D. A., and Gross, D. C. 1990. Etiology of canker and dieback of sweet cherry trees in Washington State. Plant Dis. 74:430-433.
16. Rogers, W. E. 1960. A microenvironment chamber for critical control of relative humidity. Phytopathology 56:980-982.
17. Rohrbach, K. G., and Luepschen, N. S. 1968. Environmental and nutritional factors affecting pycnidiospore germination of *Cytospora leucostoma*. Phytopathology 58:1134-1138.
18. Scharpf, R. F. 1964. A compact system for humidity control. Plant Dis. Rep. 48:66-67.
19. Schulz, U., and Schmidle, A. 1983. Zur Epidemiologie der Valsa-Krankheit. Angew. Bot. 57:99-107.
20. Spielman, L. J. 1985. A monograph of *Valsa* on hardwoods in North America. Can. J. Bot. 63:1355-1378.
21. Tekauz, A. 1972. The role of leaf scar and pruning cut infections in the etiology and epidemiology of peach canker caused by *Leucostoma* species. Ph.D. thesis. University of Toronto, Toronto.
22. Wehmeyer, L. E. 1926. A biologic and phylogenetic study of the stromatic sphaeriales. Am. J. Bot. 13:575-645.
23. Willison, R. S. 1937. Peach canker investigation. III. Further notes on incidence, contributing factors, and control measures. Can. J. Res. Sec. C. 15:324-339.
24. Wilson, E. E., and Ogawa, J. M. 1979. Fungal, Bacterial, and Certain Nonparasitic Diseases of Fruit and Nut Crops in California. Div. Agric. Sci. Univ. Calif., Berkeley, CA.

ERRATUM / Volume 79, Number 9, 1995

In the article "Furovirus Diseases of Sugar Beets in the United States" by C. M. Rush and G. B. Heidel on pages 868-875, the author biography for Ms. Heidel should read, Gretchen Heidel is a research associate in plant pathology at the Texas Agricultural Experiment Station (TAES) in Amarillo. She received a B.S. in biology and a B.A. in English from Texas A&M University in 1985 and completed an M.S. in plant pathology at Texas A&M in 1990. Since joining TAES in 1990, her research has been directed toward virus diseases of sugar beets, wheat, and corn.