Leptotrochila medicaginis: Moisture Requirements for Ascospore Discharge, Germination, and Plant Infection

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ABSTRACT

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In humidity-controlled atmospheres above water or different concentrations of aqueous NaCl solutions at 20–22 C, mature apothecia on dry, infected alfalfa leaves (*Medicago sativa*) rehydrated and discharged ascospores of *Leptotrochila medicaginis* more abundantly near 99.5 and 100% RH and less abundantly at progressively lower RH values; RH limits for discharge were 98.0% at 14.4 and 15.5 C and 97.5% at 20–22 and 26-28 C. On glass in a moist chamber (~100% RH) and on fresh water agar (WA) at 20–25 C, discharged ascospores swelled and formed short germ tubes within 4 hr, which lengthened to spore width (approximately 5 μm) within 6 hr. Ascospores on fresh potato-dextrose agar completed these two processes within 4 hr. They also completed these two processes on WA fortified with glucose, glycerol, and sucrose as humectants within 4 hr at water potentials ranging from −40.6 to −30.7 bar and within 12–24 hr at water potentials ranging from −70.8 to −63.6 bar, and on WA fortified with NaCl and KCl as humectants within 24 hr at water potentials of −49.2 and −63.6 bar, respectively. Ascospores on alfalfa stem cuttings held in a moist chamber at 20–25 C swelled within 4 hr, and their germ tubes infected leaves within 8 hr. At a constant 17 C, the minimum RH for leaf infection within 24 hr was 95%.

Additional keywords: yellow leafblotch

From 1952 to 1983, yellow leafblotch of alfalfa (*Medicago sativa* L.), induced by *Leptotrochila medicaginis* (Fuckel) H. Schüepp, occurred in varying incidences year after year in the western part of South Dakota. In the eastern part of the state where rainfall is higher, it almost ceased to exist in the early 1960s, having been abundant there during the mid-1950s. The reason for this difference was not understood, but it may have been due to differences in the rate of inoculum regeneration and dissipation as related to moisture availability. Salunska (17) performed field studies of apothecial development, ascospore production, and disease progress by the yellow leafblotch pathogen and deduced that alternating dry and wet periods favored pathogen persistence and disease occurrence, and that ambient moisture conditions above 70% relative humidity (RH) were necessary for ascospore discharge and plant infection. To assess more accurately the latter relationship for *L. medicaginis*, a determination was made of moisture needs for ascospore discharge, ascospore germination, and plant infection. A preliminary summary of the results has been reported (21).

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MATERIALS AND METHODS

Supply of mature apothecia and ascospores. Alfalfa leaves with yellow leafblotch symptoms were collected from fields in western South Dakota in early summer 1981 and exposed, as previously described (19,20,25), to local (Brookings) outdoor moisture conditions until apothecia with mature ascospores developed. Ascospores developed by early fall, whereupon the leaves were pressed flat and air-dried between moisture-absorbing paper, stored in a closed polyethylene box at 4–8 C, and used over the ensuing winter months.

Moisture required for ascospore discharge. Three or four dry leaflets with well-developed apothecia were placed on each of two 25 × 75 mm glass microscope slides contained in transparent plastic boxes (80 × 63 × 30 mm). These slides were supported horizontally and side-by-side in each box by end-wall ledges 10 mm above 60 ml of aqueous NaCl solutions (12,16) or deionized water that produced relative humidities ranging from 95 to 100% at 0.5% intervals. Other slides were supported 3 mm above the leaf-bearing slides in order to catch discharged ascospores. The boxes were covered and sealed tightly with plastic tape.

Four trials were conducted, each with a triplicate array of leaf-containing boxes with a range of RH values. In two trials, boxes were kept in a large, insulated room at a constant temperature of 14.4 ± 0.5 and 15.5 ± 0.5 C, respectively; apothecia were scored for their ascospore discharges at 2- and 4-day intervals. In the other two trials, boxes were held in temperature-controlled cabinets at 21.0 ± 1.0 and 27.0 ± 1.0 C, respectively; ascospore discharge scores were determined after 2 days.

Moisture required for ascospore germination. Dry leaflets bearing mature apothecia were rehydrated for 1–2 days at 2–5 C on a wet paper towel within a closed plastic box. Each leaflet, with apothecia facing upward, was then transferred onto a piece of wet paper towel within the well (18 mm wide × 5 mm deep) of a Syracuse dish and held at 20–25 C. Each well was covered with an ethanol-sterilized microscope coverslip

(18 × 18 mm) to catch ascospores that discharged within several minutes after leaflet transfer. When an estimated 150–250 ascospores had adhered to a coverslip, the coverslip was replaced, often as many as five times. Coverslips bearing ascospores were stored in sterile petri dishes at 4–8 C for use within the next 2 days.

