AN ABSTRACT OF THE THESIS OF

Jesus Perez Fernandez for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on April 19, 1990

Title: Rhynchosporium Orthosporum in Orchardgrass, Isolation Frequency, Colonization, Variability, and an Evaluation of Cultivar Resistance

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Ronald E. Welty

Studies were undertaken in orchardgrass (Dactylis glomerata) and the plant pathogen Rhynchosporium orthosporum to determine the seasonal development and frequency of leaf spot fungi; the effect of inoculum concentration, seedling age and cultivars on disease development; the variability in virulence among isolates; the effect of inoculation at four plant growth stages; and infection, invasion, and sporulation of R. orthosporum in orchardgrass leaves. Leaf samples taken during three years show that R. orthosporum was the most prevalent leaf spot pathogen recovered followed by Mastigosporium rubricosum. Others fungi isolated were Cercosporidium graminis, Bipolaris sorokiniana, and Puccinia striiformis. No cultivar (of 10 evaluated) was highly resistant to R. orthosporum. However, there were significant differences in disease reaction among cultivars means. Hallmark and Pennlate show a positive linear increase of

disease severity as inoculum concentration increased (from 10^1 to 10^6 conidia/ml). Small differences in disease severity were detected between cultivars for different levels of inoculum. Both cultivars show a negative linear response to increasing seedling age at the time of inoculation. Twenty isolates of R. orthosporum were evaluated for pathogenicity on clones of Hallmark and Pennlate. Highly significant differences were found among the isolates and cultivar x isolation interaction. Significant mean disease differences were found among clones of both cultivars for five isolates. Hallmark and Pennlate were inoculated with R. orthosporum at four growth plant stages in 1988 and 1989. Hallmark was more susceptible than Pennlate to the disease in both years. In 1989, the highest seed yield reduction for Hallmark occurred, when inoculum was applied at heading, and for Pennlate at flowering. Seed yield and thousand seed-weight were not correlated with disease severity. R. orthosporum conidia germinated to produced an appresoria and penetrated directly through the adaxial or abaxial leaf surfaces. After penetration the fungus formed intercellularly subcuticular mycelium. Hyphae become intracellular after disintegration and death of mesophyll cells. Hyphae were found growing in the phloem, but not the xylem. Conidia were produced on intact leaf surfaces from subcuticular mycelium on short conidiophores that extruded through stomata and cuticular holes.

RHYNCHOSPORIUM ORTHOSPORUM IN ORCHARDGRASS, ISOLATION FREQUENCY, COLONIZATION, VARIABILITY, AND AN EVALUATION OF CULTIVAR RESISTANCE

by

Jesus Perez Fernandez

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in charge of Major

Redacted for privacy

Chairperson of Department of Botany and Plant Pathology

Redacted for privacy

Dean of Graduate School

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Typed by Jesus Perez Fernandez

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RHYNCHOSPORIUM ORTHOSPORUM IN ORCHARDGRASS, ISOLATION FREQUENCY, COLONIZATION, VARIABILITY, AND AN EVALUATION OF CULTIVAR RESISTANCE

INTRODUCTION

Leaf scald or white leaf stripe, caused by *Rhynchosporium orthosporum* Caldwell, is a serious disease on orchardgrass (*Dactylis glomerata* L.) in Oregon, as well as other major orchardgrass growing areas (4, 8, 24, 30, 41). About 98% of the orchardgrass seed in the United States is produced in the Willamette Valley of Oregon. Leaf scald occurs every year and when severe, it reduces seed yield (4, 43).

Rhynchosporium orthosporum can be isolated from different gramineous hosts, but the fungus is host specific. That is, the fungus infects only the host genus from which it was isolated (4, 51, 52). Leaf scald lesions on orchardgrass leaves begin as 2.5-3.0 mm long spots, which coalesce to form irregular lesions. At first, lesions are water-soaked, later becoming gray with a brown margin. Isolates of *R. orthosporum* produce cylindrical conidia that have a median septum. They are easily distinguished from conidia of *R. secalis* which possesses an apical beak on the conidium.

R. orthosporum, along with Mastigosporium rubricosum and Cercosporidium graminis, are frequently isolated from spot lesions on leaves of orchardgrass. However, there has been no systematic determination of occurrence of fungi associated with leaf spot diseases in orchardgrass.

The literature on leaf scald in orchardgrass is limited. The disease was described by Caldwell in 1937 in Wisconsin (4). Fungicides have been tested in Oregon (50) to reduce scald severity and have been shown to control the disease depending upon the stage of plant development when the fungicides are applied. Occurrence of pathogenic variability within Rhynchosporium species on other hosts occurs quite often and is a major problem in global evaluation of resistance to leaf scald (15). Information of the pathogenic variability of R. orthosporum is lacking. This basic information is needed to provide a basis for developing a breeding program for scald resistance. Conidial spore production of Rhynchosporium secalis in barley has been controversial. Previous studies (3, 14) reported differences on the way that conidia are produced on barley leaves. Histological information on conidia development and sporulation is lacking for R. orthosporum on orchardgrass.

This research was divided into five distinct topics, all interrelated. The objectives of this research were 1) to

determine the seasonal development and frequency of occurrence of the important leaf spot fungi associated with leaf spot lesions; 2) to evaluate cultivars in controlled environments for resistance to leaf scald, and the effect of inoculum concentration and seedling age on disease severity in seedlings; 3) to evaluate isolates of R. orthosporum for variability in virulence; 4) to determine if seed production was influenced by plant growth stage at the time of inoculation by R. orthosporum; and 5) to characterize infection, invasion, and sporulation of R. orthosporum in orchardgrass leaves with histological techniques, including scanning electron and light microscopy.

The thesis is prepared as individual chapters for each study. An introduction, materials and methods, results and discussion, summary, and literature review is presented for each chapter. Observations, procedures, and data not included in the chapters appear in the appendix.

CHAPTER I

IDENTITY AND FREQUENCY DISTRIBUTION OF FUNGI CAUSING LEAF SPOTS ON ORCHARDGRASS LEAVES

JESUS PEREZ FERNANDEZ

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, USA

and

RONALD WELTY

US Department of Agriculture, Agricultural Research Service, National Forage Seed Production Research Center Corvallis, Oregon 97330, USA

SUMMARY

Leaves from 10 orchardgrass cultivars grown in Oregon were sampled in 1987, 1988 and 1989 to determine the prevalence of leaf spot fungi associated with leaf spot symptoms. R. orthosporum was isolated more frequently during the three growing seasons; it was isolated from 62%, 80% and 63% of the lesions in 1987, 1988 and 1989 respectively. M. rubricosum was recovered from 19%, 45% and 57% of the lesions in 1987, 1988 and 1989 respectively. B. sorokiniana in contrast was recovered in 1989 from 25% of the samples. Other fungi in decreasing order of prevalence identified with R. orthosporum and M. rubricosum were Cercosporidium graminis and Puccinia striiformis. No other pathogens were isolated.

This survey suggests that R. orthosporum and M. rubricosum are the principal leaf spot fungi on orchardgrass in Oregon.

INTRODUCTION

Several fungi are reported as foliar diseases of orchardgrass (Dactylis glomerata L.). Rhynchosporium orthosporum, along with Mastigosporium rubricosum and Cercosporidium graminis are frequently isolated from leaf spot lesions on orchardgrass leaves (43, 50). However, little is known about the seasonal occurrence of these pathogens in Oregon.

A list of parasitic fungi on orchardgrass was prepared by Sprague (42). However, there is no systematic recording of the seasonal occurrence or prevalence of these leaf pathogens. The purpose of this study was to identify when during the growing seasons these fungi occur in orchardgrass.

MATERIALS AND METHODS

Ten orchardgrass cultivars were sown in July 1984, in an herbicide-prepared seed bed (2.7 kg a.i./ha of diuron). Cultivars were selected to represent early, middle or late maturity according to their flowering date. Plots were 2.4 m wide by 6 m long. The distance between plots was 1.2 m. Treatments were replicated eight times. The soil was limed to correct pH, and fertilized (41.7 kg/ha of N) with urea in the spring, and in the fall with 18.1 kg/ha of NPK (16:20:0) to maintain vigorous plant growth. Leaf samples with symptoms were collected from each cultivar at frequent 15-20 days intervals during the growing season. A total of 100 leaves per sampling date were collected.

Leaf sections with leaf spots were surface sterilized by dipping in 70% alcohol for 30 sec, then into a solution of 1.5% sodium hypochlorite for 2 min, and then rinsed in sterile distilled water. They were transferred to petri dishes containing acid potato dextrose agar (aPDA), and incubated at 15 C with alternating 12-hr period of fluorescent light and darkness for 8-10 days. Identification of fungi associated with leaf spots was made by mounting in water or observing directly at x100 to x400.

Identifications were based on characteristic fungal morphology in accordance with previously published descriptions (4, 39, 40, 43).

RESULTS AND DISCUSSION

A summary of incidence of fungi associated with leaf spot symptoms are given in Table I.1. The most prevalent fungal leaf spot diseases on orchardgrass during the three seasons sampled were leaf scald, caused by Rhynchosporium orthosporum (Caldwell), eye spot, caused by Mastigosporium rubricosum (Dearn and Barth), leaf streak, caused by Cercosporidium graminis (Fuckel), leaf spot, caused by Bipolaris sorokiniana (Sacc. ex Sorok.) Shoem. (syn. Helminthosporium sativum, teleomorph: Cochliobolus sativus (Ito and Kurib) Drechs.), and stripe rust, induced by Puccinia striiformis (Westend.).

R. orthosporum was the most frequently isolated fungus from orchardgrass leaves during the survey. In 1987, it was recovered from more than 60% of the lesions collected in April, May, June and December (Fig. I.1). M. rubricosum was recovered in March and April in 1987, and R. orthosporum and M. rubricosum were frequently isolated from leaves in February through June (1988) (Fig. I.2). Precipitation was above average during the main growing season of April, May and June (Fig. I.4). Other fungi identified late in the 1987 and 1988 growing season, in decreasing order of prevalence included M. rubricosum and P. striiformis.

In 1989 R. orthosporum was isolated consistently from orchardgrass leaves in a higher frequency than other leaf spot fungi (Fig. I.3). However, the incidence of R. orthosporum was lower than in 1988. This may be due to lower amount of rain during the growing season (Fig. I.4). The amount of M. rubricosum found in 1989 was higher than the previous two years. This fungus was recovered most frequently in March, which may be due to higher infection rates due to above normal precipitation in this month. During 1989, leaf spot caused by B. sorokiniana occurred during the season and was recovered in moderate numbers (Table I.1 and Fig. I.3). In 1989 C. graminis occurred later in the growing season (Table I.1) with highest prevalence during May and June (Fig. I.3). P. striiformis was observed in all three years with the highest frequency occurring in June and July.

R. orthosporum is the principal leaf spot pathogen on orchardgrass followed by M. rubricosum. Other pathogens also occur in orchardgrass, but their presence was sporadic. The prevalence of these two principal leaf spot fungi varied from year to year and the frequency of these pathogens appears to depend on the amount of rain during the growing season. Two or more fungi frequently occurred in the same cultivar and were isolated from the same leaf lesion. Welty (personal communication) isolated or identified the same fungi in orchardgrass leaves during the growing seasons of 1984, 1985,

and 1986. Hardison first reported *P. striiformis* on orchardgrass in the Willamette Valley in 1984 (12). The data reported here indicated that leaf spot pathogens known to occur in other areas of the United States and the world, are well established in Oregon. This study was done at the Hyslop Field Laboratory near Corvallis, Oregon and is thus based on a limited area. However, the 10 cultivars sampled are those commonly grown for seed production in this region.

The high occurrence of leaf spots caused by *R. orthosporum* and *M. rubricosum* in orchardgrass suggests that these diseases may be caused yield losses. A study of the occurrence, severity, and distribution of fungal leaf spot in Oregon should be done in conjunction with yield losses studies to facilitate prediction of potential damage.

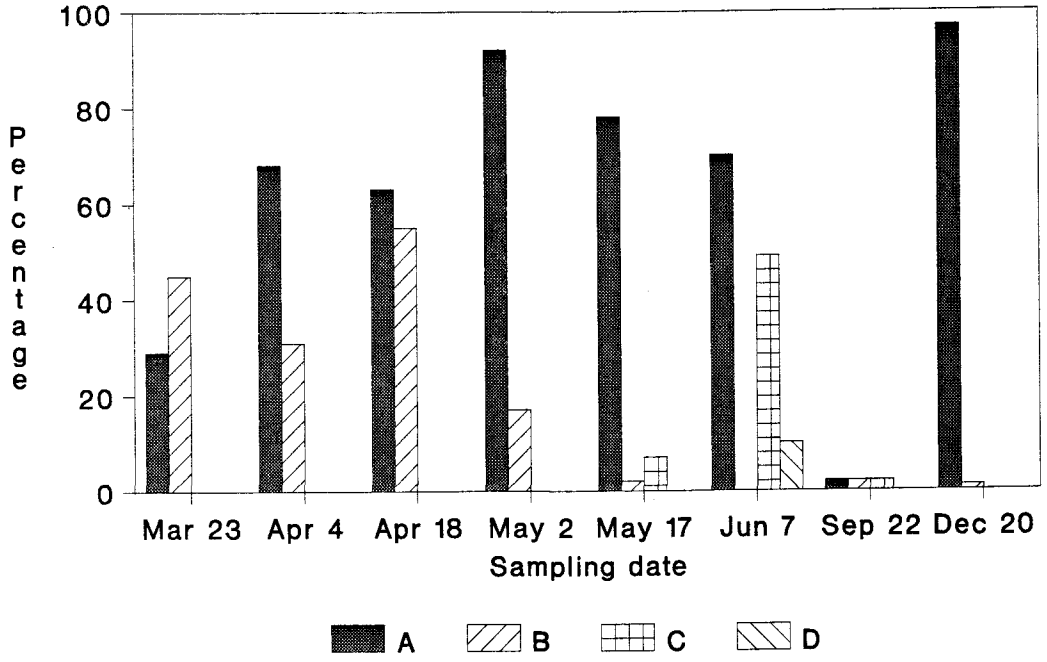

Fig. I.1 Percentage of leaves from which leaf spot fungi were recovered in 1987. A= Rhynchosporium orthosporum, B= Mastigosporium rubricosum, C= Cercosporidium graminis and D= Puccinia striiformis.

