ETIOLOGY AND MANAGEMENT

OF SPINACH WHITE RUST

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INTRODUCTION

Texas, Oklahoma and Arkansas lead the nation in spinach (*Spinacea oleraceae* L) produced for the processing market with more than 16,000 acres planted each year [5, 18, 32]. White rust, caused by *Albugo occidentalis* Wilson is an important disease in all U.S. spinach production areas east of the Rocky Mountains [14]. It is the major foliar disease of spinach in Oklahoma and is also considered a primary problem in the spinach production areas of Texas and Arkansas where it can occur in epidemic proportions [24, 44]. White rust can cause substantial yield losses through a reduction in quality of both fresh and processed spinach. White rust has not been reported in western production areas or outside the United States [14].

Spinach, native to Central Asia (most probably Iran), is a member of the Chenopodiaceae and is related to Swiss chard, sugar beet, table beet, lambs quarter, and saltbrush. The earliest record of cultivation is from China in 647 A.D. [40]. The spinach plant produces a compact rosette of leaves, which may be smooth (flat leaf) or crinkled (savoy), and bolts (produces a seed stalk) in response to warm temperatures and long photoperiods. It is dioecious, producing male and female flowers on different plants. However some plants may be monoecious with varying proportions of male and female flowers on the same plant. Male plants bolt and flower earlier than female plants, and die soon after flowering. When the plant forms a seed stalk it is considered unmarketable [40].

Spinach is typically a fall, winter, or spring crop. Only in California is spinach produced year-round. Most commercial spinach is direct seeded with production

strategies dictated by the market destination of the commodity. Most fresh-market spinach is hand harvested while spinach for processing is mechanically harvested [14].

Federal standards have defined low tolerances for weeds, grass, insects, spray residues, disease, and other contaminants in processed spinach, along with strict regulation on chemicals used for pest control. Therefore, crop management strategies aimed at maintaining an economically viable productivity of high-quality spinach while reducing sources of environmental contamination are needed [9, 35].

History of the disease:

White rust first became known as a destructive disease of spinach in March 1937, when it was found in loads of spinach received at the market in New York, N.Y., from the Winter Garden region of Texas[1]. One load had as much as 75% of the plants damaged by white rust [48]. In the 1937-1938 growing season, white rust was found in every field examined in the Winter Garden region where, in many fields, 100% of the plants were infected. In a few cases, damage was so extensive that the entire fields were not harvested [49]. The first appearance of the disease in the Lower Rio Grande Valley and the Costal Bend area of Texas came in 1941[29].

The first report of spinach white rust in Oklahoma was near Muskogee in 1943. Though this is the first record of the disease in the state, a shipper reported having seen small amounts in previous years [13]. In 1943 and 1944 the disease was observed in Wagoner, Sequayah and Leflore counties of Oklahoma [3]. In addition, the disease was reported for the first time in the areas around Laredo, Dallas and Temple, Texas [7, 8].

[1] Dimmit, Maverick, Uvalde, Zavala, and Frio counties comprise the Winter Garden region of Texas.

White rust was first reported on spinach in Arkansas near Van Buren in the fall of 1945 and near Alma in 1946 [6, 50]. Over a period of nine years white rust had spread to all the major spinach production areas in Texas, Oklahoma and Arkansas. In 1970, white rust was considered to be the foremost spinach disease problem in South Texas [44]. Today, white rust remains one of the most economically important diseases of spinach. With favorable environmental conditions, it can rapidly spread and cause dramatic decreases in the marketability of spinach.

Causal Organism:

Albugo occidentalis, a member of the Peronosporales, is an obligate fungal pathogen, with its economic host range confined to spinach [39]. It was first reported on *Chenopodium capitatum* (L.), a wild relative of spinach, in Colorado in 1901. Reports of *A. occidentalis* infecting other members of the Chenopodiaceae, including *Beta vulgaris* (L.) (sugar beet, table beet, and swiss chard) have been refuted [37, 49].

Though the biology of *A. occidentalis* is not completely understood, it is thought to resemble that of *Albugo candida* (Pers.) Kunze, the more thoroughly studied white rust pathogen of crucifers [14]. *A. occidentalis* produces both sexual (oospores) and asexual (sporangia) reproductive structures. While factors effecting disease development through sporangial-initiated infections have been well documented [18, 39, 42], no reports of oospore germination or oospore-derived infection have been reported [14].