Water agar (WA) (1 L of H_2O and 20 g of Difco Bacto agar], fortified with graded concentrations (w/w) of glucose, glycerol, sucrose, NaCl, or KCl as humectants, was steam-sterilized and petri-plated. Concentration grades corresponding to a range of RH values were derived for glucose and glycerol solutions from Norrish's formula (14) and for sucrose, NaCl, and KCl solutions from Robinson and Stoke's data (16). Before use, duplicate plates of freshly poured agar containing the same humectant and RH value were held together for 2 days within a closed polyethylene bag at 20–25 C to allow the free moisture within plates to equilibrate with the agar medium. Equilibrium RH values were inferred from the concentration of humectant in the agar. Water potential values (6) that correspond to these equilibrium RH values are reported in cases in which germination progress occurred while ascospores were in direct and sole contact with the agar medium as a moisture source.

Ascospores were observed germinating under a range of moisture conditions while on glass in a saturated atmosphere above water (100% RH) and while in direct contact with fresh WA (~−1.4 bar), fresh potato-dextrose agar (PDA) (~−4.2 bar), and fortified WA media. Two ascospore-loaded coverslips were laid, ascospore-bearing surface down, onto the agar surface within each plate. The plates were returned to the original bags and held at 20–25 C. At periodic intervals spanning 24–30 hr, the ascospores were examined in situ for germination progress. Counts were made of incipiently germinated ascospores (those with a sidewall bulge or with a short side tube <1× ascospore width). Germ tube lengths were determined for fully germinated ascospores with germ tubes ≥1× ascospore width at selected intervals within 3 days after initial contact with agar fortified with glucose and glycerol and within 30 hr, with sucrose.

Moisture required for infection. Two studies of the range of relative humidities permitting infection were conducted with leaf-bearing stem cuttings of two cloned plants of the susceptible cultivar Mesa Sirsa (25). In both studies, stem cuttings were 25–50 mm long, and each bore one or more trifoliolate leaves at the top. For inoculation, cuttings were clustered vertically in potted moist sand and showered over a 2-hr period with ascospores from a canopy of rehydrated apothecia-bearing leaves (20).

In the first study, groups of four inoculated cuttings, using clones of only one plant, with stems wrapped together in aluminum foil, were suspended over 600 ml of NaCl solutions of different concentrations within 0.95-L (1-qt) glass jars. In a first trial of this study, solutions were graded to produce humidities ranging from 94 to 99% RH at 1% intervals; in a second trial, from 93.0 to 99.5% RH at 0.5% intervals. After 24 hr at a constant 17 C for both trials, cuttings were removed from the jars, and stems were inserted in potted moist sand and held in the open laboratory (20–25 C, indirect sunlight, RH <60%) for up to 3 days to allow for possible further growth of infectious hyphae within leaves. One or more leaflets from each cutting were then decolorized in boiling 80% ethanol, stained with cotton-blue in lactophenol, and examined microscopically for fungal penetration. In the second study, 20 inoculated cuttings (10 from each of the two different plants) were placed in a moist chamber (~100% RH) at 20–25 C. At timed intervals, two cuttings from each of the two plants were removed from the chamber, and leaves were examined as before for infection progress.

RESULTS

Moisture required for ascospore discharge. After 2 and 4 days in humidity-controlled atmospheres held at 14.4 ± 0.5 C, apothecia discharged ascospores most abundantly at 100 and 99.5% RH, less abundantly at 99.0 and 98.5% RH, and in trace amounts after 4 days at 98.0% RH (Fig. 1A). No ascospores were discharged at 97.5% or lower RH values. After 4 days in relative humidities greater that 98.5%, apothecia discharged ascospores more abundantly at 14.4 ± 0.5 C than at 15.5 ± 0.5 C (Fig. 1B). The decline in ascospore discharge with

