

**Induction of Systemic Aecial Infection in Canada Thistle (*Cirsium arvense*)
by Teliospores of *Puccinia punctiformis***

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ABSTRACT

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Systemic aecial infections of secondary Canada thistle shoots were observed 4–6 weeks after inoculation of root cuttings with teliospores of *Puccinia punctiformis*. Pretreatment with a stimulatory thistle root extract sometimes increased infection. Inoculation of dormant buds was the most effective way to induce infection of root cuttings. Aqueous soil

extracts from potted Canada thistle plants stimulated teliospore germination. The active component in the soil was soluble in hexane, volatile, and was similar in nature to the hexane extract of steam-distilled Canada thistle roots. Optimum temperature range for stimulated teliospore germination on 1% agar was 16–20 C at 7 days.

Additional keywords: biocontrol, Canada thistle rust, germination stimulator, noxious weed, systemic infection.

Canada thistle (*Cirsium arvense* (L.) Scop.), a common noxious weed in many parts of the United States, is a rugged perennial, easily propagated from root cuttings or seeds. It is occasionally attacked by an endemic, macrocyclic, autoecious rust, *Puccinia punctiformis* (F. Strauss) Röhl. (3) (= *Puccinia suaveolens*, *P. obtogens*). The aecial stage of this pathogen is systemic and causes severe etiolation and necrosis of the infected leaves and stems, usually preventing flowering. Because of its devastating effects on infected shoots and its host specificity, *P. punctiformis* might provide effective biocontrol for Canada thistle. Extracts of thistle

roots stimulate the teliospore of *P. punctiformis* to germinate, producing four basidiospores which may initiate a systemic infection (8,9). Chemical and physical properties of the root extract have been reported (7). The objectives of this research were as follows: using a stimulatory thistle root extract, to measure the optimum temperature range for germination of teliospores of Canada thistle rust; to investigate the use of this germination stimulator for enhancing systemic infection by various methods of inoculating the host with teliospores; and to test the water from soil of potted thistle plants for stimulatory activity on germination of the teliospores. Our ultimate objective is to improve the efficiency of this prospective biocontrol fungus by increasing teliospore germination and determining the most efficient means of inoculation.

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MATERIALS AND METHODS

The Canada thistle plants used were asexual progeny from local plants and were grown in the greenhouse or in growth chambers in sterilized soil in 10-cm clay pots. Sections of mature roots bearing a bud 2–5 mm in length were cut and the ends waxed to protect them from root pathogens (Fig. 1). The stimulatory thistle root extract was prepared by steam distilling about 1,000 g of washed and chopped thistle roots into 40 ml of hexane in a 2-L flask. The hexane was evaporated to 0.5 ml under nitrogen (7). Since *P. punctiformis* teliospores are rarely found around Frederick, MD, they were collected in Montana in August or September (1985) and stored at 4 C. (The aecial stage of this rust is found locally in Frederick, usually in May or June.)

Optimum temperature. Optimum temperature for stimulated teliospore germination was determined on an aluminum thermogradient plate which provided a range of ten temperatures between heated and refrigerated water baths at opposite ends of the plate. The aluminum plate was covered with a 1.9-cm thick polystyrene template with ten openings large enough to accommodate three 5-cm plastic petri plates. The middle plate contained 1% agar and an iron-constantan thermocouple attached to a Brownwell Multipoint (Brownwell Elektronik, Philadelphia, PA) recorder. The teliospores were placed on 5.0 ml of 1% agar in 5-cm petri plates, with and without 0.075 μ l (15 ppm) hexane extract of steam-distilled thistle roots. Petri plates were wrapped in aluminum foil and placed on the aluminum plate, and the template was covered with a 182-cm \times 60.9-cm \times 1.9-cm sheet of polystyrene insulation to isolate the separate chambers and to prevent air circulation, thereby reducing condensation of moisture on the thermogradient plate at the colder temperatures.

Inoculation of Root Cuttings. For inoculation studies, approximately 2 mg of teliospores were evenly dispersed on 2.0 ml of distilled water in the center of Conway diffusion cells. For stimulator treatments, 0.075 μ l of hexane extract (7) was added to the water by Hamilton syringe. Cells were covered by ground glass plates and placed in the dark at 18 C for 6 days.