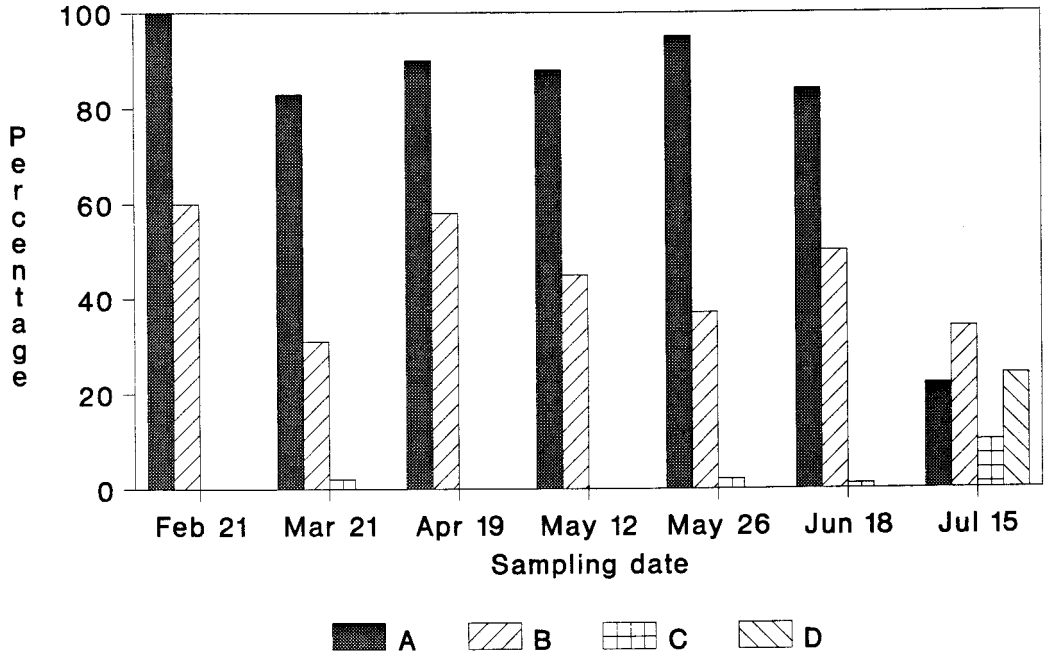

Fig. I.2 Percentage of leaves from which leaf spot fungi were recovered in 1988. A= Rhynchosporium orthosporum, B= Mastigosporium rubricosum, C= Cercosporidium graminis and series 4= Puccinia striiformis.

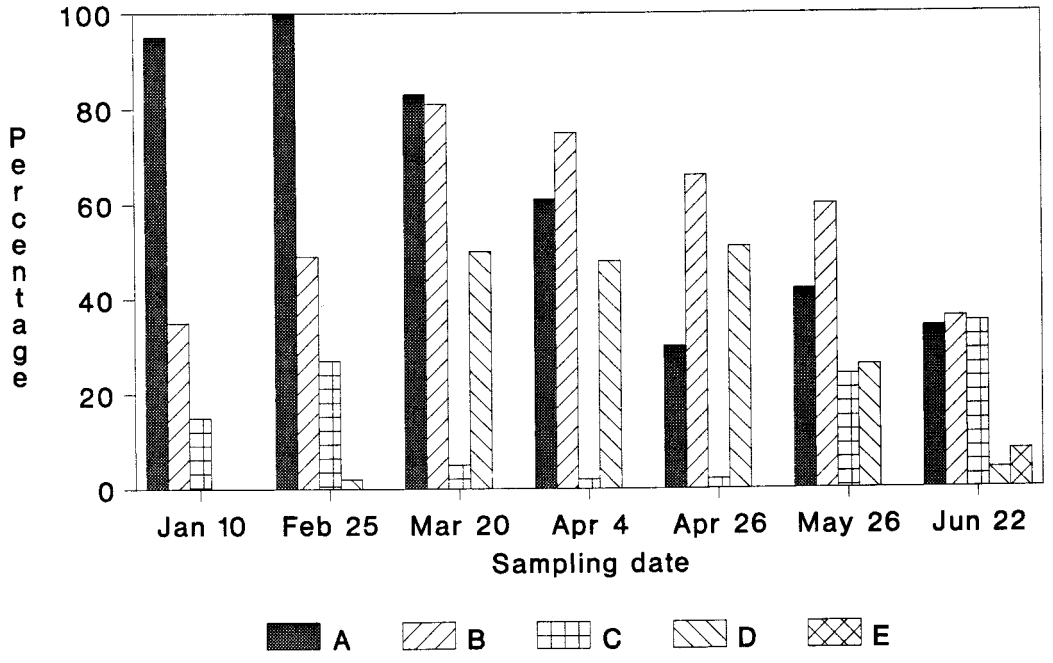

Fig. I.3 Percentage of leaves from which leaf spot fungi were recovered in 1989. A = Rhynchosporium orthosporum, B = Mastigosporium rubricosum, C = Cercosporidium graminis, D = Bipolaris sorokiniana and E = Puccinia striiformis.

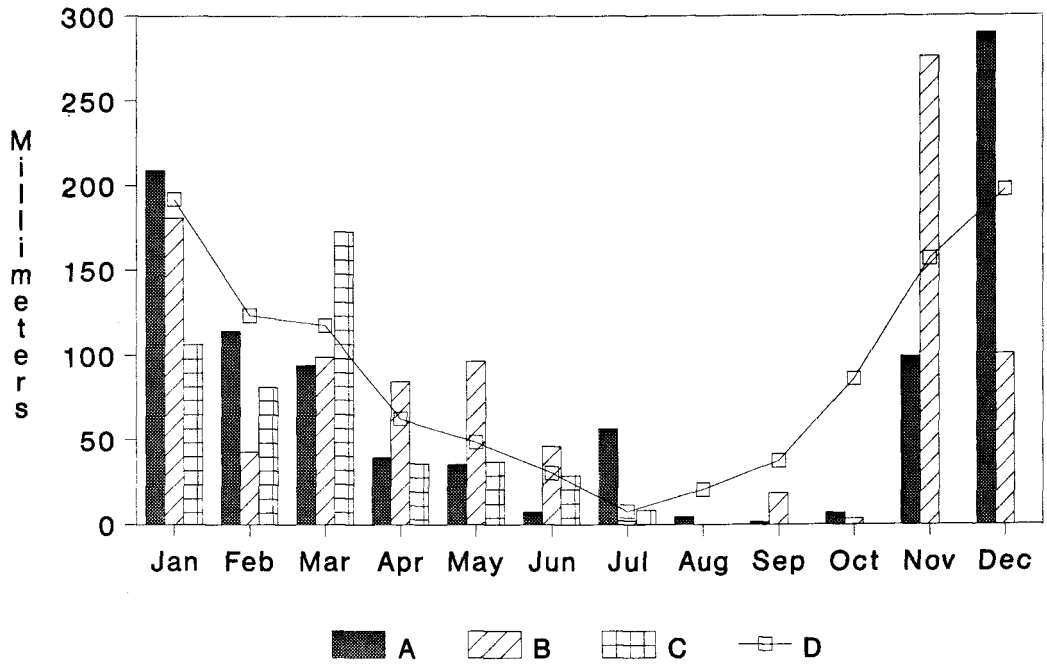

Fig. I.4 Monthly average precipitation at Hyslop Field laboratory (Corvallis, Oregon) for 1987, (A), 1988 (B), 1989 (C), and 30 year average (D).

Table I.1 Frequency (%) of fungi isolated from leaf spots in orchardgrass during 1987, 1988 and 1989[a]

Fungus	Year			
	1987	1988	1989	Mean
	[b]			
Rhynchosporium orthosporum	62.4	80.3	63.6	68.7
Mastigosporium rubricosum	19.1	45.0	57.4	40.5
Cercosporidium graminis	7.3	2.1	15.7	8.3
Bipolaris sorokiniana	-	-	25.9	8.6
Puccinia striiformis	1.3	3.4	1.3	2.0

[a] Values are based on 800, 700 and 700 leaf samples collected from 10 orchardgrass cultivars in 1987, 1988 and 1989 respectively.

[b] Percentage of leaves from which each fungus was recovered.

CHAPTER II

REACTION OF SEVERAL COMMERCIAL ORCHARDGRASS VARIETIES TO LEAF SCALD UNDER CONTROLLED ENVIRONMENTAL CONDITIONS. EFFECT OF INOCULUM CONCENTRATION AND SEEDLING AGE

JESUS PEREZ FERNANDEZ

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, USA

and

RONALD E. WELTY

US Department of Agriculture, Agricultural Research Service, National Forage Seed Production Research Center Corvallis, Oregon 97330, USA

SUMMARY

Ten cultivars of orchardgrass were evaluated for resistance to leaf scald caused by Rhynchosporium orthosporum in the greenhouse. Disease was assessed by visual disease rating based on an scale of 0 to 6 (0= no lesions, to 6= 100% of leaf blade with lesions or dead plant). The disease severity for the 10 cultivars ranged from 4.27 to 4.77. No cultivar was highly resistant to leaf scald. However, there were significant differences in disease reaction among cultivars means (P= 0.05). The effect of varying the inoculum concentration was tested on cultivars Hallmark and Pennlate. There was an increase of disease severity in both cultivars

as inoculum concentration increased. Small differences in disease severity ratings were detected among cultivars for the different levels of inoculum. There was a positive linear response to increasing inoculum levels for both cultivars. Both cultivars showed a negative linear response to increasing seedling age at the time of inoculation. There was a significant (P= 0.05) increase in disease resistance as seedling age of both cultivars increased. Seedlings of Pennlate show a lower percent infection in 12 and 14 week old-plants than did Hallmark.

INTRODUCTION

Genetic resistance is an important practical means of reducing losses caused by foliar diseases of orchardgrass (26, 55). The Agriculture Handbook number 170, "Grass Varieties in the United States" (11) describes some orchardgrass cultivars that possess general resistance or tolerance to foliar diseases. However, resistance to scald leaf spot caused by Rhynchosporium orthosporum is not specifically included in this review. In the past, natural field infection has been used to evaluate resistance to this disease (13, 18). An evaluation of resistance in field plantings can be time-consuming and inaccurate because disease severity is not similar every year and plants scored as "disease resistant" may actually be susceptible plants that escaped infection. Inoculating plants under controlled environmental conditions can avoid disease escapes (54, 55).

Sugita et al. (46) reported the importance of developing resistant varieties of orchardgrass to leaf scald by using an artificial inoculation method on seedlings. The objectives of our research were to determine the range of reaction to R. orthosporum among and within commercial orchardgrass cultivars. We also studied the effect of inoculum concentration and seedling age on the disease reaction of two

orchardgrass cultivars.

MATERIALS AND METHODS

Cultivar reaction studies. Ten plants per entry of each of ten orchardgrass cultivars (Table II.1) were established in a 1:1:1:2 (v:v:v:v) mixture of steamed peat moss, soil (sandy loam), sand, and pumice in 1.5 l pots, with drainage holes in the bottom. The 10 entries were randomized in five replicated pots, each containing 10 plants which provided 50 plants/cultivar. Seedlings were 4-wk-old when inoculated.

Four single conidial isolates of R. orthosporum, obtained from symptomatic leaves of orchardgrass growing at Hyslop Field Laboratory (Oregon State University), were used in the inoculation tests. Each isolate was grown on aPDA for 3 wk in an incubator at 15 C with 12 hr of fluorescent light and 12 hr of darkness. Conidial suspensions were prepared for each isolate by gently rubbing the culture surface in 15 ml of sterile water to free the conidia. Conidia from the four cultures were combined and filtered through four layers of cheesecloth to eliminate mycelial fragments and bulked into a flask. The final concentration was adjusted to 500,000 conidia/ml. The solution contained 1 drop/100 ml of Tween 20 as a wetting agent.

Four-week-old plants were sprayed with the conidial

suspension until run off. Plants were covered with a clear plastic bag, and placed in a growth chamber at 15 C with 14 hr of fluorescent light and 10 hr of darkness. Plants were uncovered after 4 days and remained in the growth chamber for an additional 10 days. Plants were then transferred to a greenhouse at 20-22 C with natural daylight. Disease severity was scored 17 days after inoculation. Foliage was removed, regrowth was inoculated again after 4 weeks of growth as previously described, and disease reaction was rated as before.

A scale of 0 to 6 was used to score the disease severity where; 0= no lesions; 1= very small lesions on leaf margin or leaf tips (1-5%); 2= small scattered lesions on leaf blade (6-15%); 3= 16-40% of leaf blade with lesions; 4= 41-70% of leaf blade with lesions; 5= 71-99% of leaf blade with lesions, and 6= 100% of leaf blade with lesions or dead plants.

Inoculum concentration study. The cultivars Hallmark ("field susceptible") and Pennlate ("field resistant") (unpublished data) were used in this study. Ten plants of each cultivar per entry were established in a single container and replicated five times. Isolates, inoculum preparation, inoculation procedures and disease severity rating were as described above. Four-week-old plants were

inoculated with inoculum concentrations of 1 x 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 conidia/ml. Plants were subsequently treated and scored as described in the cultivar reaction study.

Seedling age study. Seeds of the cultivars Hallmark and Pennlate were planted every two wk in a soil mixture as described for the other experiments. The two entries were randomized in four replicated containers, each containing 10 plants, and providing 40 plants/cultivar for each planting date. Seedlings 4, 6, 8, 10, 12, and 14 wk old were inoculated with the same isolates used for the cultivar reaction study. Inoculum preparation, inoculum concentration, and inoculum procedures were as described for the cultivar reaction study. After inoculation, plants were treated as described in the cultivar reaction study. Disease severity ratings were based on the percent of leaf area covered by lesions.

Statistical analysis. A completely randomized design was used for each experiment. Analysis of variance and linear regression were performed by using Michigan Statistical Analysis (version 4.0, Michigan State University). Fisher's least significant difference (P= 0.05) (6) was used to compare treatment means. For the cultivar age and inoculum concentration studies, regression methods were used to partition the main effect of seedling age and inoculum

concentration into linear sum of squares to establish the type of relationship between disease severity and predictors.

RESULTS AND DISCUSSION

Cultivar reaction study. The reactions of the 10 cultivars inoculated with R. orthosporum are shown in Table II.1. The overall mean disease severity ratings for the two inoculations ranged from 4.27 to 4.77. There were significant differences in disease reaction among cultivars means (P= 0.05). Individual plant responses within each cultivar ranged from slight disease (2 or 3) to moderately severe or severe (4 or 5) (Table II.2). There was a slight difference for the number of class and mean disease severity ratings between the first and second inoculation. A small number of plants died after clipping, however the cause was attributed to factors other than R. orthosporum.

No cultivar was highly resistant to leaf scald. Cultivars varied in numbers of plants with slight disease (2 or 3) in either test (Table II.2). This variation ranged from nine plants of Frontier and Aonami to 0 of Juno, Potomac, Able and Hallmark for the first inoculation. For the second inoculation there were fewer plants with ratings 2 or 3. It seemed some plants rated 2 or 3 in the first inoculation represented random variation in disease incidence rather than resistance.

These data provided an estimate of the disease reaction of several orchardgrass cultivars inoculated at the same age and in controlled conditions. No orchardgrass cultivar showed immunity or a high level of resistance to leaf scald. Although there were significant differences in susceptibility to leaf scald, no cultivar was rated resistant in this inoculation test. This study is an important starting point in developing inoculations methods for screening for disease resistance to R. orthosporum..

Inoculum concentration study. The effects of inoculum concentration of R. orthosporum on disease expression is shown in Fig. II.1. Disease severity increased as inoculum concentration increased. Linear regression analysis revealed a positive correlation between disease severity and inoculum concentration. Regression equations for Hallmark and Pennlate cultivars and corresponding coefficients of correlation (r) were $y= 1.25 + 0.68 x$, $r= 0.94$, and $y= 1.31 + 0.66 x$, $r= 0.94$. There was no significant difference ($P= 0.05$) in disease severity, between 1×10^5 and 1×10^6 inoculum level for either cultivar.

Our results show that disease response of 4-week-old orchardgrass seedlings, measured as disease severity on a scale from 0 to 6, progressively increased until reaching an inoculum concentration level of 1×10^5 conidia/ml for both

genotypes. Disease severity was highest at 1 x 10^5 and 1 x 10^6 conidia per ml. The concentration level to use for a severe disease development in orchardgrass leaves appears to range from 1 x 10^4 to 1 x 10^6 conidia/ml. From this study we obtained an estimate of the inoculum level that would cause severe leaf scald infection on orchardgrass leaves. These results provide a starting point in developing artificial inoculations methods for reaction to the leaf scald fungus.