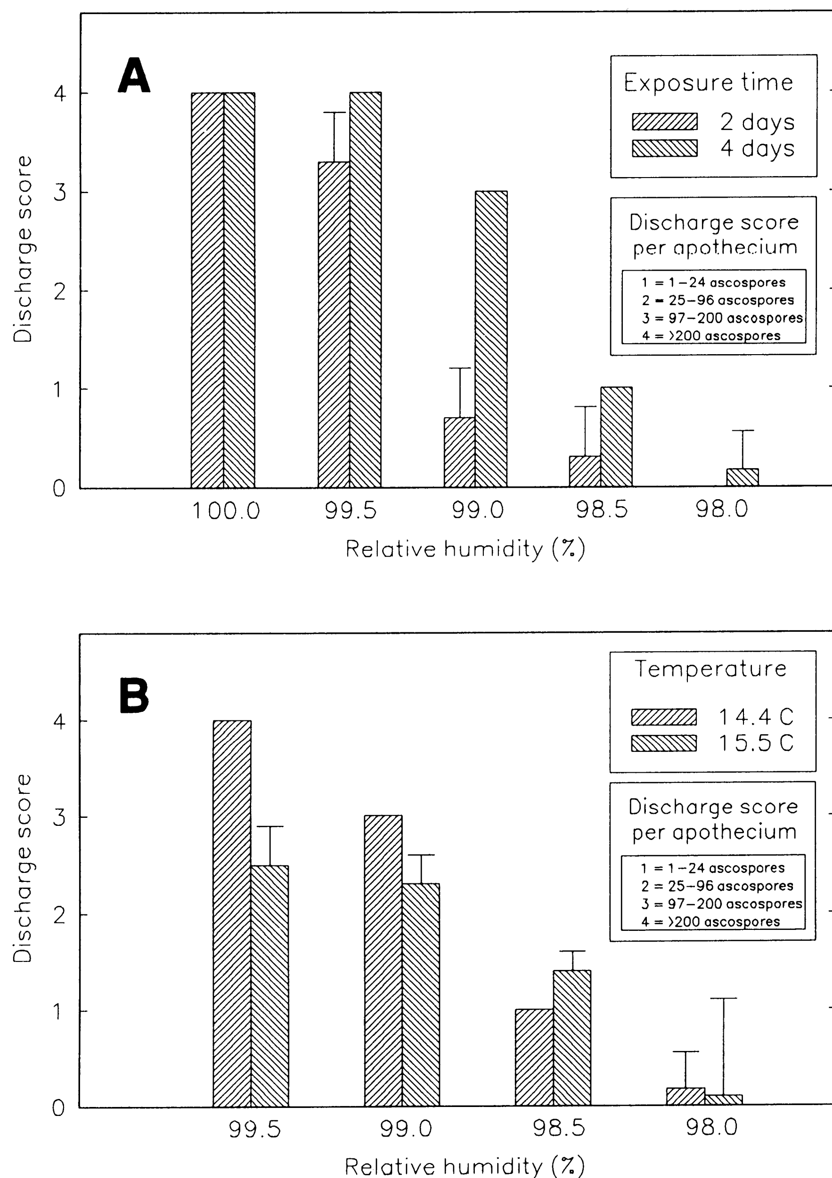

Fig. 1. Abundance of ascospores discharged per apothecium after exposure of dry apothecia to different relative humidities for **(A)** 2 and 4 days at 14.4 ± 0.5 C and **(B)** 4 days at 14.4 ± 0.5 and 15.5 ± 0.5 C. Only positive SD values are shown.

relative humidity also roughly paralleled the decline in the number of apothecia per leaflet that discharged ascospores, the abundance of ascospores per apothecium that were discharged, and the extent to which the apothecia swelled. After 2 days at 21.0 ± 1.0 and 27 ± 1.0 C in humidity-controlled atmospheres (data not shown), apothecia discharged ascospores with declining abundance as relative humidity declined from 100 and 99.5%, as in the other trials, except the minimum RH for discharge was 97.5%.

Ascospore germination on glass at high humidity and on fresh agar surfaces. Ascospore germination on glass or agar surfaces was seen in two stages: in stage 1, ascospores absorbed moisture from the atmosphere or agar and began to swell to form a bulge, or a short side tube, less than the diameter of the ascospore; in stage 2, a germ tube extended from this protuberance and exceeded the width of the ascospore. When stage 2 was complete, the ascospore was considered to have germinated. Within 4 hr at 20–25 C, about one third of the ascospores on bare glass, fresh WA, and fresh PDA surfaces germinated incipiently, with about one half of those germinating on PDA also forming germ tube lengths equal to 1× ascospore width. Within 6 hr, germ tube lengths equal to 1× ascospore width were attained by one half of 54% germinated spores on glass and on WA. Within 12 hr on glass, germ tube lengths of 1–2× ascospore width were produced by nearly one half of the 80%-germinated ascospores.