In a preliminary experiment (Exp. 1), root cuttings (one per pot) were inoculated with stimulated (five pots) or unstimulated teliospores (five pots) as follows: 1) root cuttings, including bud, dipped in floating teliospores; 2) root portion of cutting, but not bud, dipped in floating teliospores; 3) buds only, of root cutting, inoculated by placing floating teliospores on the bud surface with a 3-mm inoculation loop; 4) newly formed white roots of cuttings dipped in floating teliospores; and 5) expanding buds only, of cutting, inoculated with floating teliospores using a 3-mm loop.

In a more extensive experiment (Exp. 2), five replications of ten roots each were treated as follows: 1) buds of root cuttings were inoculated with a 3-mm loop of stimulated teliospores, as previously described; 2) root-only portions of cuttings were

inoculated with a 3-mm loop of stimulated teliospores; 3) buds were inoculated with a 3-mm loop of unstimulated teliospores; and 4) root-only portions of cuttings were inoculated with a 3-mm loop of unstimulated teliospores.

In both experiments, root cuttings were planted 2 cm deep in soil in 10-cm clay pots and kept in a dark, cold room at 16 C for 1 wk to enhance conditions for teliospore germination, basidiospore production, and infection. The pots were then transferred to the greenhouse. In experiment 2, the pots in the greenhouse were arranged in a serpentine pattern in a randomized block design. The total number of plants infected per treatment was graphed as a function of time, up to 91 days. The means of the treatments were compared using Duncan's Multiple Range test ($P = 0.05$).

In another experiment, young, potted plants (5–10 cm) were inoculated with aeciospores or urediniospores, placed in a dew chamber for 24 hr at 22 C, and then placed in the greenhouse for 9 mo. After the plants set flower buds, they were cut back to soil level and allowed to regrow. Plants were observed for systemic infection of the new growth.

The susceptibility to infection of shoots breaking through the soil was tested in other experiments. Teliospores treated 6 days at 18 C with stimulator were transferred by inoculating loop to young thistle shoots just emerging through the soil surface. The pots were placed in a growth chamber at 22–24 C, for 12-hr days, and covered by 50-ml beakers for 2 weeks.

Soil extracts. Since thistle roots produce a stimulus to teliospore germination (7,9) and root buds appeared to be a good site for infection, we examined aqueous extracts of soil containing living thistle roots for stimulatory activity. A positive response might suggest involvement of thistle root exudates in the normal infection process. Water was added to and collected from the soil of 10-cm clay pots, three pots with a 15–20-cm young thistle plant, and three pots with soil only as controls. The pots previously had been placed in a tray of water to saturate the soil. Pots were placed on filter paper in a 15-cm Büchner funnel and watered. The water percolated through the soil and was collected in a vacuum Erlenmeyer flask. Equal volumes of water (120 ml) were collected from each pot. The leachate from each pot was examined separately. Portions of the soil leachates (4 ml for each of three replicates) were mixed with 1 ml of 5% agar for bioassay. Teliospores suspended in isopentane (2-methyl butane) (0.2 ml per plate) were dispersed on the surface of 1% agar mixtures of the soil leachates, 5.0 ml per 5-cm plastic petri plate. The teliospores were incubated in darkness at 18 C. Germination counts were made at weekly intervals, 4 \times 100 counts for each of three replicates per each of six pots, three pots each containing a thistle plant, and three pots containing only soil. The data were analyzed by Waller's grouping performed on arc sine transformed germination data, $\alpha = 0.1$.



Fig. 1. Washed thistle root showing intact dormant buds and typical root cuttings with waxed ends.