The fungus obtains its nourishment by producing an intracellular haustorium that penetrates the host cells. Hyphae are most abundant in the large intercellular spaces of the spongy parenchyma where they give rise to sprangiophores that produce large numbers of basipetally formed sporangia. As sporangia are formed, they raise the

epidermis to form a sorus (pustule), the epidermis ruptures, releasing sporangia that are disseminated by air currents. With favorable environmental conditions they germinate to reinitiate the disease [2, 37]. At the time of release, sporangia are dry, discoid and measure approximately 10 x 14 μm. When hydrated the sporangia become spherical to ellipsoid and measure 10-19 x 20-22 μm. [37].

In germination, the sporangial wall swells, weakens and forms a papilla through which the cytoplasm of the sporangia is released into a thin-walled vesicle. The cytoplasm oscillates for a short time and then differentiates into zoospores. The zoospores are reniform, move by means of two flagella, and measure about 7 x 10 x 5 μm. After swimming a short time, they encyst and germinate to produce a germ tube. This germ tube can enter a stoma and incite infection. Rarely, the sporangia will germinate directly to produce a germ tube [38].

A. occidentalis also produces a sexual state that results in the formation of oospores. Sexual organs, the male antheridia and the female oogonia, arise from swellings in the ends of hyphae. The antherdia are elongated and measure about 20 x 50 μm while the oogonia are spherical and measure about 70 μm [37]. Fertilization apparently follows zonation in the oogonium although many oospores are reported to develop parthenogenetically [38]. Oospores, which serve as resting spores, are spherical, brown, finely reticulate, and measure 44-62 μm [37]. There are no descriptions of oospore germination for *A. occidentalis* [14].

Symptoms:

Initially, small chlorotic lesions develop on the leaf surface. As symptoms progress, small glassy white pustules are produced which frequently appear in concentric

rings, on the underside of the leaf and occasionally on the upper leaf surface. The pustules are blister-like and may be oval, irregularly oval, or elongated in shape, ranging in size from 0.5-2 mm in diameter and up to 4 mm in length. Lesions often coalesce and the pustules can cover the entire lower surface of the leaf. Although the pustules are usually formed on the lower leaf surface, they are occasionally produced on the upper leaf surface, petioles, and on branches and fruit coats during advanced stages of the disease [14, 39].

Higher temperatures favor production of oospores over the production of sporangia [14]. Oospores are formed in leaves, petioles, main stems, side branches, and fruit coats, often in such numbers that infected tissues are nearly black [37].

White rust often becomes systemic in plants that have bolted to seed, but rarely in vegetative plants. When systemic, sporangia and oospores are produced in all infected parts of the plant. A slight twisting of the stem and leaves may occur, but there is little or no hypertrophy or hyperplasia as in white rust of the Cruciferae caused by *A. candida* [39, 48]. Frequently the infected areas of the leaves become necrotic and when infection is severe, whole leaves are killed. Severely affected fields may appear brownish due to dead leaves [39].

Epidemiology:

Primary infections are found on lower leaves in direct contact with, or close proximity to the soil surface. Oospores that formed on previous spinach crops are considered to be the primary inoculum [44, 14], as no known alternate host for the pathogen has been found in Texas, Oklahoma, or Arkansas. Continuous cropping has led to the deposit of large numbers of oospores in commercial fields [44]. Although the

survival ability of oospores of *A. occidentalis* in the soil is not known, oospores of *A. candida* have been shown to remain viable in laboratory storage for up to 17 years [36].

Sporangia are responsible for the polycyclic nature of the disease and environmental factors affecting sporangial germination and infection are important for increase of the disease. Raabe and Pound [39] found that several environmental factors affect germination of sporangia of *A. occidentalis*. Free moisture is required for sporangial germination, but temperature is also an important variable [39, 42]. In germination studies, Raabe and Pound [39] found that temperature requirements for germination range from 2 to 24ºC with 12 to16ºC being optimal for percent as well as rate of germination. When sporangia were chilled at 12ºC for 1.5 hours germination percentages were about the same at all temperatures [37].

In plant inoculation studies Sullivan [42] showed that infection occurred and disease developed at a temperature range of 12 to 22 ºC and wetness (RH ≥ 95%) periods as brief as 3 hours. A maximum of 90% disease severity was reached within the optimum temperature range of 12 to 18ºC with an 84-hour wetness period. However, 75% disease severity was reached after a 12-hour wetness period at 12 to 18ºC. At less than optimum temperatures, longer wetness periods up to 84-hours were necessary to achieve infection. Disease severity decreased when the temperature was above or below optimum range and wetness periods were less than12 hours [42].

