Pathogenicity testing of three fungi recovered from roots and crowns of diseased alfalfa in central Alberta

Kan-Fa Chang and Peter V. Blenis[1]

The pathogenicity of three species of fungi, isolated from diseased alfalfa in north-central Alberta, was tested. A factorial design was used to determine the effect of variety (Algonquin vs. Beaver), plant age (1, 6, 10 and 14 mos.), fungal species (*Plenodomus meliloti, Cylindrocladium gracile* and *Fusarium roseum)* and chilling (chilled vs. non-chilled). Mortality was high, even among the control plants. A single isolate of C. *gracile* was used to inoculate 30 alfalfa seedlings growing in test tubes. Only one seedling, from which the fungus was not reisolated, died.

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On a vérifié la pathogénicité de trois espèces de champignons isolées à partir de plants de luzerne dans le centre-nord de l'Alberta. Pour ce faire, on a utilisé un dispositif factoriel pour déterminer l'effet de la variété (Algonquin vs. Beaver), de l'âge des plants (1, 6, 10 et 14 mois), des espèces de champignons (*Plenodomus meliloti, Cylindrocladium gracile* et *Fusarium roseum*) et du refroidissement (refroidi vs. non-refroidi). La mortalité est élevée même parmi les plants témoins. On a inoculé dans 30 plantules de luzerne cultivbes en bprouvette un isolat de C. *gracile*. Une seule plantule sur laquelle on n'a pas retrouvb le champignon est morte.

Introduction

In 1967, a disease, later referred to as alfalfa sickness, was reported in central Alberta (13). The disease was common on sites which previously had produced good crops of alfalfa. Affected stands were not understocked, but rather consisted of poorly-nodulated, yellow-green, and stunted plants, interspersed with occasional healthy plants. Since then, considerable effort has been made to determine the cause of this problem. Initial studies indicated that a biological agent was involved (13). Although the nematode ***Paratylenchus projectus*** Jenkins was more numerous in locations with poor alfalfa growth than in locations with good growth (14), subsequent inoculation experiments showed that it was unlikely to be capable of causing the disease by itself (4). Several fungi have been implicated in alfalfa sickness. Isolates of Phytophthora (including one identified as P. ***megasperma*** Drechs.) were recovered from seedlings inoculated with dilutions of the affected soils (5). Five of these Phytophthora isolates were pathogenic on 7-day-old seedlings. In a survey of four central Alberta alfalfa fields, 69 Pythium isolates were recovered from a total of 1594 plants (11).

In 1980-81 a survey of 55 alfalfa fields was conducted in central Alberta (9). ***Cylindrocladium gracile*** Bugn. (Boesew.) and ***Fusarium*** roseum (L.K.) emend. Snyder and Hansen accounted for 44.3% of the isolates obtained from diseased alfalfa roots. Symptoms of brown root rot, caused by ***Plenodomus meliloti*** Mark.-Let., were frequently seen, although this fungus accounted for only 0.3% of the isolates. ***Phytophthora megasperma*** var. ***megasperma*** was not isolated. It may be significant that the inoculations that showed the pathogenicity of P. ***megasperma*** were done on young seedlings (5), whereas this survey included fields that were up to 6 years old. Although the results of this survey might suggest that C ***gracile, F. roseum*** and ***P. meliloti*** are important in alfalfa sickness, the pathogenicity of these isolates was not reported, and thus their role in this disease seems uncertain. It was thus essential to do a follow-up experiment in which the pathogenicity of those isolates was tested. The primary objective of our research, therefore, was to determine whether isolates of C ***gracile, F. roseum,*** and P. ***meliloti,*** obtained during the 1980-81 survey, were capable of causing alfalfa sickness on inoculated seedlings. Secondary objectives were to determine whether the amount of disease caused by these isolates would be influenced by plant age and variety, and exposure to chilling temperatures.

Materials and methods

Pathogenicity testing in soil. Three isolates of C ***gracile,*** two isolates of ***meliloti,*** and three isolates of F. ***roseum*** were used. They had been collected during the 1980-81 survey and stored at 4°C for two years. They were transferred to PDA plates, and checked for viability 2 weeks before being retransferred to sterilized oat grain medium. The flasks were shaken every week to ensure uniform mycelium development in the medium. Before use, approximately equal amounts of the different isolates of the same species were combined and thoroughly mixed.

Seeds of the alfalfa cultivars Beaver and Algonquin were sown in 'Ferdinand' Rootrainer® trays (Spencer Lemaire Ind. Ltd.) in September 1983, January 1984, May 1984, and October 1984 to produce plants which were 14, 10, 6, and 1 mo. old, respectively, at the time of inoculation in November, 1984. The soil mixture used had not been sterilized and consisted of 12 kg soil, 3 kg peat, 12 kg sand, 25 g superphosphate, and 9 g calcium nitrate. The plants were kept in a greenhouse at 18°C and fertilized with 20-20-20 fertilizer (200 ppm) every two weeks.