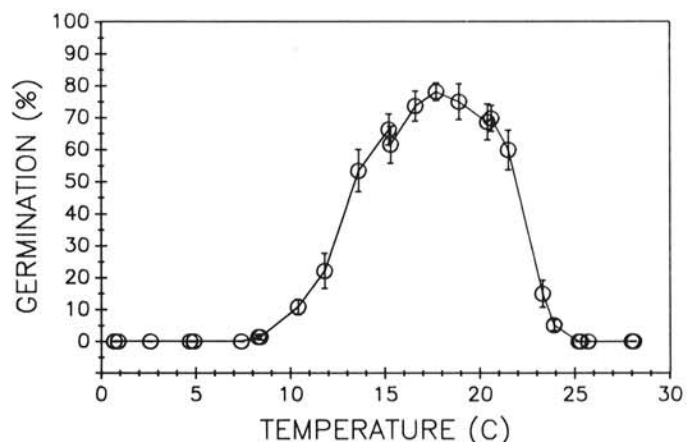


Fig. 2. Optimum temperature range for germination of stimulated teliospores of *Puccinia punctiformis* at 7 days. Average of three experiments, 10 temperatures in each; 8 \times 100 spores counted per temperature, SD at each point is indicated by bar.

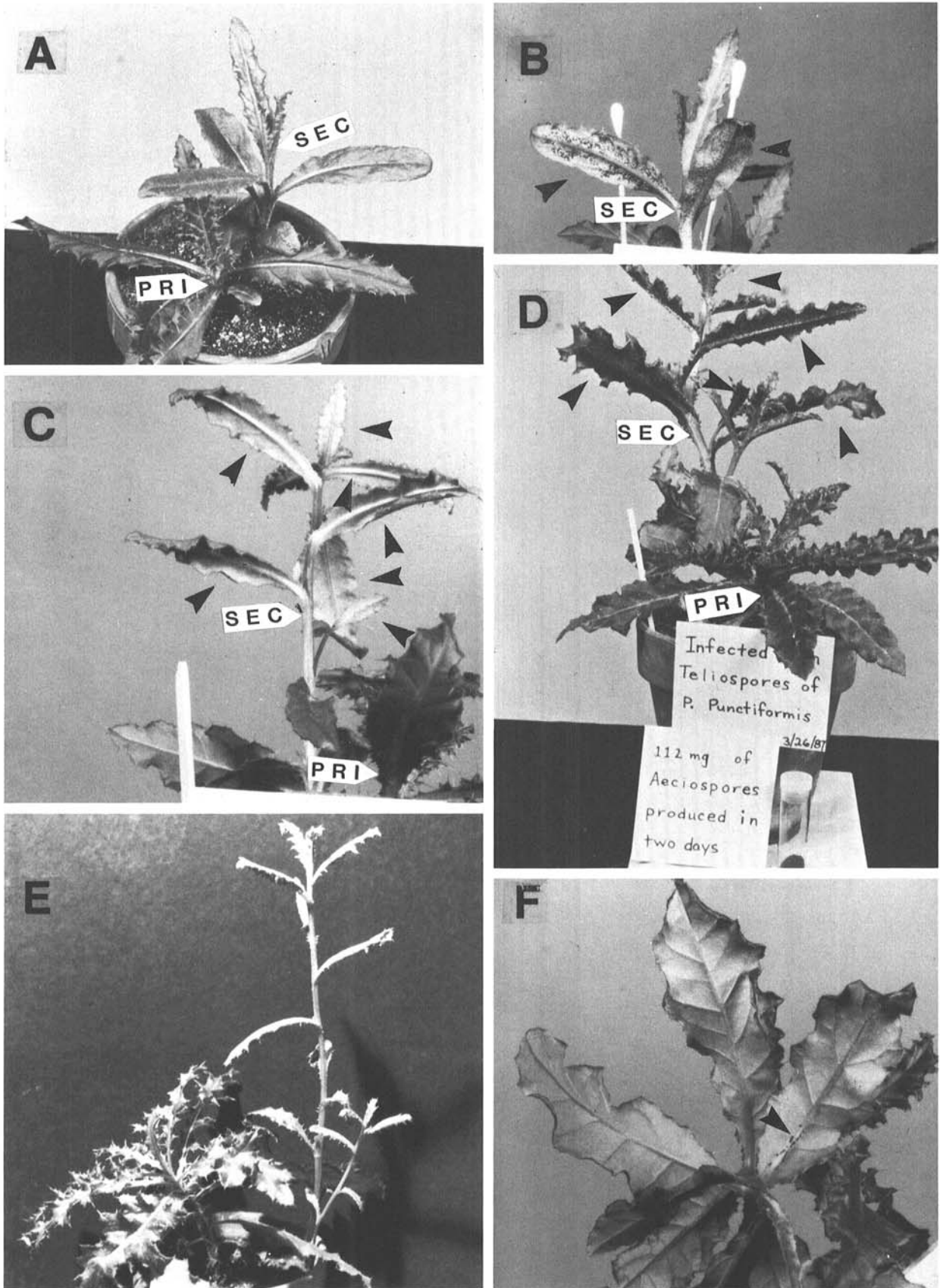


Fig. 3. Symptoms of infection of Canada thistle with *P. punctiformis*. (PRI = primary shoot; SEC = secondary shoot.) **A**, Shoots from inoculated root cutting, teliospores placed on root bud. Primary healthy shoot, left, and infected secondary shoot on right ($\times .44$) at 4 wk. **B**, Same plant, showing aecia and spermatogonia on abaxial leaf surface (arrows) ($\times .44$). **C**, Another secondary shoot bearing masses of orange spermatogonial structures on abaxial leaf surfaces (arrows) ($\times .4$). **D**, Secondary shoot producing great amounts of aeciospores (arrows) ($\times .4$), 112 mg/2 days. **E**, Canada thistle plant originally infected with urediniospores, showing systemically infected secondary shoot ($\times .35$). **F**, Plant inoculated with stimulated teliospores after green shoot broke through soil surface. Spermatogonial and aecial infections on abaxial midrib of upper right leaf (arrow) ($\times .8$). A small systemically infected shoot later grew from this leaf node. Systemic infection did not spread to other plant parts.

To compare the stimulatory activity in the soil leachates with that of the distilled thistle root extract (7), each of the leachates was extracted with hexane and bioassayed. Sodium chloride (17.5 g to give 50% saturation) was added to each of the remaining 108 ml of leachates to facilitate extraction. The leachates were extracted with 1) 100 ml of hexane, and 2) 20 ml of hexane, and the pooled 120 ml of hexane was evaporated to 0.5 ml on a cold plate (0 C) under nitrogen. Hexane extract (150 μ l) was placed in 5 ml of warm, 1% liquid agar, thoroughly mixed, and poured into 5-cm plastic petri plates, three replicates per each extract. Germination tests were run and analyzed as described above.