Ascospore germination and germ tube growth in relation to moisture stress. On WA fortified with graded concentrations of glucose, glycerol, or sucrose as humectants at 20–25 C (Fig. 2), less than 15% of the ascospores germinated within 4 hr at water potentials below −40.6 bar, with <5% of the ascospores on each agar medium producing germ tube lengths of 1× ascospore width. Within 12 hr, the lowest water potential for attaining germ tubes of 1–2× ascospore width was −40.6 bar with glucose, −54.9 bar with glycerol, and −49.2 bar with sucrose as humectants. At higher water potentials within this 12-hr time span, some germ tube lengths were as much as 6× ascospore width. Over 24–72 hr, ascospore germination percentages did not improve significantly over those attained within 12 hr, but germ tube lengths increased at different rates, depending upon the water potential of the agar medium (Fig 3). The standard deviation of germ tube lengths during the first 72 hr increased linearly by a factor of 2.47 and 2.71 on glucose and glycerol agars, respectively, with increases in average germ tube lengths. Within this time interval, minimum water potential values (in bars) for ascospores to generate germ tubes were −67.9 (glucose), −86.9 (glycerol), and −63.6 (sucrose). Above these water potential values on glucose, sucrose, and glycerol agars, ascospores attained nearly 100% germination over a 2-wk period, and germ tubes branched and increased in length. After 5 days on sucrose agar, germ tubes grew well at water potentials of −42.0 bar and above, slowly with some degeneration at −49.2 bar, and poorly or not at all at −56.4 bar and below. When a culture derived from ascospores was transferred to yeast-malt agar (50 ml of H_2O, 0.2 g of yeast extract, 0.5 g of malt extract, and 1.0 g of agar) at 20–25 C, mycelium grew well in the medium fortified with 20 g ofsucrose (∼−30.7 bar) but did not grow when the medium was fortified with 30 g of sucrose (∼−50.6 bar).

Within 24 hr on WA fortified with NaCl or KCl (Fig. 4,) only a few ascospores germinated at water potential values below −49.2 (NaCl) and −63.6 bar (KCl). Spores swelled without forming germ tubes at water potentials lower than these, but germ tubes that were formed at −20.9 bar and at higher

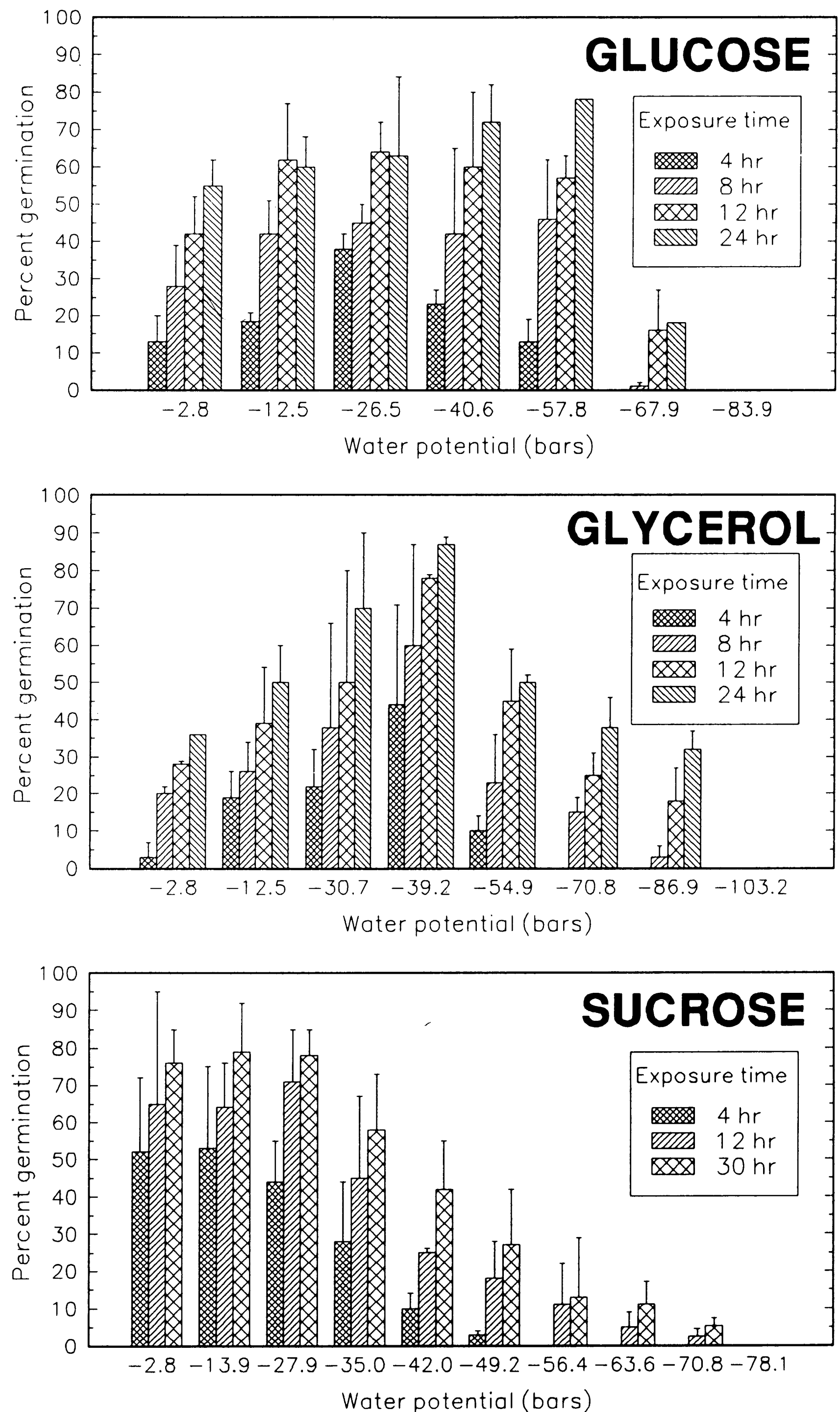

Fig. 2. Percentage of ascospores germinating over time on plain agar fortified with different concentrations of glucose, glycerol, or sucrose equivalent to a range of water potential values. Only positive SD values are shown.