Seedling age study. The response of seedling age on disease severity in Hallmark and Pennlate are given in Fig. II.2. There was a decrease in disease severity as seedling age increased for both cultivars. Disease scores for both cultivars were similar when 4-to-6 wk old and 8-to-10 wk old seedlings were inoculated. For both cultivars, the lowest level of disease was reached when the cultivars were 14 wk old. Pennlate seedlings show a lower percent infection for 12 and 14 wk old plants than Hallmark, a reaction similar to that has been observed in the field for these cultivars.

Linear regression analysis revealed a negative correlation (P= 0.05) between disease severity and seedling age (Fig. II.2). Regression equations for Hallmark and Pennlate cultivars and corresponding coefficients of correlation (r) were y= 90.4 - 2.2 x , r= -0.88, and y= 95.3 - 3.2 x, r= -0.92.

These results corroborate those of Sugita et al. (1987) (46), who found that the degree of resistance to leaf scald increased with increasing seedling age at inoculation. The response of orchardgrass of varying age, measured as percent of infection, was linear for both genotypes. The reason for this linear response to leaf scald is unknown. The lower percent infection observed in Pennlate corroborate those of field studies (50), where Pennlate had less disease severity under severe leaf scald infection. The higher scald resistance observed in Pennlate represents a way to maintain seed yield under conditions suitable for disease development.

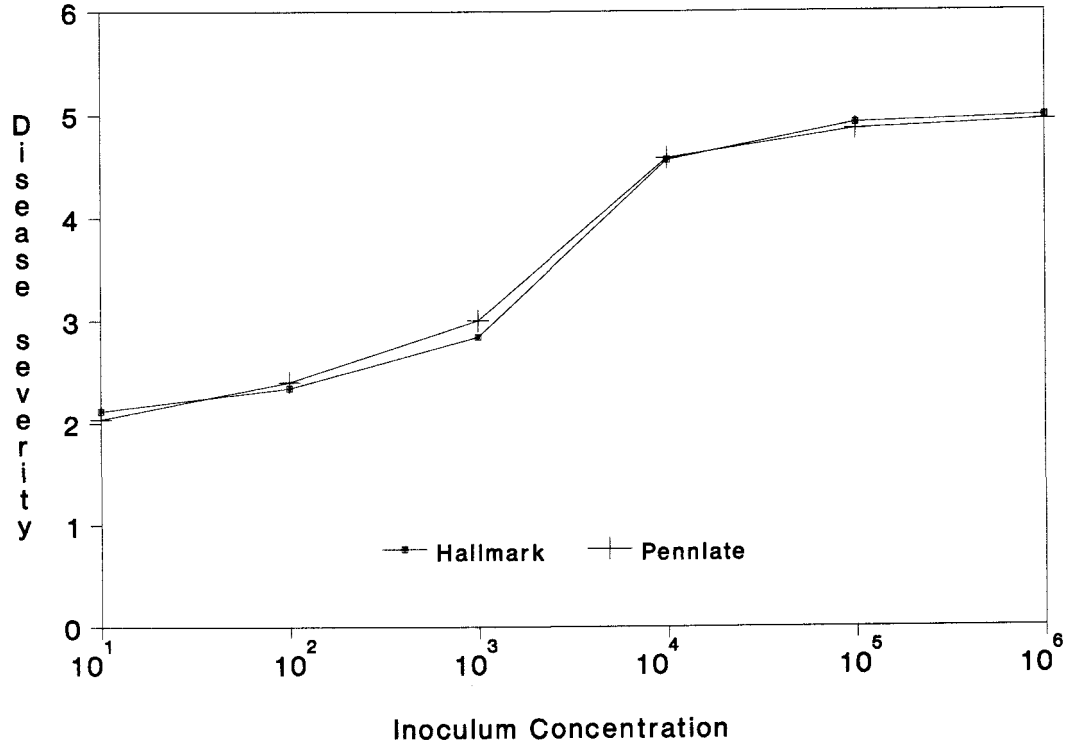

Fig. II.1 Relationship between disease severity and inoculum concentration of Rhynchosporium orthosporum for two orchardgrass genotypes. Regression equations: Hallmark, y= 1.25 + 0.68 x, r= 0.94; Pennlate, y= 1.31 + 0.66 x, r= 0.94.

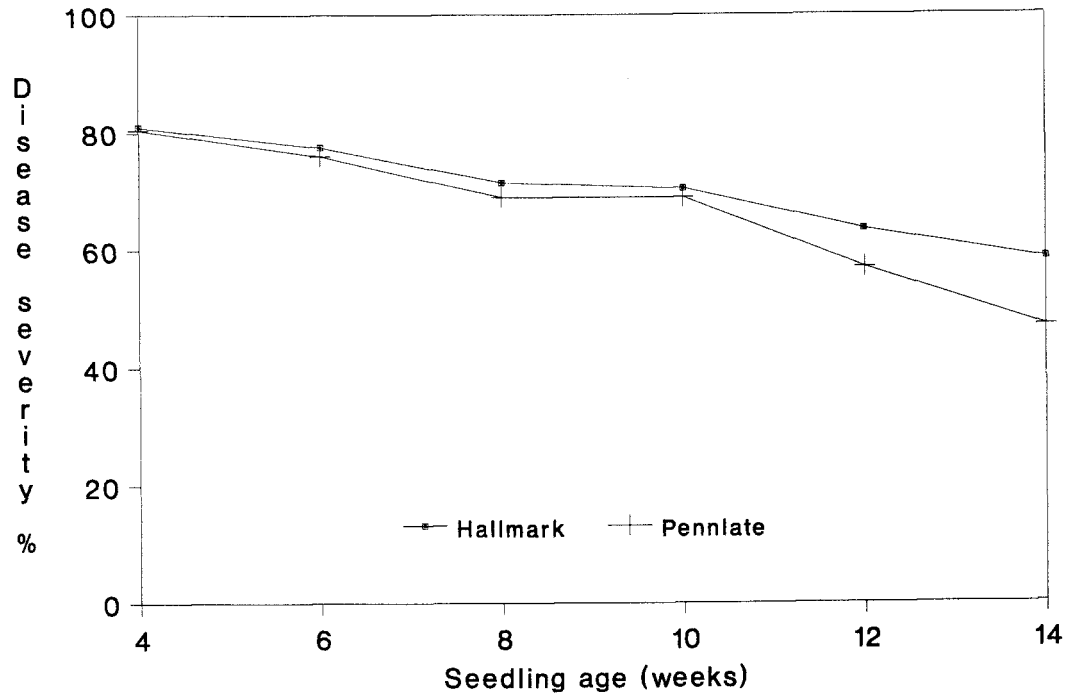

Fig. II.2 Relationship between disease severity and seedling age for two orchardgrass genotypes inoculated with Rhynchosporium orthosporum. Regression equations: Hallmark, y= 90.4 - 2.2 x, r= -0.88; Pennlate, y= 95.3 - 3.2 x, r= -0.92.

Table II.1 Leaf scald severity rating for 10 orchardgrass cultivars inoculated with Rhynchosporium orthosporum

Cultivars	First inoculation[2]		Second inoculation[2]		Total disease severity rating
	total plants	mean disease ratings	total plants	mean disease ratings	
Frontier	50	4.10	47	4.40	4.27 a[1]
Aonami	50	4.00	45	4.50	4.27 a
Latar	50	4.28	44	4.55	4.42 ab
Pennlate	50	4.36	37	4.65	4.51 bc
Cambria	50	4.72	40	4.36	4.54 bcd
Sterling	50	4.60	45	4.54	4.57 bcd
Juno	50	4.74	41	4.56	4.59 bcd
Potomac	50	4.80	49	4.63	4.72 cd
Able	50	4.82	37	4.63	4.73 cd
Hallmark	50	4.76	39	4.77	4.77 d
Total or mean	500	4.52	424	4.57	4.54

[1] Means not having a letter in common are significantly different from each other at 0.05 probability level according to the Fisher's protected LSD test. LSD 0.05= 0.23.

[2] The same plants were tested in both inoculations. Ratings were made 17 days after inoculations. Scale: 0= no lesion to 6= 100% of leaf blade with lesions or dead plant.

Table II.2 Distribution of number of plants in each rating class for ten orchardgrass cultivars inoculated with Rhynchosporium orthosporum

Cultivars	First inoculation[a]							Second inoculation[a]						
	0	1	2	3	4	5	6	0	1	2	3	4	5	6
Frontier	0	0	2	7	25	16	0	0	0	0	2	23	22	0
Aonami	0	0	1	8	30	11	0	0	0	0	0	19	26	0
Latar	0	0	0	1	34	15	0	0	0	0	0	19	25	0
Pennlate	0	0	0	3	26	21	0	0	0	0	0	12	25	0
Cambria	0	0	0	2	12	36	0	0	0	0	1	25	14	0
Sterling	0	0	0	1	18	31	0	0	0	0	0	21	24	0
Juno	0	0	0	0	13	37	0	0	0	0	0	16	25	0
Potomac	0	0	0	0	11	39	0	0	0	0	0	18	31	0
Able	0	0	0	0	9	41	0	0	0	0	0	12	25	0
Hallmark	0	0	0	0	12	38	0	0	0	0	0	8	31	0
Total	-	-	3	22	190	285	-	-	-	-	3	174	248	-
% of total	-	-	0.6	4.4	38	57	-	-	-	-	0.7	41	58.5	-

[a] The same plants were tested in both inoculations. Ratings were made 2 weeks after inoculations. Scale: 0= no lesion to 6= 100% of leaf blade with lesions or dead plant.

CHAPTER III

VARIABILITY AMONG ISOLATES OF *RHYNCHOSPORIUM ORTHOSPORUM* ON ORCHARDGRASS

JESUS PEREZ FERNANDEZ

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 973331, USA.

and

RONALD E. WELTY

US Department of Agriculture, Agricultural Research Service, National Forage Seed Production Research Center Corvallis, Oregon 97330, USA.

SUMMARY

Pathogenic variation in *Rhynchosporium orthosporum* was evaluated using clones of orchardgrass cultivars of Hallmark and Pennlate cultivars and 20 fungal isolates. There were significant differences (P=0.01) among the isolates and a significant cultivar x isolate interaction. The mean disease severity ranged from 49.0% to 27.8% and from 48.0% to 26.1% on Hallmark and Pennlate. There were significant differences (P=0.05) for the mean differences in disease severity for each isolate between cultivars among five isolates. Inoculation of 20 single lesion isolates on clones of two orchardgrass cultivars resulted in the identification of pathogenic variability of the fungus in the Willamette Valley

of Oregon. This is the first report of the pathogenic variability of R. orthosporum on orchardgrass by artificial inoculation.

INTRODUCTION

Rhynchosporium spp. is a common pathogen of barley, orchardgrass and other grasses in many parts of the world (4, 43). It is an imperfect fungus (33), with uninucleate hyphal and conidia (10, 17), and hence has apparently limited mechanisms for the generation of variability. Nevertheless, variation between isolates of R. secalis has been noted with respect to several characters. Newman (28), reported isozyme polymorphism from mycelial extracts of single spores isolates. Newman (29), also recovered recombinant isozyme phenotypes after inoculating barley with pairs of isolates, demonstrating in vivo asexual recombination.

A high degree of specialization within R. secalis has been described in the United States (2, 15, 37) and several other countries (1, 10, 31, 32). The occurrence of pathogenic variability within Rhynchosporium species represent a major problem in global evaluation to resistance to leaf scald (15). In the past, physiological race identification was done on selected cultivars (differential cultivars), that react differentially with regard to resistance and susceptibility to isolates of the fungus.

Information about the pathogenic variability of R.

orthosporum, causal agent of leaf scald on orchardgrass, has not been investigated because differential cultivars have not been identified for evaluating pathogenicity of R. orthosporum. Therefore, this study was undertaken to determine if pathogenic variability exists in R. orthosporum on orchardgrass in Oregon. We sought this information to provide a basis upon which to develop breeding programs for scald resistance.

MATERIALS AND METHODS

A representative sample of fungal isolates were recovered from symptomatic plants of orchardgrass in Benton and Linn counties, Oregon during the spring and summer of 1988. Leaves with fresh lesions were collected from orchardgrass fields and brought to the laboratory for isolation of the fungus. Twenty isolates, five from each of four locations, were used in this study. Isolates were named on the basis of location of collection.

Leaf pieces with lesions were surface-sterilized in 70% alcohol for 30 sec, 1.5% sodium hypochlorite for 2 min, and rinsed in sterile distilled water. The surface-sterilized sections were plated on acid potato-dextrose agar (aPDA). The plates were incubated at 15 C with alternating 12 hr period of fluorescent light and darkness to induce sporulation of the fungus. Spores then were scraped from the lesion or medium with a needle and transferred onto plates of aPDA. The plates were incubated at 15 C for 1 wk to allow the spores to germinate. Spores from a clean single lesion culture were scraped with a needle and transferred to fresh plates of aPDA to establish the single-lesion isolates.

Clones from a 1-year-old single plant of the cultivars

Hallmark and Pennlate were established in a 1:1:1:2 (v:v:v:v) mixture of steamed peat moss, soil (sandy loam), sand and pumice in 1.5 l pots, with drainage holes in the bottom. Clones were grown in a greenhouse at 20-22 C with natural daylight for four months. Tillers bearing four leaves, were separated from the soil and washed under the sink with tap water. Before inoculation each tiller was placed in a test tube (20 x 150 mm) containing 20 ml of a aqueous nutrient solution (5) (see Appendix). Test tubes with tillers and nutrient solution were placed in plastic racks and randomized in 10 replicated test tubes, giving a total of 200 tillers/cultivar for the 20 isolates. After inoculation, nutrient solution was added to the test tubes as needed to maintain plant growth and vigor.

Conidial suspensions, from 3-wk-old cultures, were prepared by gently rubbing the conidia free of the culture with 15 ml of sterile water. Inoculum was filtered through four layers of cheesecloth to eliminate mycelial fragments. A final concentration of 500,000 conidia/ml was used to which 1 drop of Tween 20 per 100 ml was added. Preliminary tests showed this concentration adequate for uniform infection.

Tillers were inoculated by spraying with the conidial suspension until run off. Tillers were then covered with a clear plastic bag and placed in a growth chamber at 15 C with

14 hr of fluorescent light and 10 hr of darkness. Tillers were uncovered after 4 days and transferred to the greenhouse 10 days later. Disease severity was rated 2.5 wk after inoculation. Disease severity ratings were based on the percent of leaf area covered by lesions.

A two x two factorial experiment with a complete randomized design was used in this study. Main effects were cultivar (two), and isolates (twenty). The treatments were replicated ten times. Disease severity mean value of each set of 4 leaves/tiller was calculated. Disease severity means were analyzed by analysis of variance using Michigan Statistical Analysis System (version 4.0, Michigan State University). Fisher's least significant difference (P=0.05), was used to compare mean differences among isolates and cultivars (34, 47). Because the disease severity percentage covered a wide range of values, the arc sin square root of the percent of infection was performed before analysis (45).

RESULTS AND DISCUSSION

Mean squares from the analysis of variance of the mean disease ratings of 20 isolates of R. orthosporum on clones of Hallmark and Pennlate (Table III.1) showed highly significant differences (P=0.01) among the isolates and a highly significant cultivar x isolation interaction. Further analysis indicated that these interactions were due to three isolates showing a different magnitude in response of disease severity, and two isolates contributing a significant difference in rank. The mean disease severity of scald for the 20 isolates ranged from 49.0% to 27.8% on Hallmark, and from 48.0% to 26.0% on Pennlate (Table III.2). On Hallmark clones the more aggressive isolates were I-25 and H-19 and the least aggressive were T-9 and I-29. On Pennlate clones disease was more severe with B-11 and B-14 and the least severe with B-17. There were significant differences (P=0.05) for the mean disease severity differences for each isolate between cultivars among isolates T-4, B-11, B-14, B-17 and I-25.