Volatility. In order to obtain significant data regarding the volatility of the germination stimulator in thistle soil, 200 ml of leachate was collected from each pot of four groups of four 15–20 cm tall, 7-mo-old potted thistle plants. The leachate was collected in 1-L glass beakers and drawn through glass wool and activated C-18 Seppak preparative chromatographic columns by house vacuum. Three C-18 Seppaks were used for the 800-ml leachate collected from each group. Hexane (2.0 ml) was passed through each C-18 Seppak and the 6.0-ml total was concentrated to 0.5 ml at 0 C under nitrogen. Fifty μ l portions of this hexane extract were placed on 16-mm Whatman No. 42 filter paper discs placed in the center of inverted lids of three 5-cm plastic petri plates. Teliospores suspended in isopentane were dispersed on the surface of 10-ml 1% agar in the base of the petri plates. Control plates using 50 μ l of pure hexane were included. The inverted plates with volatile samples on the lids were placed at 18 C in darkness, and percent germination was determined at 14 days. Standard controls consisting of 1% water agar \pm 50 μ l/L of the hexane extract of distilled thistle roots were also prepared in triplicate. Four hundred counts on each of three replicates were made on the hexane extracts of leachates from four groups of four plants, and from the appropriate controls. Statistical analysis was carried out as described above.

RESULTS

Optimum temperature. After 7 days of incubation, the optimum temperature range, where at least 90% of the maximum germination occurred, was 16–20 C (Fig. 2). Germination of at least fifty percent of maximum occurred between 13 and 23 C. No germination occurred below 8 C or above 25 C. At 5 days, germination was less than 1%, and as high as 80% at 7 days. At 25 C or above, contamination obscured germination at 14 days. Teliospores not treated with stimulator did not germinate.