One hundred and twenty plants in each variety × age combination were divided into four groups of 30 plants. Each group received one of the following four treatments: inoculation

[1] *Department of Plant Science, University of Alberta, Edmonton, Alberta T6G 2P5*

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with *P. meliloti,* C. gracile, *F.* roseum or sterilized oat grain medium as an uninoculated control. Replicates were obtained by having three people inoculate 10 plants in each of the treatment × variety × age combinations. The plants were removed from their trays and the soil was washed from their roots. The crown areas of the 14- and 9-month-old plants were wounded by a 22G 1/2 syringe needle. The 6-month- and 1-month-old plants were wounded twice and once, respectively, by a 26G 3/8 syringe needle. After wounding, the plants were transferred to 'Tinus' Rootrainers®. Nonsterilized soil, prepared as described above, was poured around the plants to a depth of 5 mm below the wound. A layer consisting of 10 ml of oat grain inoculum was placed around the wounded area. The inoculum was covered with a thin layer of soil to prevent desiccation of the inoculum.

In January 1985, half of the plants were exposed to a chilling treatment. These plants were kept at a temperature of 4°C for one month before they were returned to the greenhouse.

In April 1985, mortality was assessed for those plants that had not received the chilling treatment. In early May 1985, isolations were made from three plants in each of the 16 temperature treatment × inoculation treatment x variety combinations. The plants were chosen by randomly selecting one of the three replicates of each of the 16 combinations and then randomly selecting three plants within that replication. Modified Nash-Snyder medium (7) was used for the isolation of *C.* gracile and *F.* roseum whereas PDA was used for the isolation of P. *meliloti* and for isolations from the uninoculated controls. The isolations were repeated in mid-May, except that 1/10 strength PDA was used instead of PDA. In addition, another 38 plants, taken without particular regard to treatment, were dissected and preserved in FAA to permit visual comparison of symptoms.

Pathogenicity testing in test tubes. A second experiment was conducted in which alfalfa seedlings growing in agar in test tubes were inoculated with a single isolate of C. gracile. Seeds of the variety Algonquin were surface sterilized in a solution of 0.3% NaOCl, 70% ethanol, and 29.7% water for 3 min before being rinsed in sterile water and plated on PDA. Three days later, those germlings that were sterile were transferred to 18 cm test tubes containing 0.5% agar and Hoagland's solution. After growing for one month under room conditions, 30 seedlings were inoculated with a 3-wk-old isolate of *C.* gracile and 30 seedlings were left as uninoculated controls. All seedlings were placed in a growth chamber with a night temperature of 15°C, a day temperature of 20°C. and a 10-hr photoperiod. The seedlings were re-examined one month later.

Results and discussion

Many of the plants that had been grown in soil, and not exposed to chilling temperature, died (Table 1). The reason for this mortality is uncertain since many of the control plants died as well. None of the pathogens were isolated from the plants into which they had been inoculated, and there were no obvious differences in root symptoms between any of the treatments. Of the sixty seedlings grown in test tubes, only one, which had been inoculated with C. gracile, died. The pathogen could not be reisolated from this single specimen.

The pathogenicity of *Cylindrocarpon ehrenbergii* Wr. (later rejected as a *nomen confusum* [1]), P. *meliloti,* and *Fusarium* spp. on alfalfa have been demonstrated by other workers, but not all pathogenicity tests with these fungi have been successful. C. *ehrenbergii* and P. *meliloti* have been shown to be pathogenic to alfalfa (2, **3,** 10) but there is evidence to indicate that they are more pathogenic on plants during the spring, after overwintering, than they are during the summer, when the plants are growing actively (3, 10). Nonetheless, pathogenicity of the latter fungus was demonstrated on young seedlings growing on agar medium in Ehrlenmeyer flasks (8). Similarly, although three isolates of F. roseum 'Acuminatum' caused rot on alfalfa cultured by the slant-board technique, a majority of the Fusarium spp. isolates initially tested caused no decay (6). This same species was 'weakly virulent' when inoculated into the crown of 3- to 4-month old seedlings in a separate experiment (12).

Table 1. Number of dead seedlings seven months after inoculation (non-chilled treatment)

	Seedling Age (mos)	Algonquin*	Beaver*	Total for Treatment**
P. *meliloti*	14	**6**	7	
	10	**3**	1	**23**
	1	**3**	2	
C. gracile	14	10	10	
	10	**2**	1	
	6	**3**	4	**36**
	1	**3**	**3**	
F. roseum	14	6	11	
	10	2	2	
	6	1	**7**	**38**
	1	4	5	
Control	14	9	**8**	
	10	1	**3**	
	6	4	**0**	**30**
	1	**2**	4	

*Out of 15; **out of 60

Although our experiments failed to demonstrate the pathogenicity of any of the three fungi tested, the results must be evaluated with caution. The high rate of mortality among the uninoculated controls in the first experiment confounds interpretation of those data. Furthermore, the isolates used were several years **old** and may have declined in pathogenicity before they were used. Further experimentation, with fresh isolates and a wide range of environmental conditions, will be necessary to expand our knowledge of the role of these fungi in alfalfa sickness.

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