The characterization of 20 isolates on clones of two orchardgrass cultivars resulted in the identification of pathogenic variability of the fungus. This is not surprising when one considers the widespread area of orchardgrass

production, the number of orchardgrass cultivars growing in the region, and the large amount of genetic variability found for R. secalis, the cause of leaf scald of barley (15). The isolates and clones used in this research determined the presence of variability of leaf scald isolates in the region. However, the establishment of a set of "differential" host cultivars is needed as a means for further quantification of the genetic variability in the pathogenicity of this fungus and identification of physiological races. At present, knowledge of genetic variability for resistance in orchardgrass is unknown, and differential cultivars have not been selected.

The nutrient solution used in this study was adequate to maintain and stimulate growth of orchardgrass tillers through and two weeks beyond the disease evaluation period. The present work is the first report of the pathogenic variability of R. orthosporum on orchardgrass by artificial inoculation. This research gives some indication of the variation of the fungus in the Willamette Valley of Oregon. The documentation of the pathogenic variability of the casual organism will be important in development a breeding program to control the disease through conventional breeding methods.

Table III.1 Analysis of variance of mean disease ratings of 20 isolates of Rhynchosporium orthosporum on two orchardgrass cultivars

Source	df	Mean square[a]
Cultivars	1	0.54
Isolates	19	317.07 **
Cultivars x isolates	19	285.14 **
Error	360	87.87

[a] **= significant at P= 0.01.

Table III.2 Mean disease severity ratings for clones of Hallmark and Pennlate when inoculated with 20 isolates of Rhynchosporium orthosporum

Isolates[a]	Cultivars		Mean differences
	Hallmark	Pennlate	
	b	b	c
T-1	37.48	36.97	0.51
T-3	37.95	39.63	1.68
T-4	39.90	28.49	11.41 *
T-8	35.82	40.60	4.78
T-9	28.43	35.99	7.56
B-11	35.16	48.01	12.85 *
B-12	32.95	35.86	2.91
B-14	29.65	41.87	12.22 *
B-15	29.54	29.99	0.45
B-17	39.79	26.07	13.72 *
I-28	29.44	37.62	8.18
I-27	36.73	38.82	2.09
I-25	49.03	36.49	12.54 *
I-29	27.82	30.04	2.22
I-30	36.90	40.13	3.23
H-19	42.64	36.86	5.78
H-20	32.29	27.82	4.47
H-21	37.40	32.11	5.29
H-22	33.89	30.93	2.96
H-23	35.08	35.44	0.36
LSD (P= 0.05)	7.68	8.82	

a Isolates were collected from four locations : Tangent, Botany Farm, International Seed Experimental Field, and Hyslop Field Lab. in Benton and Linn counties, Oregon.

b Mean disease severity ratings based on the arc sin square root of the percent of infection of 10 replicates.

c Mean differences followed by an asterisk are significantly different at P=0.05 (LSD= 8.22)

CHAPTER IV

RELATIONSHIP BETWEEN SEVERITY OF LEAF SCALD AND YIELD COMPONENTS OF ORCHARDGRASS

JESUS PEREZ FERNANDEZ

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, USA.

and

RONALD E. WELTY

US Department of Agriculture, Agricultural Research Service, National Forage Seed Production Research Center Corvallis, Oregon 97330, USA.

SUMMARY

Two cultivars of orchardgrass, Hallmark and Pennlate, differing in their field susceptibility to leaf scald caused by Rhynchosporium orthosporum and in their flowering behavior, were grown outdoors in pots and inoculated with a mixture of four single spore isolates of the fungus at jointing, boot, heading or flowering in 1988 and 1989. Measurements included disease severity on flag leaves, first and second leaves below the flag leaves; seed yield; and thousand-seed weight. Disease severity ratings for flag leaf, first, and second leaves were highly correlated with total disease severity averaged over all leaves for both years. A low correlation was found between disease severity, seed yield, and thousand-seed weight for both years. Hallmark was

more susceptible to the disease in both years. In 1989, Hallmark had the highest mean seed yield reduction, when inoculum was applied at the heading stage and Pennlate had the highest seed yield reduction when inoculated at the flowering stage of plant growth.

INTRODUCTION

A major impediment to the evaluation of fungicide control of leaf scald in orchardgrass is a lack of quantitative information between disease severity and seed yield loss. Seed yield losses are associated with an increase in scald and other leaf diseases, but the response of seed yield and thousand seed-weight under natural or artificial leaf scald infection has not been investigated. Orchardgrass cultivars that are grown for seed production in the Willamette Valley have a range of flowering times, a trait included in cultivar descriptions. Fungicides that reduce scald severity and increase seed yield in Oregon have shown that a single application of a fungicide is more effective when applied at the boot or heading stage than at the jointing or flowering stage in cultivars that flower early in the spring (50).

Two cultivars of orchardgrass were chosen based on their field responses to R. orthosporum, and flowering behavior. Cultivar "Hallmark" was selected because it is highly susceptible to leaf scald and flowers early. "Pennlate" is more resistant to scald and flowers late (unpublished data). The purpose of this study was to investigate relationships between disease severity, seed yield, and thousand seed-

weight when R. orthosporum inoculum is applied at four well spaced morphological growth stages: jointing, boot, heading or flowering. These controlled inoculations also allowed for a comparison of disease resistance of both cultivars.

MATERIALS AND METHODS

Field-greenhouse experiments were conducted in 1988 and 1989 at the National Forage Seed Production Research Center, Oregon State University. On September 20, 1987, five plants per entry of cultivars "Hallmark" and "Pennlate" (giving a total of 160 plants/cultivar) were established from seed in a 1:1:1:2 (v:v:v:v) mixture of steamed peat moss, soil (sandy loam), sand and pumice in 19.4 l pots with drainage holes in the bottom. After 3 months in the greenhouse plants were placed outdoors for vernalization and arranged in a completely randomized design. During this period, pots were covered with soil to protect roots and crowns from winter injury. A three-way factorial experiment with four replicates was used in this study. The treatments were: cultivar (Hallmark / Pennlate), inoculation time at four morphological stages of cultivar development (jointing, boot, heading or flowering; corresponding to cereal growth stages (GS): GS-6, GS-9, GS-10.5 and GS-10.52 of the Feeke's scale, respectively (22)), and inoculation with the leaf scald fungus (inoculated and noninoculated).

Plants in designated pots were inoculated with a conidial suspension of a mixture of four single spore isolates of R. orthosporum in distilled water. The isolates

were obtained from leaf spots on orchardgrass growing at Hyslop Field Laboratory (Oregon State University) in Corvallis, OR. Cultures of the fungus were grown on acid potato dextrose agar for 3 wk in an incubator at 15 C with 12 hr photoperiod supplied by fluorescent light. Conidial suspensions of each isolate were prepared by gently rubbing the conidia free of the culture into 15 ml of sterile water. Inoculum was filtered through four layers of cheesecloth to eliminate mycelial fragments and combined in a flask. A final concentration of 500,000 conidia/ml was used to which 1 drop/100 ml of Tween 20 was added.

Leaves were inoculated by spraying with the conidial suspension until thoroughly wet. Plants in the noninoculated treatments were sprayed with sterile water. After inoculation, plants were covered with a plastic bag and moved into the growth chamber at 15 C with 14 hr photoperiod supplied by fluorescent light. Bags were removed after 4 days and plants were transferred outdoors 10 days later.

Disease severity was assessed on the flag leaf (DS f-g), and first (DS f-1) and second leaf (DS f-2) below the flag leaf, as the percentage of leaf area for each tiller affected by the disease (7). Disease severity mean (DS mean) was derived by taking the average percent infection of DS f-g, DS f-1, and DS f-2. Both cultivars were evaluated at the milky

growth stage of seed development (GS-10.1). Seed was harvested by removing the panicles from each plant. Samples were air-dried, threshed individually and total seed weight (seed/w), and thousand-seed weight (1000/w) were determined.

During the study, plants were fertilized every 4 wk with 500 ppm of a soluble fertilizer (20:20:20, NPK) to maintain vigorous growth. Plants were clipped during the summer of 1988 and the regrowth was used to repeat the experiment in 1989. Data were analyzed as a three-way factorial with a completely randomized design for each experimental variable. Also, an analysis of variance was used to examine data combined over years, which were in a 2 x 4 x 2 x 2 factorial (two cultivars, four stages of plant growth, inoculation, and two years). Analyses of variance were performed using the Michigan Statistical Analysis System (version 4.0, Michigan State University). The relation between seed yield, thousand seed weight and disease parameters were investigated by correlation analysis.

RESULTS AND DISCUSSION

The analysis of variance of the three-way factorial of mean disease severity of leaf scald for the three leaves combined (DS mean) and yield components for Hallmark and Pennlate in 1988 and 1989 are shown in Tables IV.1 and IV.2 (see appendix for analysis of variance of mean disease severity of f-g, f-1, and f-2). Highly significant differences (P= 0.01) were found for DS mean for cultivar x inoculation, and cultivar x stages interactions (1988); cultivar x stages x inoculation was highly significant for DS mean in 1989. Separate analysis of data from 1988 indicated that these interactions were due to an increase in magnitude of disease severity for both inoculated and control treatments, and to an increase of disease severity for stages of plant growth on Hallmark. Further analysis indicated that the cultivar x stages x inoculation interaction (1989) was attributed to an increase in magnitude of disease severity on Hallmark, when inoculated at the heading stage, and on Pennlate when inoculated at the boot stage of plant growth.

Seed weight was significant (P= 0.05) for cultivar x stages x inoculation (1988), and for cultivar x stages and main effect inoculation (1989). In 1988 there was an inconsistent rank of treatments for mean seed yield that make

it difficult to explain the second order interaction. In 1989, however, the interactions were attributed to an increase in magnitude of mean seed yield on Hallmark at the heading stage of plant growth. In addition, there was an increase of total mean seed yield for the noninoculated plants. Thousand-seed weight was highly significant for cultivar x stages (1988), and stages of growth. Separate analysis of data indicate that these interactions were attributed to an increase in magnitude of mean thousand seed-weight on Hallmark at heading and flowering stages (1988), and to an increase on both cultivars at the jointing stage of plant growth in 1989.

Analysis of variance of the four-way factorial (see appendix for analysis of variance) demonstrated that the cultivar x inoculation x year , and cultivar x year interactions were highly significant (P= 0.01) for DS mean and seed yield. In addition, cultivar x year x stages was significant (P= 0.05) for thousand seed-weight. The highly significant interactions were attributed to a higher mean disease severity on Hallmark in inoculated plants, and to an increase in the number of tillers per plant for both cultivars in 1989. There was not a consistent trend to explain the cultivar x year x stages interaction for thousand seed-weight.

The linear correlation coefficients presented for both years (Table IV.3) indicate the relationship that existed among disease severity ratings and yield components. There was a high correlation among disease severity ratings and the total disease severity for both years. A low correlation was found between disease severity and components of yield for both years. In 1989, however, DS mean and thousand seed-weight were significantly correlated (P= 0.05).

The total mean disease severity ratings of leaf scald and stages of growth x inoculation level interaction are shown for 1988 and 1989 (Tables IV.4 and IV.5), and summarizes the disease assessments. Controls were not completely free of infection by leaf scald. However, scald symptoms in controls never reached the percentage of disease of the inoculated plants. The cultivar Hallmark had higher disease on the leaves than Pennlate for both years. In 1988 and 1989, Hallmark reached its highest mean infection at the heading stage of plant development and Pennlate at the boot stage. In 1989 there was a higher disease intensity on Hallmark than 1988. Pennlate had a lower leaf scald infection for both inoculated and control treatments for both years. Seed weight yield and stages of growth x inoculation level interaction are shown for 1988 and 1989 (Tables IV.6 and IV.7). The data show a low seed yield in 1988. In general seed production in perennial orchardgrass is low during the

first year. During the first year of this study, time for floral induction was reduced, because plants were moved outdoors by the end of December. Onset of flowering may have been modified by the short time plants were exposed to the winter in 1988, reducing the numbers of fertile tillers and thus seed yield. This reduction may be partially explained by the mean numbers of tillers per pot, which in 1988 was 4.1 for Hallmark and 3.5 for Pennlate. In 1989, Hallmark had 12.5 tillers per pot and Pennlate 11.1 tillers per pot. In 1989, when disease severity was higher, mean seed yield was reduced by 44% on Hallmark, when the inoculum was applied at the heading stage and 20% on Pennlate at the flowering stage of growth. The total mean seed yield production for the inoculated plots was decreased by 24.4% on Hallmark and by 8.4% on Pennlate. Little differences were found in 1988 in total mean seed- weight between inoculated and noninoculated plots.

Effects of stages of growth and inoculation on thousand-seed weight are summarized (Tables IV.8 and IV.9) for Hallmark and Pennlate for 1988 and 1989. The variability of the data for thousand seed-weight show non consistent pattern between inoculated and control treatments and stages of growth on both cultivars. There was an increase of mean thousand seed-weight on Hallmark at heading and flowering stages of plant growth (1988), and an increase of the total

mean thousand seed-weight at the jointing stage of plant development in 1989.

Under the conditions of this study, Hallmark ("field susceptible cultivar") was more susceptible to leaf scald than was Pennlate. In both years, Hallmark had the highest disease severity values (Tables IV.4 and IV.5) at the heading stage of growth and the largest decrease of seed yield (Tables IV.6 and IV.7). Pennlate had the highest disease severity values (Tables IV.4 and IV.6) at the boot stage of growth and the largest decrease of seed yield at the flowering stage (1989) (Table IV.7). These results agree with fungicide trials conducted in the Willamette Valley (Oregon) to increase seed production on orchardgrass, where an increase in seed production was reached when fungicides were applied to an early cultivar at the heading stage of development (50). As suggested for another perennial grass (53) the number of tillers per plant was important in seed production. In 1989, an increase in number of tiller per pot resulted in an increase of seed yield for both cultivars. Seed production loss and reduced kernel weight have been shown to be related to disease severity on the upper leaves at specific stages of growth for other leaf spots diseases on annual crops (16, 27, 44). In our study, for the two perennial orchardgrass cultivars, there was a lack of correlation between disease severity ratings , seed yield,

and thousand seed-weight for both years. In annual crops, the plant dies with the maturity of reproductive tillers. In perennial grasses, plants do not die after death of reproductive tillers. As reported by Langer (20), perenniality depends on the alternation of tillers, and only part of tillers turned reproductive (48). Therefore an individual orchardgrass plant was composed of both vegetative and reproductive tillers. A perennial orchardgrass plant might compensate for the lack of photosynthate area in the upper leaves with the vegetative tillers, and this may be associated with seed production (35).

In summary, this study demonstrated genetic differences between Hallmark and Pennlate in relative ability to yield under disease pressure at different stages of plant growth. However, seed yield, and 1000 seed-weight were not very consistent as a measure of yield under different severities of leaf scald on orchardgrass.