Raabe and Pound [39] showed that sporangial germination varied with pustule development. In germination tests, sporangia collected from pustules in very early development, failed to germinate. Sporangia collected from young, unopened pustules and pustules that had just opened showed 3.2% and 16% germination, respectively. A

sample of sporangia that had fallen from the leaf within a period of 24 hours had 24.6%, germination and in inoculation studies, produced more infections than sporangia collected from pustules at any other stage of development. Sporangia that had fallen from the leaf over a period of one week failed to germinate. The researchers concluded that either maturity or moisture content of the sporangia was responsible for the increase in germination with age of the pustule [37, 39].

In an attempt to separate the effect of sporangial maturation from the effect of moisture content of the sporangia, Raab and Pound selected leaves with a large number of pustules just beginning to open. Leaves were allowed to dry on the laboratory bench, and at timed intervals, were reweighed and the sporangia collected. As a control, sporangia were collected from leaves that were intact on the same plants and from pustules in the same apparent stage of development. The researchers concluded that with an increase in water loss from the leaves, there was an increase in the germination percentage up to a certain point, after which the germination dropped considerably and in some experiments stopped [37]. Evaluating the germination of sporangia from wilted leaves, Raab and Pound demonstrated that the loss of water from the leaves affects germination. Sporangia collected from plants that had not been watered and were wilted germinated much better than sporangia collected from turgid plants [39].

Disease Management:

Because *A. occidentalis* is an obligate parasite, in-vitro studies are difficult, and little is known about its sexual cycle or the biology of the oospores which are assumed to be the cause of primary infections. Without an understanding of oospore germination,

viability, and survival, the principles of integrated management cannot be fully utilized. Biological control methods aimed at reducing oospore survival in soil cannot be developed, primary inoculum levels cannot be assessed, and effective crop rotation intervals cannot be defined. Therefore, chemical control, alone or in combination with host plant resistance has been the foundation of white rust management [12, 15, 18].

Resistance:

A cooperative effort between the U.S. Department of Agriculture and Texas A&M University to develop spinach cultivars with resistance to white rust was initiated in 1960. The partially resistant cultivars Wintergarden, Jewel, and Crystal were released in 1975 as a result of this breeding program. A significant reduction in white rust severity occurred on these cultivars compared to susceptible cultivars. In 1972, a breeding program was initiated at the University of Arkansas [11]. Using a field selection process to further develop field, or horizontal resistance to white rust, several breeding lines were developed. The cultivars, Fall Green, Ozarka, Greenvalley, Wintergreen, and F 380 have been released which have varying levels of horizontal resistance. Fall Green and F 380 have the highest levels of white rust resistance among commercial cultivars [10, 30, 31].

Several genes that influence a range of physiological processes in the plant control horizontal resistance. Therefore, horizontal resistance is durable and effective against all races of an individual pathogen. However, horizontal resistance does not provide complete protection from infection, but rather slows the rate of disease increase. Therefore, resistant cultivars can become severely diseased under favorable environmental conditions and high inoculum pressure [14]. In addition, effective levels

of resistance are lacking for long-standing cultivars useful for production in the overwinter and spring production seasons. Many resistant cultivars are open-pollinated and do not yield as well as hybrids [16, 42].

Chemical Control:

Protective fungicides such as the ethylene bisdithiocarbamates (EDBCs), maneb, zineb, and mancozeb were registered for use on spinach in 1955. These fungicides provided good control of white rust when applied on a 7-day schedule [34]. However, the Environmental Protection Agency (EPA) issued a Rebuttal Presumption Against Registration (RPAR) for EDBCs in 1977 [34]. A residue tolerance of 10 ppm was issued in the U. S. in 1982. In 1980, Canadian markets restricted residues of EDBCs allowed on imported spinach exceeding 0.1 ppm. Canada consumed about 50% of the fresh market spinach and a significant proportion of the processing spinach produced in the Winter Garden region of Texas [34]. Thus, EDBCs were eliminated from many spinach disease control programs. In 1992 the EPA revoked the registration of the EDBCs on spinach and other some vegetable crops in the U.S.

Copper compounds (copper sulfate and copper hydroxide); metalaxyl; a pre-mix of metalaxyl and copper sulfate; and fosetyl-aluminum became the only fungicides registered for control of white rust [42]. Copper compounds are phytotoxic, and injury caused by them may be as damaging as foliar disease. In Texas, soil applications of metalaxyl are effective when used in-furrow, as a seed treatment, and in combination with foliar applications of metalaxyl and copper sulfate [17, 19]. In Oklahoma, soil applications of metalaxyl and foliar applications of metalaxyl and copper sulfate have

been ineffective [20, 22]. However, soil applications of metalaxyl followed by foliar applications of metalaxyl and copper sulfate in alternation with copper hydroxide significantly reduced disease incidence and severity [20, 22, 23]. Metalaxyl and metalaxyl with copper sulfate are expensive treatments. Fosetyl-aluminum has shown to reduce disease incidence and severity with minimum phytotoxic effects [20, 27].