Infection of root cuttings. Primary shoots from inoculated root cuttings emerged after 2 wk, eventually producing healthy, disease-free shoots at 4 wk. Rust symptoms usually appeared only on secondary shoots (Fig. 3A, B). All plants which eventually became

diseased produced rusted secondary shoots which grew out of the soil close to the primary shoot. The earliest symptoms of infection occurred at 27 days, when the secondary shoots were about 3–8 cm tall.

In experiment 1, all of the cuttings in which the bud and the root plus bud were inoculated with stimulated teliospores showed massive systemic infections of the secondary shoots (Table 1). The heavily infected secondary shoots were 10–30 cm tall. The root plus bud inoculations with unstimulated teliospores showed two plants with secondary shoots bearing spermatogonia. One cutting of five in which only the root portion was inoculated with stimulated teliospores was infected. No infection resulted from inoculations of roots, expanding buds, or white roots growing from cuttings. The inoculation of dormant buds (Table 1, Exp. 1) was the most successful infection technique, since 16 of 20 cuttings in treatments in which dormant buds were inoculated became infected.

In a more extensive test (Table 1, Exp. 2), in which only buds or root portions of cuttings were inoculated, bud inoculation again was most effective.

The first sign of infection in this experiment was observed after 40 days (Fig. 4). The number of infected plants increased in bud-inoculated cuttings up to 91 days. Buds inoculated with stimulated teliospores were 52% (26:50) infected at 91 days (Table 1). Buds inoculated with unstimulated teliospores were 26% (13:50) infected. Both treatments were significantly different from root inoculations, 0% (0:50) infection for stimulated, and 2% (1:50) for unstimulated teliospores. A significant increase in infection resulted from the use of stimulated versus unstimulated teliospores in this and in the preceding experiment. In other experiments there was no significant increase in infection using stimulated teliospores. The possible reasons for this will be discussed later.

The first visible sign of infection, following secondary shoot production, was the appearance of orange-colored spermatogonia on the abaxial leaf surfaces (Fig. 3C). A sweet fragrance, similar to that of Canada thistle flowers, but with a more camphoraceous odor, accompanied the orange coloration. The fragrance disappeared with the appearance of the dark brown aeciospores. The growth of the primary shoots slowed markedly when the infected secondary shoots began to grow. The infected secondary shoots were much taller, spindly, yellowish green, with small narrow leaves (Fig. 3A–E) resembling those treated with gibberellin. Aeciospore production, preceded by spermatogonia production, occurred primarily on the abaxial leaf surface, in many cases giving the lower leaf surface an almost completely black coloration (Fig. 3D). Aeciospore production also was noted along the stem, and very sparsely on the adaxial leaf surfaces. One plant produced 112 mg of aeciospores in 2 days (Fig. 3D).

Occasionally plants initially infected with aeciospores or urediniospores developed a systemic infection of a secondary shoot (Fig. 3E). The original plant was cut back and a typical uninfected

TABLE 1. Effect of type of inoculation of thistle root cuttings on infection of plants in the greenhouse

Type of treatment	Experiment 1 No. of plants infected/50 47 days	Experiment 2 No. of plants infected/50 91 days
Root + bud + stimulator	5	
Root + bud – stimulator	2	
Bud only + stimulator	5	26 c ²
Bud only – stimulator	4	13 b
Root only + stimulator	1	0 a
Root only – stimulator	0	1 a
Expanding buds + stimulator	0	
Expanding buds – stimulator	0	
White roots + stimulator	0	
White roots – stimulator	0	

²The letter following each average indicates Duncan's new multiple range groupings. Averages followed by the same letter do not differ significantly at the .05 level.