Table IV.1 Analysis of variance of mean severity of leaf scald, total seed weight, and 1000-seed weight for two orchardgrass cultivars in 1988

Source of variation	df	F-value[a] DS mean	Seed/w	1000/w
Cultivars	1	199.5**	0.4	10.9**
Stages of growth	3	0.11	2.2	8.7**
Cult x stages	3	6.1**	2.3	5.1**
Inoculation	1	292.8**	0.8	0.4
Cult x inoc	1	15.6**	0.1	0.7
Stages x inoc	3	2.3	1.3	1.2
Cult x stag x inoc	3	2.4	4.0*	1.1
Error	48			

*= Significant at the 0.05 level.
**= Significant at the 0.01 level.
[a] F-value for total disease severity, total seed weight and thousand-seed weight.

Table IV.2 Analysis of variance of mean severity of leaf scald, total seed weight, and 1000-seed weight for two orchardgrass cultivars in 1989

Source of variation	df	F-value[a] DS mean	seed/w	1000/w
Cultivars	1	276.4**	1.4	0.8
Stages of growth	3	0.6	2.0	4.5**
Cult x stages	3	9.94**	3.5*	2.6
Inoculation	1	447.4**	7.1*	0.1
Cult x inoc	1	45.7**	1.9	0.6
Stages x inoc	3	3.9*	1.0	2.7
Cult x stag x inoc	3	6.3**	2.5	1.7
Error	48			

*= Significant at the 0.05 level.
**= Significant at the 0.01 level.
[a] F-value for overall disease severity, total seed weight and thousand-seed weight.

Table IV.3 Simple correlation coefficients for total seed weight (seed/w), 1000-seed weight (1000/w), and disease severity ratings for two orchardgrass cultivars in 1988 and 1989

Disease severity[b]	Disease severity[b]					
	DS fg	DS f1	DS f2	DS mean	Seed/w	1000/w
DS-fg	a	0.86*	0.73*	0.90*	-0.10	-0.06
DS-f1	0.91*	-	0.87*	0.97*	-0.10	-0.05
DS-f2	0.75*	0.88*	-	0.94*	-0.10	-0.03
DS-mean	0.92*	0.98*	0.94*	-	-0.20	-0.04
Seed/w	0.09	0.13	0.23	0.20	-	0.10
1000/w	0.40*	-0.03	0.14	0.30*	0.10	-

[a] All values below the diagonal line are simple correlation coefficients for 1988. All values above the diagonal line are simple correlation coefficients for 1989.

[b] Disease severity on each plot was based on the mean percent of leaf infection on flag leaf, first and second leaves below flag leaf and overall mean disease severity.

* Correlation coefficients = 0.25 are significant (P= 0.05).

Table IV.4 Total mean disease severity ratings of leaf scald and stages of plant growth x inoculation level interaction for two orchardgrass cultivars in 1988

Stages of growth	Cultivar Hallmark			Cultivar Pennlate		
	Inoc	Cont	Means	Inoc	Cont	Means
	[a]		[b]			[b]
Jointing	55	28	41	33	18	26
Boot	50	27	38	40	17	28
Heading	59	30	44	27	15	21
Flowering	50	33	41	31	19	25
	[c]			[c]		
Means	54	30		33	17	

[a] Mean disease severity ratings based on the percent average of leaf scald infection on flag leaf and first and second leaves below flag leaf.

[b] Cultivar x stages: LSD= 4.6 (P= 0.05).

[c] Cultivar x inoculation: LSD= 3.3 (P=0.05).

Table IV.5 Total mean disease severity ratings of leaf scald and stages of plant growth x inoculation level interaction for two orchardgrass cultivars in 1989

Stages of growth	Cultivars			
	Hallmark		Pennlate	
	Inoc	Cont	Inoc	Cont
	ab			
Jointing	64	27	30	14
Boot	60	26	47	14
Heading	75	25	24	11
Flowering	62	32	31	15

a Mean disease severity ratings based on the percent of leaves infected by leaf scald on flag leaf and first and second leaves below flag leaf.

b Cultivar x stages x inoculation: LSD= 7.7 (P= 0.05).

Table IV.6 Seed weight and stages of plant growth x inoculation level interaction for two orchardgrass cultivars in 1988

Stages of growth	Cultivars			
	Hallmark		Pennlate	
	Inoc	Cont	Inoc	Cont
Jointing	1.6[ab]	1.3	1.0	1.4
Boot	1.6	1.4	2.0	1.3
Heading	1.6	2.0	1.6	1.1
Flowering	1.7	1.5	1.5	1.5

[a] Seed weights in grams were calculated using the means of four replicated pots.

[b] Cultivar x stages x inoculation: LSD= 0.6 (P= 0.05).

Table IV.7 Seed weight for cultivar x stages of plant growth, and inoculation level in 1989

Stages of growth	Cultivars: Hallmark Inoc	Hallmark Cont	Hallmark Means	Pennlate Inoc	Pennlate Cont	Pennlate Means	Inoculation Inoc	Inoculation Cont
		[a]			[b]			
Jointing	7.6	8.6	8.1	8.9	8.8	8.8		
Boot	7.8	8.9	8.3	8.3	9.6	8.9		
Heading	8.9	15.8	12.3	8.4	8.2	8.3		
Flowering	8.9	9.6	9.2	8.1	10.1	9.1		
						[b]	[c]	
Total mean	-	-	-	-	-	-	8.3	9.9

[a] Seed weights in grams were calculated using the means of four replicated pots.

[b] Cultivars x stages: LSD= 2.4 (P= 0.05).

[c] Inoculation: LSD= 1.2 (P= 0.05).

Table IV.8 Thousand seed weights and stages of plant growth x inoculation level interaction for two orchardgrass cultivars in 1988

Stages of growth	Cultivars					
	Hallmark			Pennlate		
	Inoc	Cont	Means	Inoc	Cont	Means
	a		b			b
Jointing	0.90	0.91	0.90	0.94	1.05	0.98
Boot	0.94	0.95	0.94	0.85	0.76	0.89
Heading	1.12	1.10	1.11	0.98	0.97	0.97
Flowering	1.20	1.05	1.12	0.95	0.95	0.95

a Thousand seed weights in grams were calculated using a sample of the total seed weight of four replicated pots.

b Cultivars x stages: LSD= 0.1 (P= 0.05).

Table IV.9 Thousand seed weights and stages of growth x inoculation level interaction for two orchardgrass cultivars in 1989

Stages of growth	Cultivars				
	Hallmark		Pennlate		
	Inoc	Cont	Inoc	Cont	Means
	[a]				[b]
jointing	1.02	1.09	0.96	1.21	1.07
Boot	0.88	0.84	1.04	0.92	0.92
Heading	1.01	0.91	1.05	0.89	0.99
Flowering	0.87	0.91	1.05	0.89	0.93

[a] Thousand seed weights in grams were calculated using a sample of the total seed weight of four replicated pots.

[b] Stages of growth: LSD= 0.09 (P= 0.05).

CHAPTER V

HISTOPATHOLOGY OF ORCHARDGRASS INOCULATED BY RHYNCHOSPORIUM ORTHOSPORUM

JESUS PEREZ FERNANDEZ

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, USA.

and

RONALD E. WELTY

US Department of Agriculture, Agricultural Research Service, National Forage Seed Production Research Center Corvallis, Oregon 97330, USA.

SUMMARY

Penetration, colonization and conidia production on leaves of orchardgrass by Rhynchosporium orthosporum were investigated by light, fluorescent and scanning electron microscopy. Conidia germinated on orchardgrass leaves to produce germ tubes and appressoria. Penetration occurred directly on the adaxial or abaxial surface of the leaf. After penetration the fungus formed subcuticular mycelium that grew intercellularly. Hyphae grew from this area into and throughout the mesophyll cells. Following disintegration and death of the mesophyll cells, the hyphae became intracellular. At this stage, symptoms are evident on leaf blades as irregular lenticular lesions. Hyphae were found

colonizing phloem cells, but not the xylem elements. Conidia were produced on intact leaf surfaces from the subcuticular mycelium on short conidiophores that extruded through stomata and cuticular holes. In older lesions, the surface of the leaf eventually breaks down releasing masses of conidia that were formed beneath the cuticle.

INTRODUCTION

Leaf scald, caused by Rhynchosporium orthosporum, is associated with irregular, lenticular lesions on orchardgrass leaf blades and sheathes. The development of Rhynchosporium scald on barley leaves has been studied microscopically by Caldwell (4). Previous studies examined the method of conidia production of the barley leaf scald fungus, Rhynchosporium secalis. Results have been controversial. Ayesu-Offei et al. (3) and Caldwell (4), suggested conidia develop under the protection of the cuticle and masses of conidia are released only after the cuticle ruptures. Howllett et al. (14), reported that conidia of R. secalis are produced on the intact leaf surfaces with conidiogenous mycelium protruding through cuticular holes. Information on penetration, colonization and conidia development is lacking for R. orthosporum on orchardgrass.

The present study was undertaken to examine the characteristic penetration, colonization, and conidia production on orchardgrass leaves by R. orthosporum using light, fluorescent, and scanning electron microscopy.

MATERIALS AND METHODS

R. orthosporum was isolated from orchardgrass growing at Hyslop Field Laboratory near Corvallis, OR. Conidial inoculum was produced by growing the fungus for 2 wk on acid potato dextrose agar in an incubator at 15 C with alternating 12 hrs period of fluorescent light and darkness. Conidial suspensions were prepared by gently rubbing the culture surface to free the conidia in 15 ml of sterile water and filtered through four layers of cheesecloth to eliminate mycelial fragments. The final concentration was adjusted to 500,000 conidia/ml, and the solution contained 1 drop of Tween 20 per 100 ml as a wetting agent.

Five plants of the cultivar Hallmark were established in a 1:1:1:2 (v:v:v:v) mixture of steamed peat moss, soil (sandy loam), sand and pumice in 1.5 l pots with drainage holes in the bottom. Seedlings were 4-week-old when inoculated. Leaves were sprayed with the inoculum suspension until run off, covered with a clear plastic bag, and placed in a growth chamber at 15 C with 14 hrs of fluorescent light and 10 hrs of darkness. Plants were uncovered after 4 days and transferred to the greenhouse 10 days later. One expanded leaf was harvested from each plant 2, 3, 4, 5, 6, 8, 10, and 12 days after inoculation. Several pieces excised from each

leaf at each harvest were examined in whole mounts and transverse sections. Whole mounts were made of fresh plant samples in water; other samples were cleared in saturated chloral hydrate aqueous solution and stained in lactophenol aniline blue as suggested by Shipton (38). Other samples were fixed in formaldehyde alcohol (FAA),dehydrated in tertiary butyl alcohol series and embedded in Paraplast. Blocks were sectioned at 5 microns at room temperature with an American Optical rotatory microtome.

For observation of induced fluorescence, a technique by Schans (36) was used. Sections were stained for 2 min with a 0.5% aqueous malachite green or methyl green solution. Then sections were counterstained for 20 min with 0.002% acridine orange in a boric acid-borax buffer, pH 8.6. After washing in buffer, sections were air-dried and mounted in immersion oil. Sections were examined with a Carl Zeiss Axioskop microscope equipped with an epi-fluorescence condenser, a high pressure mercury lamp and neofluor objectives. Stained sections were observed through Zeiss filters 09 and 02 consisting of exciter filters B 450-490 and G 365. Photomicrographs were prepared by using Kodachrome 64 film.

The method of spore production was examined using scanning electron microscope (SEM). Natural lesions were obtained from field plots of orchardgrass. The material was

fixed in FAA and dehydrated in a graded water/acetone series and acetone/trichlorotrifluroethane (TF) solutions. From 100% TF, samples were critical point dried in a Balzer (CPD020) drier. After mounting on aluminum planchets, they were coated with 200 nm of 60/40 wt% Au/Pd alloy in a Varian VE-10 vacuum evaporator, and examined in a AmRay 1000 A SEM. Photomicrographs were prepared by using Polaroid Type 55 film.

RESULTS AND DISCUSSION

Descriptions of leaf scald development in orchardgrass leaves were compiled from combined results of observation of leaf sections from natural and artificial inoculations using whole leaf mounts, microtome sections, and SEM. The methyl green and acridine orange stains induced a good fungal fluorescence in leaf sections. With this technique hyphae fluoresced red-orange and cells walls light yellow. A cross section of a noninfected orchardgrass leaf consists of elongated epidermal cells, enlarged bulliform cells, mesophyll cells, a thicker wall bundle sheath surrounded the vascular bundles, a strongly developed sclerenchyma and vascular bundles (xylem and phloem), (9) (Fig. V.1).

Penetration occurs on either the adaxial or abaxial epidermal surface. After 24 hrs, conidia germinated and formed a germ tube with a small, rounded structure at the end, resembling an appressorium. The fungus penetrated the leaf from an appressorium by two routes; 1) epidermal cells were penetrated directly (Fig.V.2) frequently at the junction between contiguous epidermal cells and 2) guard or subsidiary cells were penetrated directly (Fig. V.3). Penetration through open or closed stomata pores was never observed. Conidia produced one or two germ tubes (Fig. V.4) Two or

three days after penetration subcuticular mycelium was established that grew intercellularly between the cuticle and the epidermal cells (Fig. V.5). Five-to-six days after inoculation, mycelium invaded the mesophyll cells. About 7-10 days after inoculation, macroscopic symptoms of scald were observed and symptoms were easily seen on the leaf blade. At this stage the mesophyll cells were completely colonized and disintegrated by intracellular mycelium (Fig. V.6). Following death of mesophyll cells, subcuticular mycelium continued to grow forming a closely packed hyphal mat between the upper and lower epidermis and under the cuticle of a leaf. About 12 days after inoculation there was an extensive colonization and complete break-down of the mesophyll cells (Fig. V.7).

Although the fungus extensively colonized the mesophyll cells, there was little movement through the vascular system. Mycelium was found plugging the phloem (Fig. V.8), but mycelium was not observed in the xylem. About 10 -12 days after inoculation, conidia were produced outside intact leaf surfaces (Fig. V.9), on conidiogenous mycelial extensions rising from the subcuticular mycelium. Conidiogenous mycelium, presumed to be shortened conidiophores, protruded through the stomata pores, leaf cuticle or along the margins of leaf hairs (Fig. V.10 to V.14.). Conidia produced from the subcuticular mycelium were cylindrical with a median septum and delimited at the conidiogenous mycelium by a small

constriction (Fig. V.15). Subcuticular mycelium could be followed along the leaf by observing conidia produced on the leaf surface (Fig. V.16). In older lesions, the surface of the leaf and the entire mesophyll disintegrated and leaf tissues died (Fig. V.17).

The isolate of R. orthosporum used in this study penetrated leaves in ways described for the leaf scald fungus of barley, R. secalis (3, 4). No direct penetration was observed through the stomatal pores, but germ tubes penetrated guard cells and contiguous epidermal cells. This work showed extensive colonization of mesophyll cells by the mycelium which resulted in the collapse of mesophyll cells beneath the subcuticular hyphae. It was not determined if cell collapse was a response to a toxin or enzymes produced by the hyphae. Further research is needed to determine what occurs when mesophyll cells collapse. Attempts to isolate R. orthosporum distally or proximally 2.5-5 cm from a visible lesion on orchardgrass leaves were unsuccessful. The disease is not systemic in orchardgrass plants. It seems possible that the mycelium spreads from lesions to healthy tissue by slow growth of mycelium as subcuticular mycelium or through the phloem with expansion of the mycelium and lesion. The presence of hyphae in the phloem may have a significant importance with the interference with the movement of organic nutrients from the leaf cell to the phloem or with their

translocation through the phloem elements. It may be possible that the hyphae interferes with carbohydrate movement from the leaves as shown for other leaf pathogens in orchardgrass (23).