The fungicides azoxystrobin and pyraclostrobin are synthetic analogs of a naturally occurring compound, called strobilurin, produced by *Strobilurus tenacellus*, an inconspicuous woodland basiciomycete [4, 51]. Azoxystrobin was recently approved for use on spinach and has been shown to provide a high level of white rust control. Over six trials, weekly applications of azoxystrobin resulted in an average of only 0.2% disease severity with no with phytotoxic effects [21, 22, 25-28]. Pyraclostrobin, an experimental fungicide not yet registered for use on spinach, has been evaluated for spinach white rust control. In field trials under moderate to severe disease pressure, pyraclostrobin provided almost complete disease control [22, 23].

Advisory System:

Calendar-based fungicide programs are normally initiated at the first true leaf stage and continue on 7-day intervals until just before harvest. Spring-planted spinach may require up to six applications, while in fall-planted, over-wintered spinach in the Winter Garden region of Texas, up to thirteen applications may be necessary [18, 42]. Because weekly spray programs can lead to a considerable increase in the costs of spinach production, weather-based spray advisories have been developed that permit the timing of fungicide sprays to coincide with weather conditions favorable for infection.

Applications are not made during unfavorable periods thus reducing cost and potential environmental impacts of fungicide programs.

Dainello [18] demonstrated that a reduction of 25 to 39% in the number of fungicide applications compared to a calendar schedule could be achieved without impacting white rust control by timing the applications to follow 12 continuous hours of leaf wetness. Sullivan [43] developed a similar weather-based advisory program based on the observed disease response to temperature (T) and wetness duration, the period of relative humidity $\geq$ 95%, (W). Beginning when the first true leaves were fully expanded, wetness durations were weighted for temperature (T*W), accumulated over time, and sprays were applied when T*W values ranged from 3 to 36 hrs. Advisory programs using effective T*W thresholds of 3, 6, and 12 hr resulted in a reduction in the number applications by 1.4, 2.7, and 3, respectively, compared to 6 sprays per season for the 7-day program, while providing similar disease control [43].

Determining the most appropriate time to initiate a fungicide program may provide further improvement in the efficiency of fungicide usage for white rust. Raabe [37] found young leaves to be very resistant to localized infection. However, when plants became systemically infected, even the youngest leaves showed symptoms. Thus, the resistance of young leaves appears not to be against fungal growth, but rather to penetration of the leaf by the fungus. This might be attributed to the stomata remaining closed until the leaves are fully developed [37]. Because white rust has often been observed to develop to severe levels late in the season, it may be possible to omit one or more fungicide applications early in the season without sacrificing disease control.

Advisory systems are limited in that infection can take place within the first 12 hours of favorable conditions and fungicide applications may be delayed by the weather conditions that triggered the advisory system. Therefore, systemic fungicides that have postinfection activity and can provide disease control when applied to plants that have already been infected are valuable to an advisory-based, disease control program. The postinfection activity of azoxystrobin and pyraclostrobin against spinach white rust has been documented [42]. For azoxystrobin, no disease developed when the fungicide was applied within one day after inoculation and continued to show postinfection up to three days after inoculation. Pyraclostrobin showed postinfection activity at least four days after inoculation and provided complete disease control up to three days after inoculation.

While white rust is considered the most economically important foliar disease of spinach in Oklahoma, the crop can be attacked by a number of foliar diseases that can reduce quality and marketability [5]. Other major foliar diseases include blue mold (*Peronospora effusa*), Cercospora leaf spot (*Cercospora beticola*), anthracnose (*Colletotrichum spinacicola* and *C. spinaciae*) and Cladosporium leaf spot (*Cladosporium macrocarpum*). Symptoms of Cladosporium leaf spot have been observed on vegetative spinach under very wet conditions and can be very damaging to foliage of mature plants grown for seed production [14]. Cercospora leaf spot is one of the most serious and widely distributed foliar diseased of sugar beet worldwide [41] and is considered an economically important disease of spinach [14]. Favorable environmental conditions for infection of Cercospora leaf spot of sugar beet have been determined [46, 47]. Temperatures between 24 and 29º C with relative humidity >90% are optimum for infection. Infection increased sharply when inoculated plants were

exposed to these conditions for 48 hours or more. In Texas, Cercospora leaf spot affects approximately 50% of the spinach acreage and a 5% infestation can eliminate the first spinach cutting in fall and early winter fresh market fields [33]. The leaf spot disease causes lesions (3 to 5 mm in size) on older spinach leaves. During periods of warm temperatures and high humidity or leaf wetness, tan necrotic spots on lower leaves will turn gray and lower the quality of leaves or make them unmarketable.