water potentials generated sporidia on both agar media. Germination at −56.4 (NaCl) and −63.6 bar (KCl) over 4 days, however, did not proceed further, and germ tubes that were already formed as well as ungerminated ascospores were degenerating. Slow germ tube growth was maintained at −49.2 bar on NaCl agar and at −63.6 bar on KCl agar, and growth was faster on each of these agars at progressively higher water potentials. A mycelial culture of the pathogen produced no growth on yeast-malt agar fortified with 1 g of sucrose and 2.5 g of NaCl (~−39.2 bar).

Infection progress on alfalfa leaves in a moist chamber. Infection progress on two plants of cv. Mesa Sirsa are depicted in Figure 5, with plant B appearing to be more susceptible to the pathogen than plant A. The figure depicts bulged-out ascospores with narrow, cotton-blue–stained edges within a 2-hr exposure of inoculated leaves to high humidity (~100% RH), and the same bulging but with broader-stained edges within a 4-hr exposure to the same high humidity. Within an 8-hr exposure, vesicles containing granular matter were detected within the host cell directly beneath the ascospore. Within 24 hr, vesicles had become walled and had generated other walled cells, which elongated by apical growth into the next host cell. By the third day, hyphae extended linearly into a third host cell.

Relative humidity supporting infection. In two trials, a leaflet on each of four alfalfa stem cuttings became infected within 24 hr within the range of 95.0–99.5% RH. There was no infection below 95.0% RH, except for a single infection at 94.5% RH on one leaflet of one cutting in the second trial. The proportion of ascospores on a leaflet that produced infection was not determined.