At early stages during sporulation, the cuticle remains intact and conidia protrude through the stomatal pores, leaf cuticle and near a leaf hair, similar to R. secalis (14). The subcuticular mycelium remains intact and is protected from adverse conditions during most of the period of spore production. As the lesions age and the cuticle is cracked, masses of conidia are released. This observation could explain the existing differences concerning sporulation of R. secalis in barley as reported earlier (3, 4, 14).

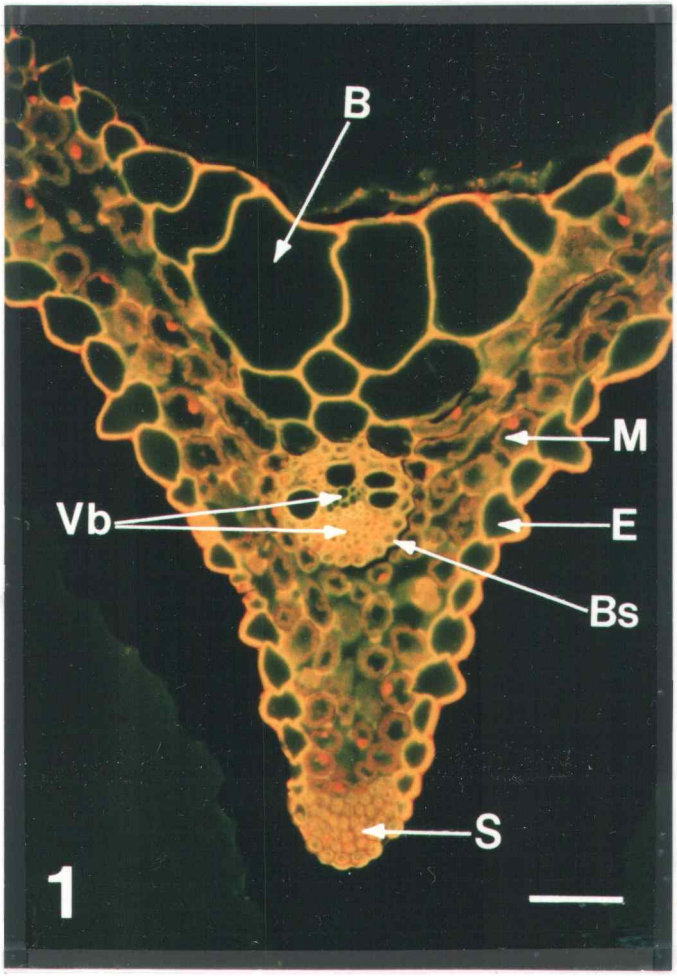

Fig. V.1 Fluorescence microscopy of a cross section of a noninfected orchardgrass leaf. E, epidermal cells; B, bulliform cells; M, mesophyll cells; Bs, a bundle sheath surrounded the vascular bundles; Vb, vascular bundles; S, sclerenchyma. Bar= 50 microns.

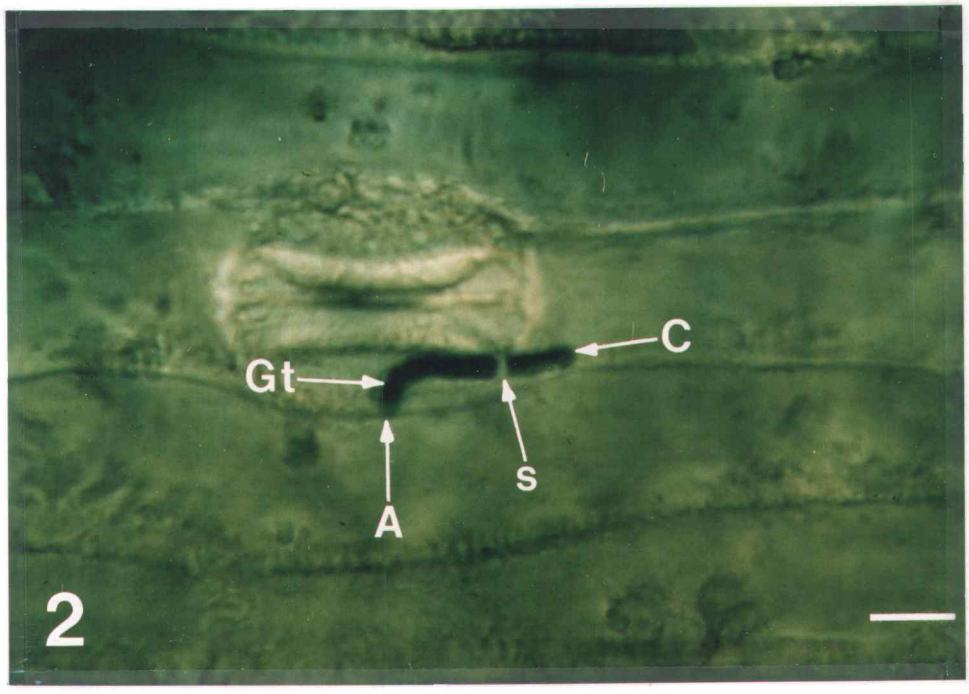

Fig. V.2 Whole leaf mount showing a R. orthosporum conidia on an orchardgrass leaf. Conidia germinating and penetrating the cuticle of leaf 24 hrs after inoculation. A, appressorium; C, conidium showing a septum (s); Gt, germ-tube. Bar= 10 microns.

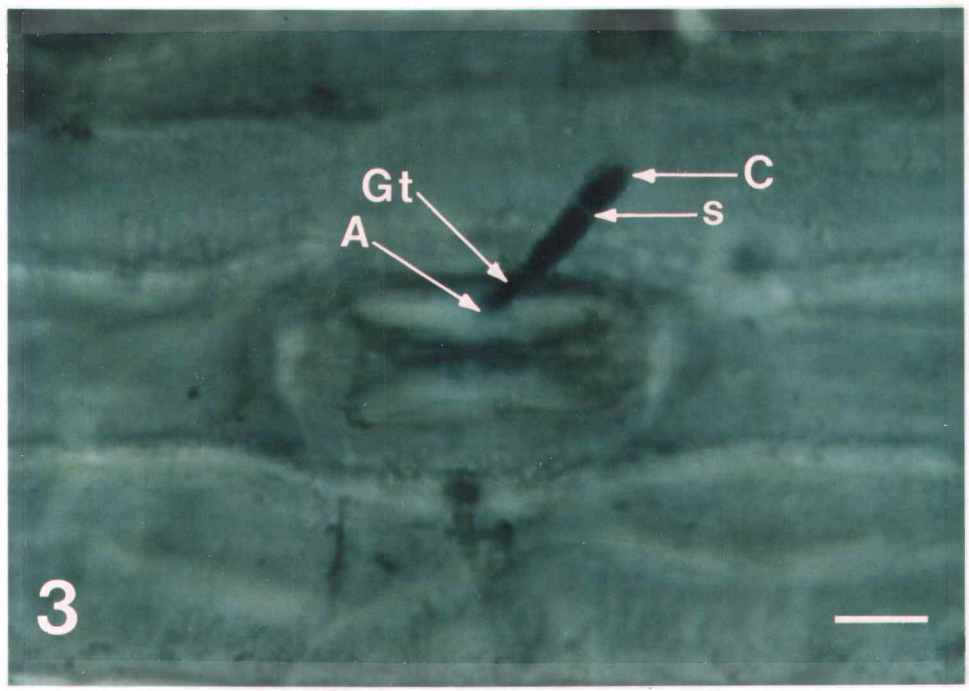

Fig. V.3 Whole leaf mount showing a R. orthosporum conidia germinating and penetrating the cuticle of an orchardgrass leaf via the subsidiary cell 24 hrs after inoculation. A, appressorium; C, conidium showing a septum (s); Gt, germ-tube. Bar= 10 microns.

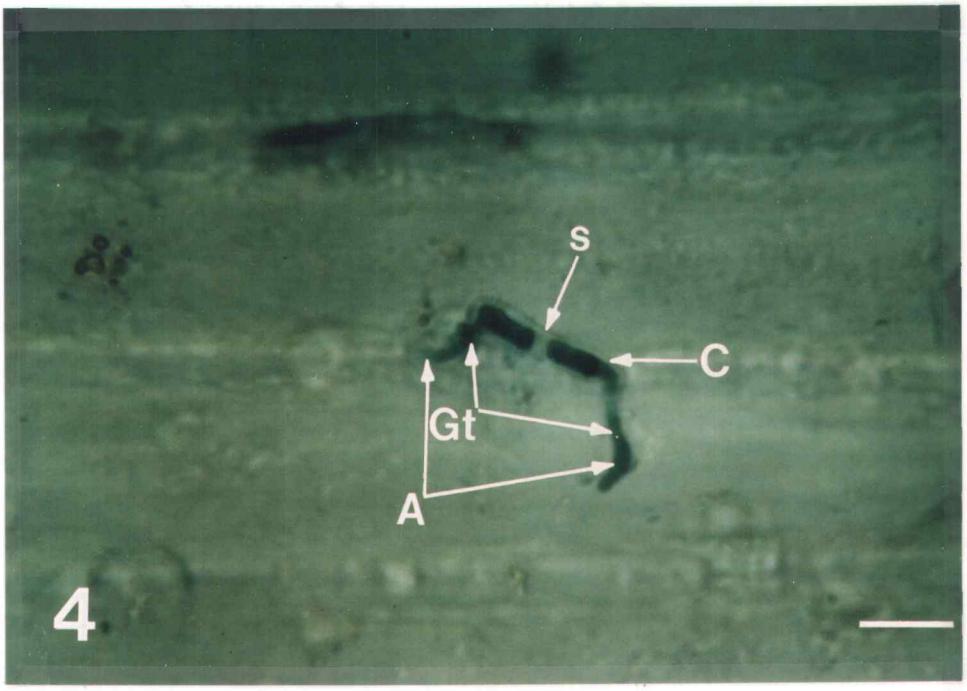

Fig. V.4 Whole leaf mount showing a R. orthosporum conidia germinating from both cells and penetrating the cuticle of an orchardgrass leaf 72 hrs after inoculation. A, appressorium; C, conidium showing a septum (s); Gt, germ-tube. Bar= 10 microns.

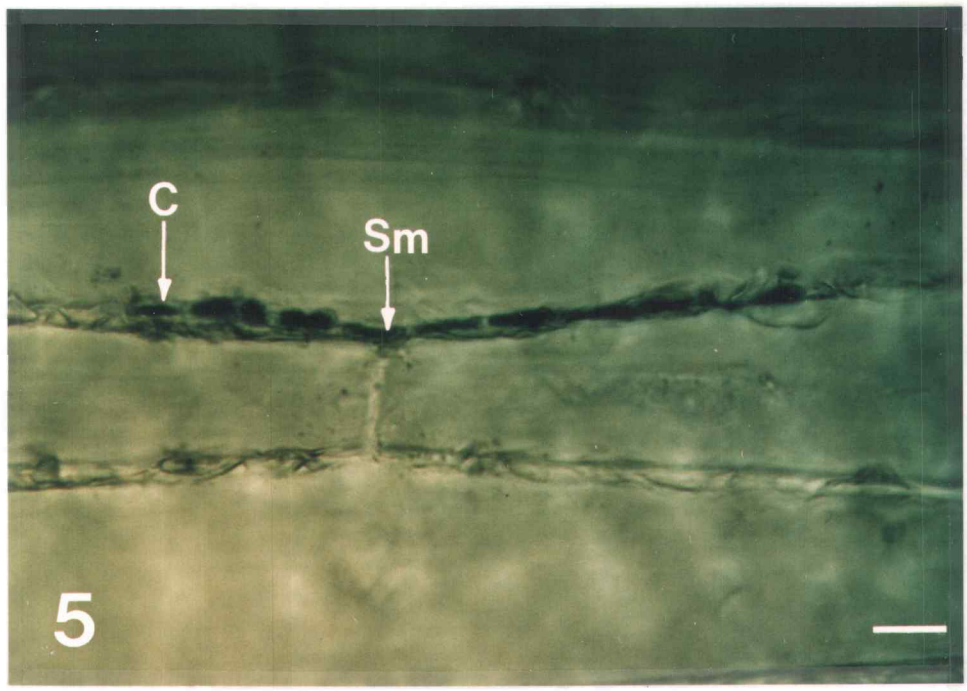

Fig. V.5 Whole leaf mount showing a R. orthosporum conidia with a subcuticular mycelium growing between lateral walls of adjacent epidermal cells 4 days after inoculation. C, conidium; Sm, subcuticular mycelium. Bar= 15 microns.

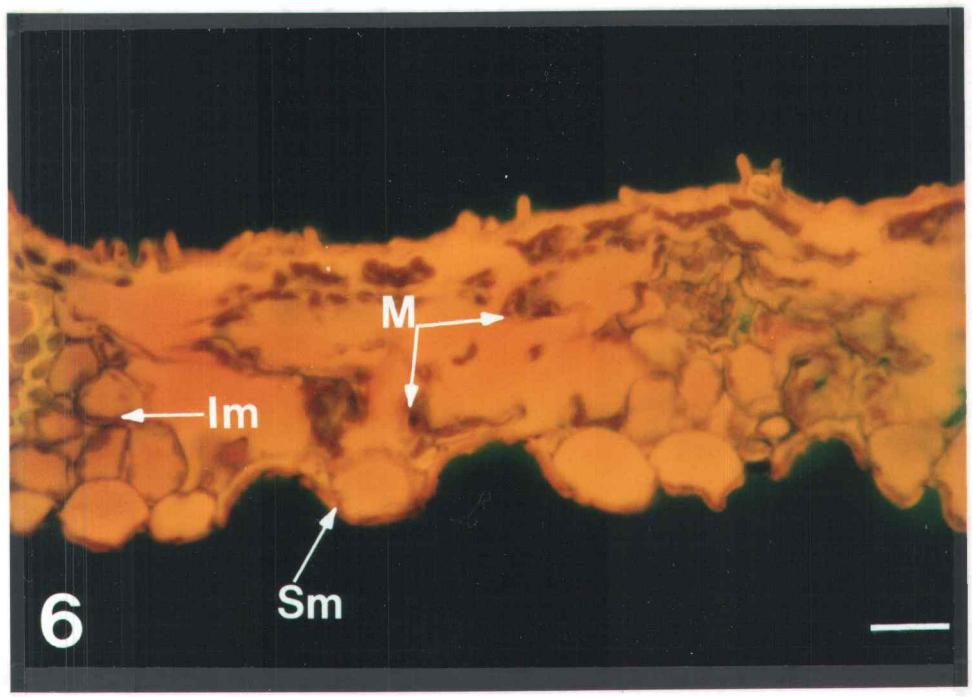

Fig. V.6 Fluorescence microscopy of a transverse section of infected orchardgrass leaf 10 days after inoculation. M, collapsed mesophyll cells; Im, intracellular mycelium; Sm, subcuticular mycelium beneath the cuticle. Bar= 34 microns.