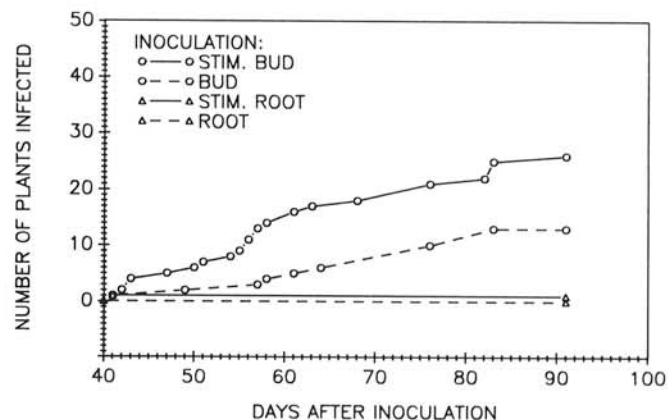


Fig. 4. Effect of the type of inoculation of Canada thistle root cuttings with teliospores of *Puccinia punctiformis* and pretreatment with thistle root extract on the time required for the appearance of systemic infection.

rosette was produced, followed by the infected shoot. The urediniospores used may have contained some teliospores, but plants inoculated with urediniospores or aeciospores were only kept in dew 24–48 hr. This would not have been long enough to permit teliospore germination, and infection of mature leaves by teliospores was never observed.

In other experiments, stimulated teliospores transferred by inoculating loop to young thistle shoots emerging through the soil surface produced some foliar symptoms after several weeks. Very few plants were affected. The symptoms consisted of yellowish areas on the leaf or on the midrib, where limited numbers of orange spermagonia appeared, and subsequently some aeciospores/teliospores were produced (Fig. 3F). Vigorous systemic infections did not develop immediately from this type of infection from teliospores. After several weeks, a small systemically infected shoot grew from the node at the base of the infected leaf of the plant shown in Fig. 3F. The infection was limited to this shoot. Infection of this type was rare, and the mechanism was not known.

Soil extracts. Water leached directly from potted thistle plants induced germination, 17% in teliospores of *P. punctiformis* at 14 days in the dark at 18 C (Table 2). Water leached from pots of soil without thistle plants was not stimulatory (0% germination). When the leachates from pots of thistle plants were extracted with hexane then concentrated to small volume, 48% germination was observed.

Volatility. The germination stimulant from thistle soil leachate collected on C-18 Seppak columns and concentrated in hexane diffused from the lids of inverted petri plates to significantly increase teliospore germination on agar (Table 3). Germination induced by substances diffusing from the hexane extract of thistle soil leachate was 17%, compared to 0% for pure hexane alone. This indicated that the hexane-soluble substances in the soil extract were volatile, and similar in this characteristic to the standard steam-distilled hexane extract of thistle roots.

DISCUSSION

In early attempts to infect Canada thistle, petri plates with germinating teliospores were suspended over plants in dew chambers or in water-sprayed plastic tents in growth chambers, expecting that the released basidiospores would infect leaves, stems, or shoot apices. In another approach, germinating teliospores were placed on the root ball of potted thistle plants or were watered into a hole made in the soil near the center of the pot. No infection was observed with any of these methods. These experiments were performed before our optimum temperature studies on stimulated teliospore germination were made, and before we found references to Buller's work (2). No consideration had been given to the importance of temperature to infectivity, and the soil may have been too warm to permit extensive teliospore germination.

Placing teliospores, stimulated or not, directly on root buds was the most efficient technique for inducing systemic infection. This may resemble the natural infection process which still is

TABLE 2. Stimulation of germination of teliospores of *Puccinia punctiformis* by soil leachates and hexane extracts of soil leachates from potted thistle plants after 14 days at 18 C

Type of extract	Germination ^z (%)
From potted thistles:	
Aqueous soil leachate, average of three pots	17.8 b
Concentrated hexane extract of soil leachate, average of three pots	48.6 c
From potted soil only:	
Aqueous soil leachate, average of three pots	0.0 a
Standard controls: Water	0.0 a
50 µl/L hexane extract of distilled thistle roots	49.2 c

^zMeans followed by the same letter are not significantly different as determined by Waller's grouping, performed on arc sine transformed data, $\alpha = 0.01$.

not fully understood. Emergence of the primary shoot, completely free of rust symptoms, from inoculated buds of cuttings is difficult to explain. Perhaps the bud first inoculated is inhibited and a secondary bud is activated which emerges first and is normal in appearance and supplies energy for the infected bud, but we have no direct evidence for this. As the disease progresses, the infected shoot grows vigorously, becoming pale green in color, tall, and thin, a response characteristic of gibberellin treatment. Bailiss and Wilson (1) have shown that systemically infected thistle shoots have high gibberellin and auxin contents. In a few of our experiments, some primary shoots became infected, and the plants produced no secondary shoots and quickly died. We have no explanation at this point for the difference in response.