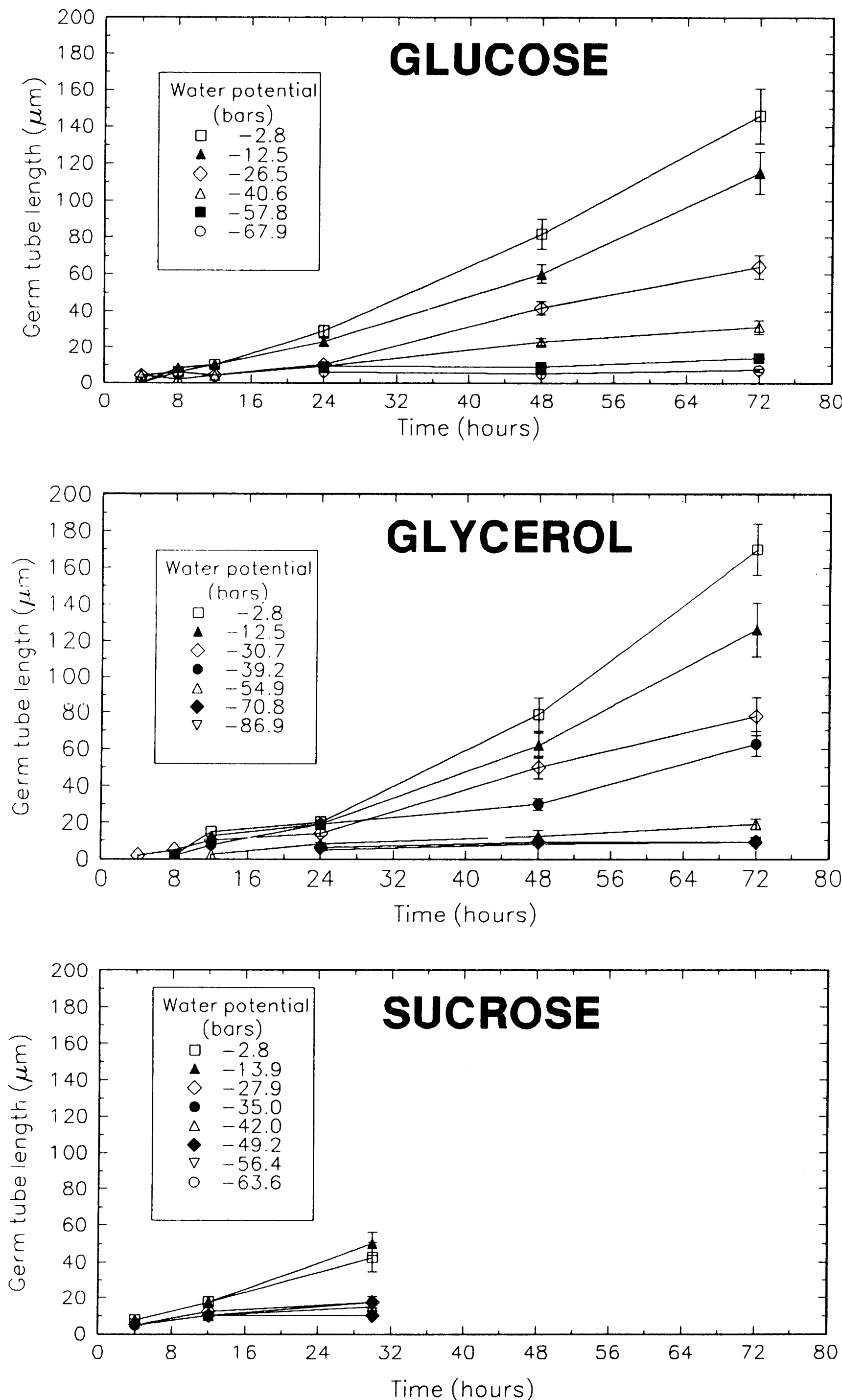

Fig. 3. Growth of ascospore germ tubes over time on plain agar fortified with different concentrations of glucose, glycerol, and sucrose equivalent to a range of water potential values. SD values are reduced by a factor of 4.

DISCUSSION

Moisture limits for ascospore discharge, ascospore germination, and plant infection reported here for *L. medicaginis* fall well above the low limit of the moisture range Salunska (17) deemed necessary (>70% RH, ambient) for field propagation of the pathogen. The requirement of 95% RH and above for these processes to occur is similar to those reported for *Pseudopeziza medicaginis* (Lib.) Sacc. (22,23), a widely studied pathogen of alfalfa. These two pathogens, which may appear separately or together within a region (1,2,5,7–9), are similar in the variable germination of their ascospores; in penetration of the host directly beneath or aside ascospores; and in infection of field plants during late spring, summer, and early fall from infected leaves fallen to the ground (10,11). They differ in rate of apothecial development, in apothecial structure, and in ability to repropagate themselves on growing plants. These differences, along with a possible differential impact of weather on production, loss, and survival of their respective inocula, may account for changes in their cohabitation. Salunska (17) has proposed a survival mechanism that may be applicable to both pathogens. Moisture shortage slows, and thereby prolongs over summer, fall, and winter, the functional life of developed, sporuliferous apothecia, whereas a moisture excess hastens and thereby shortens that life.

An additional feature to a differential survival of the two pathogens may be the relative ability of their ascospores to quickly infect plants during periods of high humidity. Observations presented elsewhere for *L. medicaginis* (20) suggest an interaction with the host that begins within 2–4 hr and is followed by host penetration within 8 hr at 20–25 C. The nature of the early interaction is not known, but it appears to confer permanence to the association, since only 2–6 hr of high moisture conditions at 18–22 C were necessary to establish an

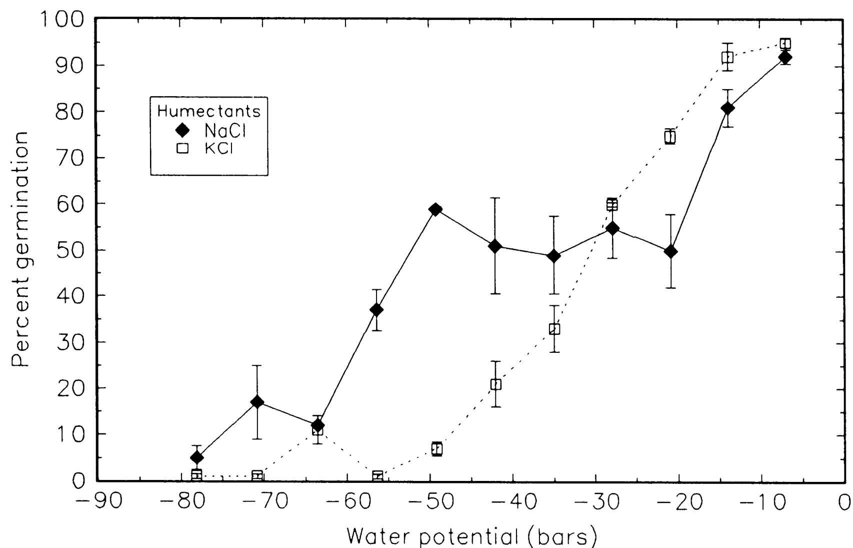

Fig. 4. Percentage of germination of ascospores after 24 hr on plain agar fortified with different concentrations of NaCl or KCl for a range of equivalent water potential values. SD values are reduced by a factor of 2.