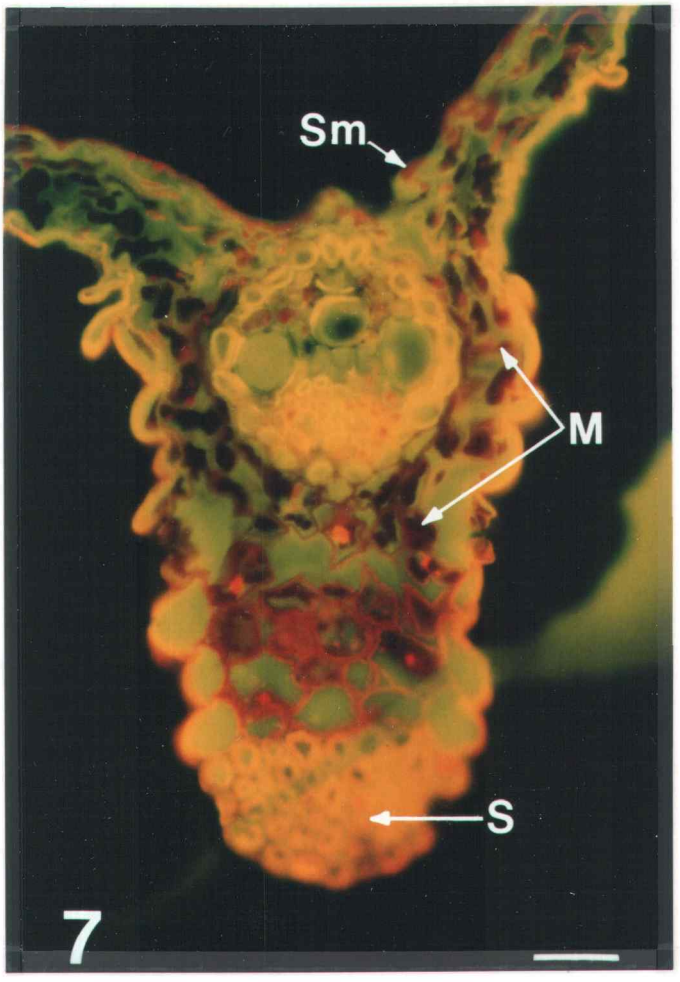

Fig. V.7 Fluorescence microscopy of a transverse section of infected orchardgrass leaf 12 days after inoculation. M, extensive colonization and breakdown of the mesophyll cells; Sm, subcuticular mycelium beneath the cuticle. S, conspicuous sclerenchyma tissue in the apex of the keel. Bar= 34 microns.

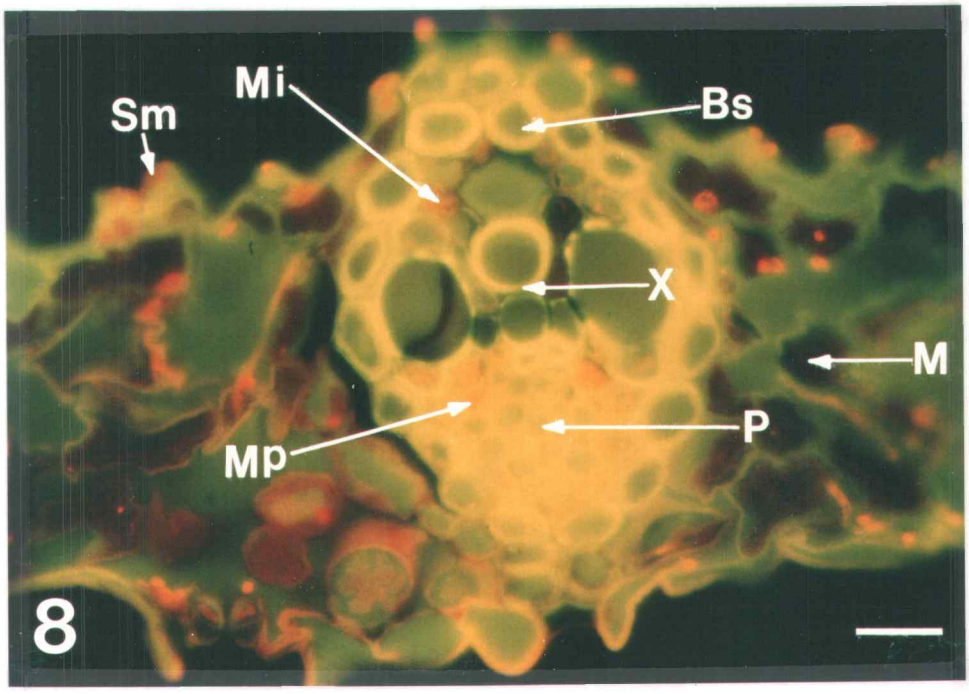

Fig. V.8 Fluorescence microscopy of a transverse section of infected orchardgrass leaf 12 days after inoculation. X, xylem; P, phloem; Bs, a bundle sheath surrounded the vascular bundles; Mp, mycelium showed in the phloem cells; Mi, mycelium showed on inner bundle sheath; Sm, subcuticular mycelium. Bar= 18 microns.

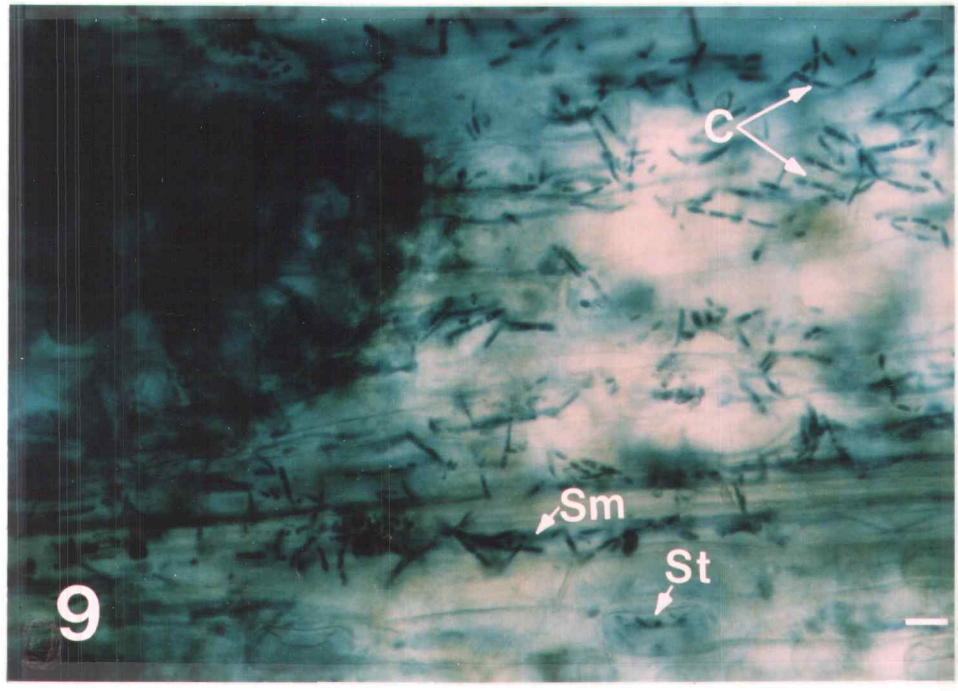

Fig. V.9 Whole leaf mount showing conidia produced above intact leaf surface. C, cylindrical shaped conidia; Sm, subcuticular mycelium; St, stomata with protruding conidia. Bar= 16 microns.

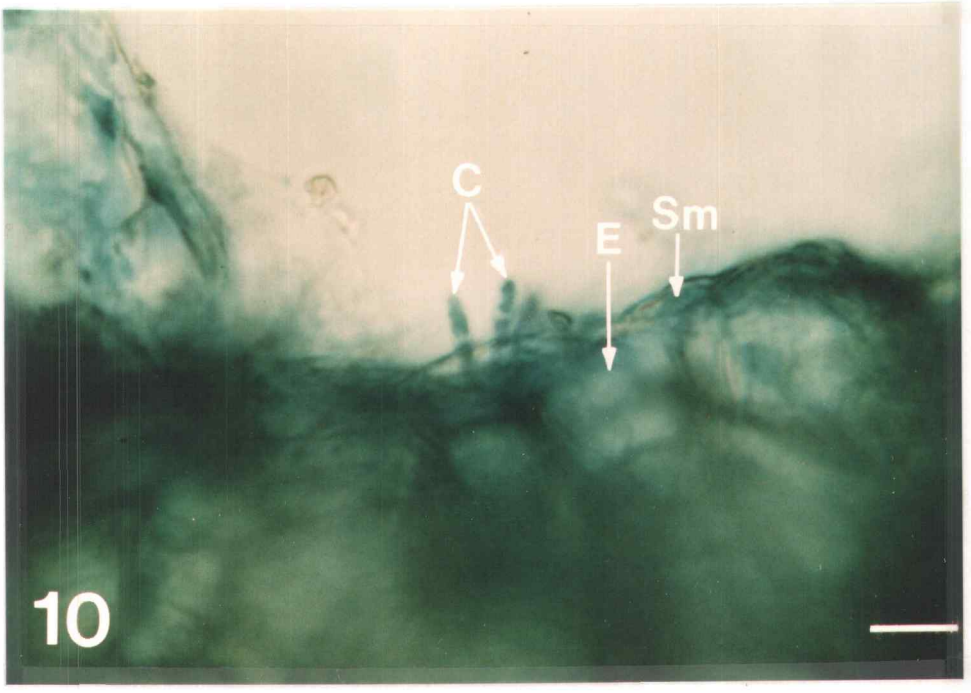

Fig. V.10 Light microscopy of a cross section of a orchardgrass leaf stained with lactophenol aniline blue. C, protruding R. orthosporum conidia; Sm, subcuticular mycelium; E, epidermal cells. Bar= 13 microns.

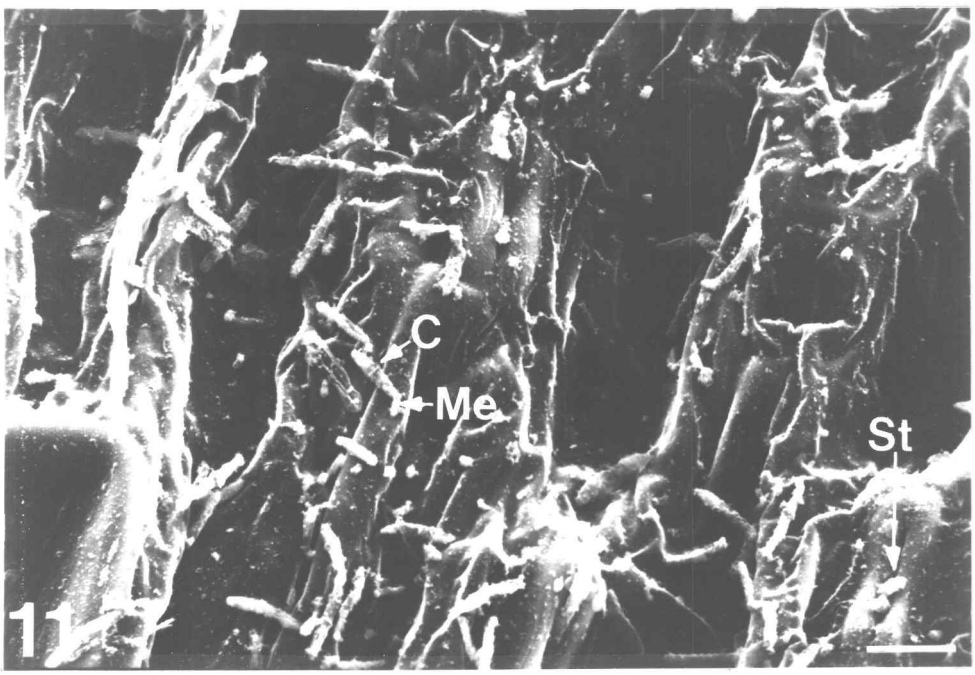

Fig. V.11 Scanning electron microscopy of a natural lesion showing R. orthosporum conidia at different stages of development. C, cylindrical shaped conidia; Me, mycelial extension; St, conidia being extruded through stomata. Bar= 10 microns.

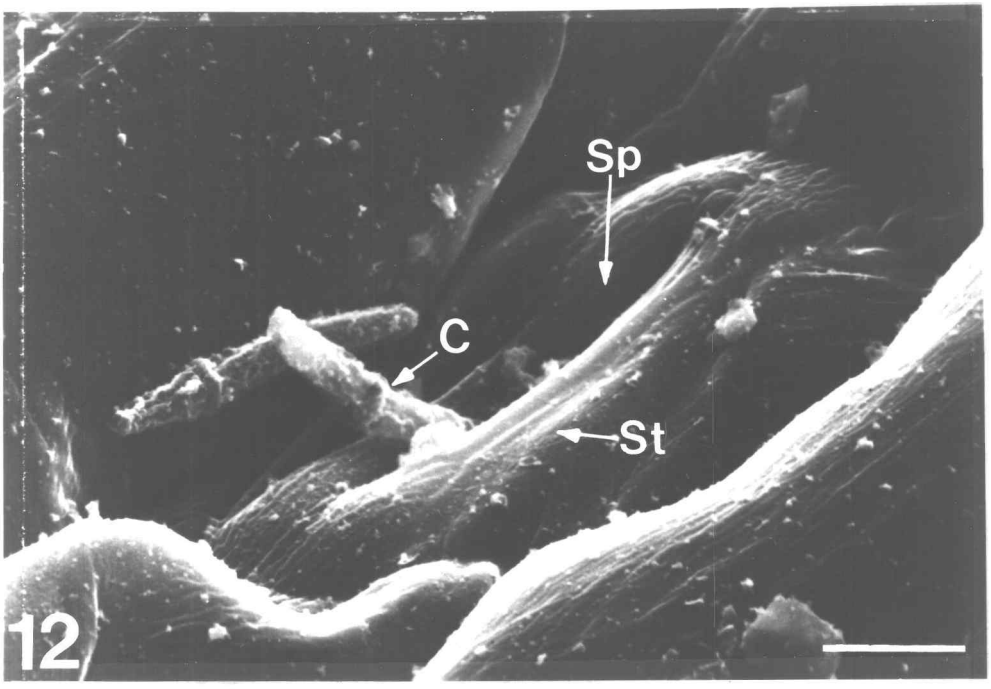

Fig. V.12 Scanning electron microscopy of a natural lesion showing R. orthosporum conidia protruding through stomata. C, conidia; St, stomata; Sp, stomata pore. Bar= 5 microns.

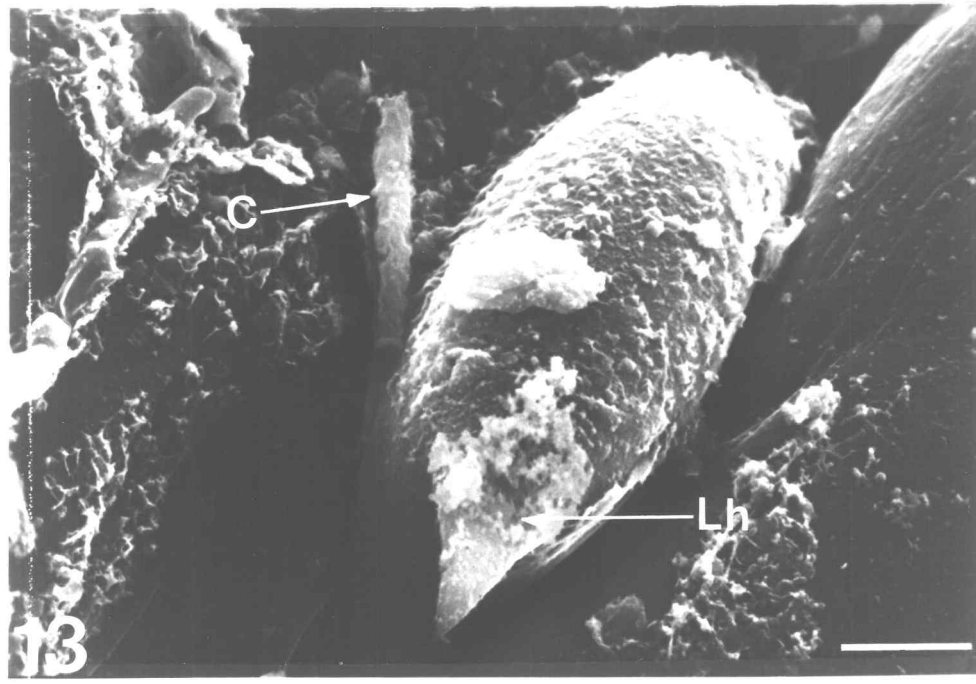

Fig. V.13 Scanning electron microscopy of a natural lesion showing a R. orthosporum conidia protruding through a natural opening close to a leaf hair. C, cylindrical shaped conidia; Lh, leaf hair. Bar= 5 microns.