In several experiments, including the two described herein, a positive effect of using stimulated teliospores was observed. In others there was little difference in the percent of plants infected between those receiving the stimulatory hexane extract and those that did not. The stimulator is an endogenous component of the root, as we have shown, and it may be released naturally to initiate teliospore germination. The washing of root cuttings or the diffusion of the active volatiles from root cuttings during different storage conditions before use may have altered residual levels of endogenous stimulators, thereby affecting teliospore germination and infection. The variability in the infection response from prestimulated or unstimulated teliospores might be explained by such factors.

For many years the mechanism of the induction of the systemic infection of Canada thistle rust was not well understood, at least until the research by Buller at the University of Manitoba, Canada. He had shown that the systemic aecial stage can arise long after previous uredinial infections of leaves; and he generally described the various infection processes in Vol. VII, *Researches on Fungi*, published posthumously (2). He also reported systemic infection from root buds inoculated with urediniospores. We also have observed this (6), but in checking our samples, we found an appreciable number of teliospores which could have been responsible for the infection. As previously mentioned, we have observed systemic infection after several months in plants in which the leaves had been inoculated with urediniospores, and held in dew chambers 48 hr. These conditions are favorable for urediniospore germination. However, this is too short a time to permit teliospore germination. Van Den Ende et al (10) also have reported the infection of the underground parts of thistle plants by teliospores.

We have reported previously on stimulation of germination of urediniospores of *P. punctiformis* by 5 methyl-2-hexanone (4) and the stimulation of spores of many other rust species by a variety of volatile aroma or flavor compounds (5). The volatile stimulator from thistle roots has physical properties very similar to the other chemical stimulators we have reported. These include

TABLE 3. Volatility test of the hexane extract of soil leachate from potted thistle on teliospore germination of *Puccinia punctiformis* after 14 days at 18 C

Type of extract	Germination ^z (%)
Concentrated hexane extracts from C-18 chromatographic columns through which 800 ml of aqueous thistle soil leachate was passed.	
Average of four pots:	
Group 1	16.7 b
2	17.4 b
3	16.6 b
4	18.2 b
Average of 16 pots	17.2
Hexane control	0.0 a
Standard controls: Water	0.0 a
50 µl/L hexane extract of distilled thistle roots	44.0 c

^zMeans followed by the same letter are not significantly different as determined by Waller's grouping, performed on arc sine transformed data, $\alpha = 0.01$.

volatility at room temperatures, solubility in organic solvents, low solubility in water, and ability to be steam distilled.

In our research we have shown that 90% or more of the maximum germination of teliospores of *P. punctiformis* occurred between 16 and 20 C, and no germination occurred below 8 C or above 25 C, at 7 days. Although the same response may not be obtained with other spore samples differing in age, pathovar, or prior environmental exposures, this information defines the temperature parameters required for the infection process by first getting teliospores to germinate. If teliospore germination does not occur, certainly infection will not. We have also provided evidence, for the first time, that dormant buds on root cuttings are a very effective site for initiating systemic aecial infection. In addition, we have presented data showing that thistle roots release an allelochemical into the soil that is capable of stimulating teliospore germination. We have found the thistle root extract to be very useful in stimulating germination of teliospores, particularly in the absence of thistle roots. This stimulation also may be useful for increasing infectivity. We are proceeding with our efforts toward identification of the stimulator(s) and with studies of infection mechanisms in order to enhance the possibilities of successfully using *Puccinia punctiformis* as a biocontrol agent of Canada thistle.

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