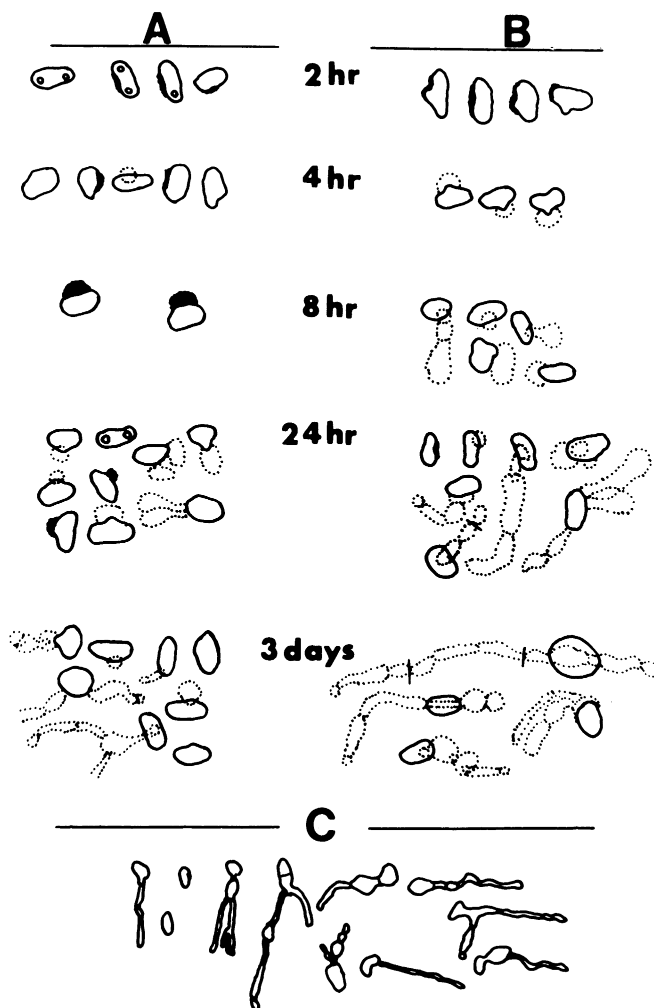

Fig. 5. Drawing of germinating *Leptotrochila medicaginis* ascospores at 20–25 C on leaves of two alfalfa plants (**A, B**) (*Medicago sativa* L. cv. Mesa Sirsa) held within a moist chamber (~100% RH) over a 72-hr period. Ascospores on leaves are outlined in solid lines; their hyphae, which appeared only within epidermal leaf cells beneath the spore, have dotted outlines. (**B**) Solid cross-line (at 24 hr and 3 day) shows passage of a hypha through a neighboring cell wall. Fuzzy spore edges shown (**A**) at 2, 4, 8, and 24 hr and (**B**) at 2 hr are cotton-blue–stained material between spore and host surface. (**C**) Ascospore germination on surface of potato-dextrose agar beneath glass coverslip after 3 days. Approximate magnification: (**A, B**) ×1,050 and (**C**) ×450.

ongoing, continuous infection process (20). A possible basis for such an association may be taken from that offered by Morgan and Parbery (13) for the protection of *P. medicaginis* ascospores on leaves from phylloplane microorganisms: namely, that ascospores secrete substances during germination that dissolve cuticular wax that encrusts and protects the germinating ascospore.

The experimental data show agreement between the low moisture limit (−70.8 to −63.6 bar, roughly corresponding to 95.0–95.5% RH) supporting ascospore germination on agar surfaces at 20–22 C and that supporting plant infection (95.0% RH) within 24 hr at 17 C. This agreement, unaccountably, was closer with the use of glycerol than glucose or sucrose as a humectant. The information, provided by the data, that germ tubes grow progressively faster as humidity increases above the minimum has relevance mainly to the prepenetrating phase of the pathogen. It has little relevance to the parasitic growth phase, because the internal moisture potential of the parasitized, living leaf is likely to be equivalent to 99.3% RH (−9.3 bar) or higher at 20 C (4).

The variable vigor with which ascospores germinate and germ tubes elongate (10,15) was further emphasized in a limited study (G. Semeniuk, *unpublished*) of *L. medicaginis*, in which only four of 24 germinated single ascospores yielded growing colonies on oatmeal agar. Germ tubes of the other 20 ascospores ceased growth within 1 wk as tiny, barely visible colonies. This variability suggests a variable potential among ascospores to infect host tissue (3,24) and to produce typical yellow leafblotch symptoms (25). Such variable interactions with the host have been noted with *P. medicaginis* (18), whose ascospores germinate and germ tubes elongate in a similarly variable manner (11).