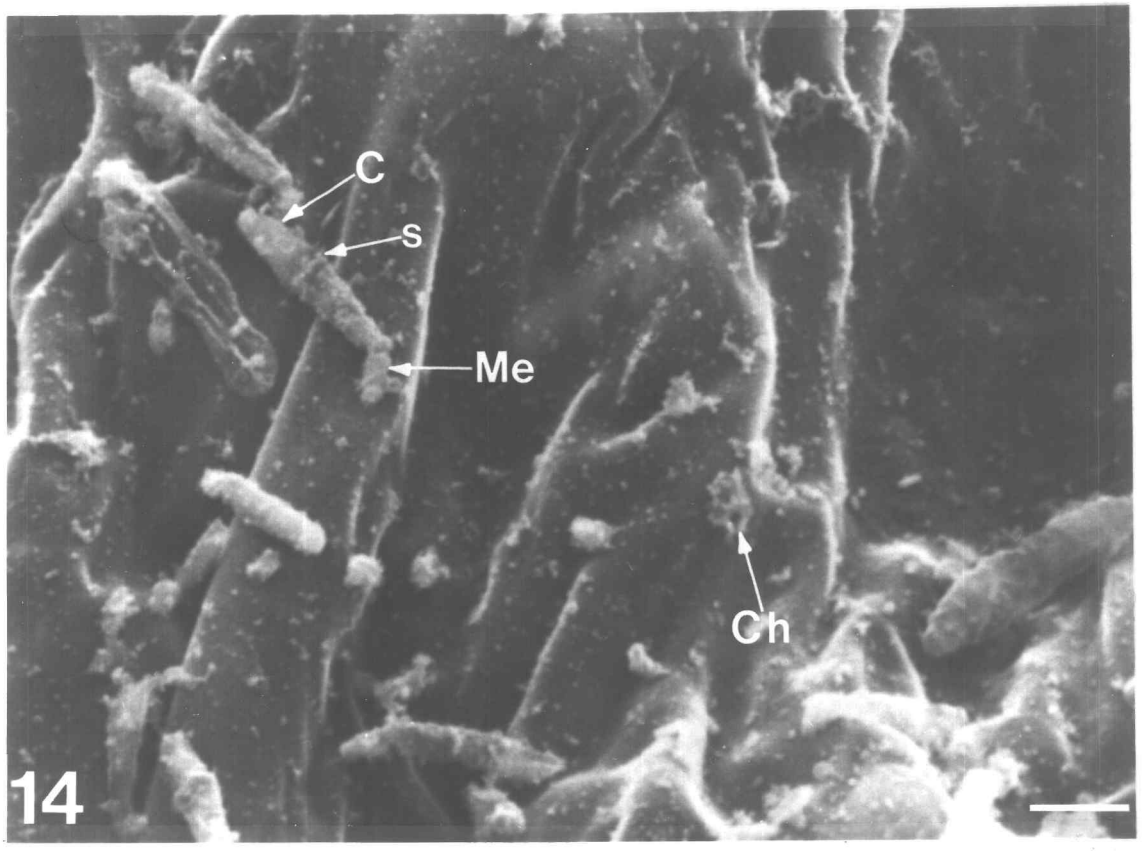

Fig. V.14 Scanning electron microscopy of a natural lesion showing the leaf cuticle with a mass of conidia and detached mycelial extension. C, conidia with a median septum (s); Me, mycelial extension; Ch, cuticular hole. Bar= 5 microns.

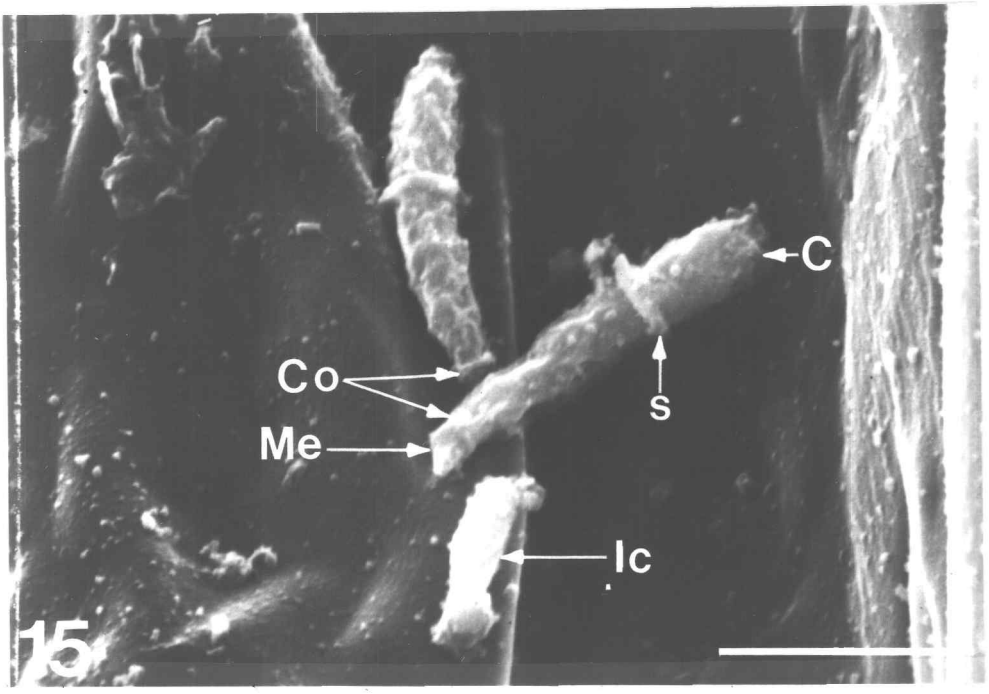

Fig. V.15 Scanning electron microscopy of a natural lesion showing R. orthosporum conidia protruding through a leaf cuticle at two different stages of development. C, cylindrical shaped conidia with a median septum (s); Me, mycelial extension; Co, constrictions; Ic, immature conidia. Bar= 5 microns.

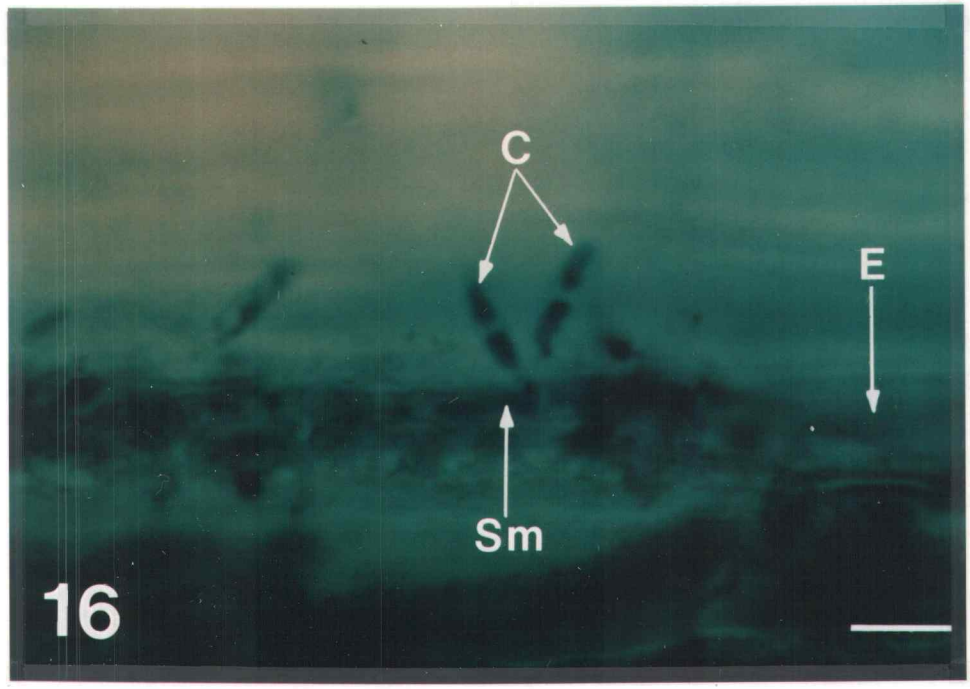

Fig. V.16 Whole leaf mount showing conidia produced from the subcuticular mycelium. C, conidia; Sm, subcuticular mycelium; E, epidermal cells. Bar= 9 microns.

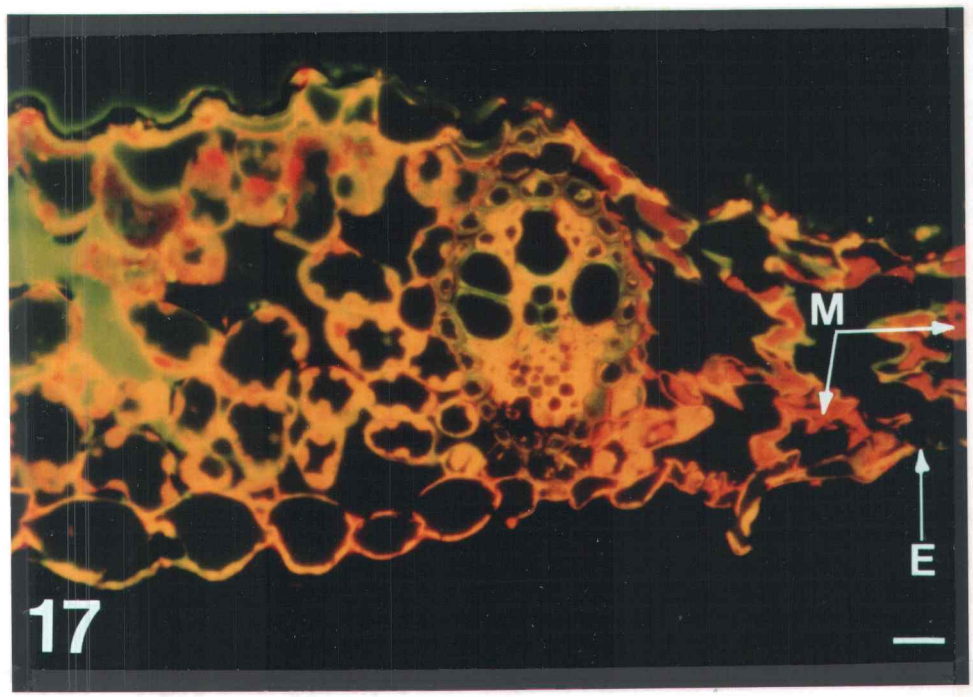

Fig. V.17 Fluorescence microscopy of a transverse section of infected orchardgrass leaf four weeks after inoculation. M, extensive destruction of the mesophyll cells; E, breakdown of the epidermis and cuticle of the leaf. Bar= 16 microns.

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APPENDICES

APPENDIX A: A NUTRIENT SOLUTION FOR TILLER GROWTH

STOCK SOLUTIONS

A. $Ca\ Cl_2 \cdot 2\ H_2O$ 3 g
$Co\ Cl_2 \cdot 6\ H_2O$ 1.0 mg
KI 10.0 mg
add water up to 500 ml.

B. $K\ H_2\ PO_4$ 3 g
$K_2\ H\ PO_4$ 0.5 g
$Na_2\ MO_4 \cdot 2\ H_2O$ 10 mg
add water up to 500 ml.

C. $Mg\ SO_4 \cdot 7\ H_2O$ 3 g
$Cu\ SO_4 \cdot 5\ H_2O$ 1 mg
$Mn\ SO_4 \cdot 4\ H_2O$ 200 mg
$Zn\ SO_4 \cdot 7\ H_2O$ 100 mg
add water up to 500 ml.

D. $H_3\ BO_3$ 100 mg
add water up to 500 ml.

E. $K\ NO_3$ 20 g
$NH_4\ NO_3$ 10 g
add water up to 500 ml.

F. 1. $Fe\ SO_4 \cdot 7\ H_2O$ 557 mg + 20 ml of water
2. Na EDTA 745 mg + 20 ml of water. Heat and filter sterile, mix with 1., over stirrer.
add water up to 100 ml. (store in dark bottle).

Final solution: mix 25 ml of stock solution A, B, C, D, and 5 ml of F. Add water up to 1000 ml.

Appendix Table 1 Analysis of variance of mean disease severity of R. orthosporum on two orchardgrass cultivars in 1988

Source of variation	df	F-value[a] DS f-g	DS f-1	DS f-2
Cultivars	1	240.6**	109.8**	120.1**
Stages of growth	3	7.4**	0.08	4.5**
Cult x stages	3	12.2**	5.8**	4.0*
Inoculation	1	231.3**	207.7**	198.8**
Cult x inoc	1	64.0**	18.7**	0.1
Stages x inoc	3	6.6**	1.4	3.5*
Cult x stag x inoc	3	4.7**	1.6	1.9
Error	48			

*= Significant at the 0.05 level.
**= Significant at the 0.01 level.
[a] F-value for disease severity on flag leaf, first and second leaves below flag leaf, respectively.

Appendix Table 2 Analysis of variance of mean disease severity of R. orthosporum on two orchardgrass cultivars in 1989

Source of variation	df	F-value[a] DS f-g	DS f-1	DS f-2
Cultivars	1	205.3**	123.2**	151.7**
Stages of growth	3	6.6**	0.8	5.6**
Cult x stages	3	19.6**	5.0**	3.8**
Inoculation	1	265.1**	284.8**	205.8**
Cult x inoc	1	101.6**	50.9**	1.3
Stages x inoc	3	11.1**	2.8	4.0*
Cult x stag x inoc	3	14.1**	1.6	3.2*
Error	48			

*= Significant at the 0.05 level.
**= Significant at the 0.01 level.
[a] F-value for disease severity on flag leaf, first and second leaves below flag leaf, respectively.

Appendix Table 3 Analysis of variance of total mean disease severity of leaf scald, total seed weight, and 1000-seed weight for two orchardgrass cultivars

Source of variation	df	F-value[a] DS mean	Seed/w	1000/w
Cultivars	1	475.92**	1.66	1.86
Inoculation	1	739.76**	6.03*	0.01
Cult x Inoc	1	59.43**	1.75	0.01
Year	1	1.67	629.34**	0.00
Cult x year	1	12.57**	1.13	7.92**
Inoc x year	1	25.67**	7.69**	0.46
Cult x Inoc x year	1	6.79**	1.97	1.27
Stages	3	0.50	2.30	6.77**
Cult x stages	3	15.84**	4.11*	3.24*
Inoc x stages	3	5.64**	1.05	3.76*
Cult x inoc x stag	3	8.31**	3.25*	1.51
year x stages	3	0.30	1.73	5.60**
Cult x year x stag	3	0.80	2.88	3.93*
Inoc x year x stag	3	0.78	1.01	0.40
C x I x Y x S	3	0.95	1.77	1.38
Error	96			

*= Significant at the 0.05 level.
**= Significant at the 0.01 level.
[a] F-value for total disease severity, total seed weight and thousand-seed weight.