LITERATURE CITED

1. Berkenkamp, B. 1971. Losses from foliage diseases of forage crops in central and northern Alberta in 1970. Can. Plant Dis. Surv. 51:96-100.
2. Berkenkamp, B. 1974. Losses from foliage diseases of forage crops in central and northern Alberta, 1973. Can. Plant Dis. Surv. 54:111-115.
3. Berkenkamp, B., and Meese, J. 1979. Resistance of alfalfa cultivars to yellow leaf blotch. Can. J. Plant Sci. 59:873-874.
4. Brown, P. W., and Tanner, C. B. 1981. Alfalfa water potential measurement: A comparison of pressure chamber and leaf dew-point hygrometers. Crop Sci. 21:240-244.
5. Gray, F. A. 1983. Assessment of foliage diseases in Wyoming. Plant Dis. 67:1156-1158.
6. Griffin, D. M. 1969. Soil water in the ecology of fungi. Biol. Rev. 38:289-310.
7. Hansen, E. W. 1953. Relative prevalence and severity of the diseases of forage legumes in Wisconsin, 1946-1952. Plant Dis. Rep. 37:467-472.
8. Harding, H. 1969. Diseases of alfalfa and other forage legumes in Saskatchewan in 1968 and 1969. Can. Plant Dis. Surv. 49:126-127.

9. Harding, H. 1972. Foliage diseases of alfalfa in northern Saskatchewan: A note on the 1972 survey and the differential reactions of nine varieties. Can. Plant Dis. Surv. 52:149-150.
10. Jones, F. R. 1918. Yellow-leafblotch of alfalfa caused by the fungus *Pyrenopeziza medicaginis*. J. Agric. Res. 13:307-329.
11. Jones, F. R. 1919. The leaf spot disease of alfalfa and red clover caused by the fungi *Pseudopeziza medicaginis* and *Pseudopeziza trifolii*, respectively. U.S. Dep. Agric. Agric. Bull. 759.
12. Linge, K. 1929. Der Dampfdruck über wässerigen Lösungen von Chlornatrium, Chlormagnesium und Chlorcalcium. Z. Gesamte Kälte-Indus. 36:189-193.
13. Morgan, W. C., and Parbery, D. G. 1977. Ascospore liberation, germination and attachment to host surface by *Pseudopeziza medicaginis*. Aust. J. Agric. Res. 28:777-784.
14. Norrish, R. S. 1966. An equation for activity coefficients and equilibrium relative humidities of water in confectionery syrups. J. Food Technol. 1:25-39.
15. Perišić, M., and Stojanović, D. 1966. Contribution to the study of the biology of *Pseudopeziza jonesii* Nannf. (*Pyrenopeziza medicaginis* Fuck.), the causal agent of the yellow leafblotch of lucerne. (In Croatian, with English summary.) Zast. Bilja 17(91-92):183-190.
16. Robinson, R. A., and Stokes, R. H. 1958. Electrolyte Solutions. (Table 1, Appendix 8.3.) Rev. ed. Butterworth, London.
17. Salunska, N. I. 1965. Predicting yellow leafblotch on alfalfa in relation to its cyclical development and build-up. (In Ukrainian.) Zahist Roslin 2:76-85.
18. Schmiedeknecht, M. 1958. Untersuchungen zur Spezialisierung von *Pseudopeziza medicaginis* (Lib.) Sacc. Phytopathol. Z. 32:431-450.
19. Semeniuk, G. 1971. Diseased leaves as a source of ascospores of *Leptotrochila medicaginis* for alfalfa inoculations. (Abstr.) Phytopathology 61:910.
20. Semeniuk, G. 1982. Time required for alfalfa infection by *Leptotrochila medicaginis* Proc. S.D. Acad. Sci. 61:107-113.
21. Semeniuk, G. 1982. *Leptotrochila medicaginis*: Pattern and distance of ascospore discharge; moisture required for ascospore discharge and germination. (Abstr.) Proc. S.D. Acad. Sci. 61:182.
22. Semeniuk, G. 1984. Common leafspot of alfalfa: Ascospore germination and disease development in relation to moisture and temperature. Phytopathol. Z. 110:281-289.
23. Semeniuk, G. 1984. Common leafspot of alfalfa: Ascospore discharge and plant infection in the field. Phytopathol. Z. 110:290-300.
24. Semeniuk, G., Adams, M. W., and Rumbaugh, M. D. 1978. Heritable reaction in two alfalfa populations in field nurseries to the yellow leafblotch disease. Proc. S.D. Acad. Sci. 57:73-80.
25. Semeniuk, G., and Rumbaugh, M. D. 1976. Reaction of some perennial and annual *Medicago* species and cultivars to the yellow leafblotch disease caused by *Leptotrochila medicaginis*. Plant Dis. Rep. 60:596-599.