It is known that most fungal pathogens need periods of free moisture to germinate, infect, and cause disease. Therefore, the white rust advisory may be effective in predicting outbreaks of foliar diseases other than white rust. By quantifying the postinfection activity and efficacy of various fungicides against the range of foliar diseases of spinach, producers can make better decisions when choosing fungicides.

Advisory systems have been shown to accurately predict the increase of white rust due to favorable conditions for sporangial germination and infection. However, the type and source of primary inoculum for disease onset is still unclear. It has been speculated that the primary inoculum for the spinach white rust disease are the oospores from previous spinach crops that survive in the soil to re-initiate the disease in the next cropping season [14, 44]. Oospores are known to be the primary inoculum for diseases caused by other members of the Peronosporales. In downy mildew of grape caused by *Plasmopara viticola*, the oospores survive in dead leaf lesions and shoots [1]. The oospores germinate during rainy periods in the spring and produce sporangia and zoospores that are disseminated by wind or water to wet leaves, which they infect through the stomata. Oospores of *Albugo candida* the causal agent for white rust of Cruciferae, have been shown to germinate after washing on a rotary shaker or allowing

water to slowly drip onto a filter where oospores have been distributed [45]. This suggests that germination in nature is influenced by the leaching action from melting snow or rain showers. Determining the conditions for germination of *A. occidentalis* oospores could be beneficial in better predicating the onset of spinach white rust.

Three chapters of this thesis are written in journal manuscript format. Chapter II, entitled "Evaluation of Fungicides and Timing the Initiation of Spray Programs for Control of Spinach White Rust" describes the effects of various fungicides and spray program initiation dates for control spinach white rust and Cladosporium leaf spot. Chapter III, entitled "Biology of Oospores of *Albugo occidentalis*" describes the role of oospores in the initiation of the spinach white rust disease. Chapter IV, entitled "Post Inoculation Activity of Fungicides Against Cercospora Leaf Spot of Spinach" describes studies under controlled conditions where the activity of these fungicides was evaluated at various periods after inoculation.

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CHAPTER II

Evaluation of Fungicides and Timing the Initiation of Spray Programs for Control of Spinach White Rust

ABSTRACT

The effects of various initiation dates for a weather-based advisory program and a 7-day calendar program using different fungicides were evaluated for control of spinach white rust caused by *Albugo occidentalis* in four field trials. The advisory program accumulated periods of temperature and wetness ((RH ≥ 90%)T*W hours) favorable for white rust development. The calendar program and the accumulation of T*W hours were initiated at the first true leaf stage (early), 7 days after the first true leaf stage (middle), and 14 days after the first true leaf stage (late). The fungicides zoxamide, pyraclostrobin, azoxystrobin, and azoxystrobin alternated with zoxamide were used in trials 1, 2, and 4; and azoxystrobin alternated with fosetyl-aluminum was used in trial 3. In trials 1-3 levels of white rust were severe in the untreated controls. Disease incidence (percentage of leaves with symptoms) averaged over 60% and disease severity (percentage of leaf area with symptoms) was over 20%. All fungicides reduced white rust levels in trials 1, 2, and 3 compared to the untreated control. Across spray programs and initiation dates, pyraclostrobin had significantly lower levels of white rust with 5.1% disease incidence and 0.4% disease severity compared to 18.9% disease incidence and 2.6% disease severity averaged over the three other fungicides in trials 1 and 2. Across spray programs and initiation dates, azoxystrobin alternated with fosetyl-aluminum provided a high level of disease control with 10.6% disease incidence and 0.6% disease severity in trial 3. Within early, middle, and late initiation dates, the number of sprays was reduced by 2-3

per season for the advisory program compared to the calendar program. In trials 1 and 2, the early and middle calendar programs had lower disease incidence and severity compared to the late calendar program, and all of the advisory initiation dates which had similar levels of white rust. However, the late initiation dates had higher levels of disease. In trial 3 there were no differences in disease incidence between initiation dates of the spray programs, but disease severity was higher for the late than for the early initiation date. Overall, disease incidence and severity were higher for the advisory program compared to the calendar program in all 3 trials. While statistically significant, differences in disease severity were small and may not be economically important. White rust was not observed in trial 4. However, Cladosporium leaf spot developed and the efficacy of spray programs, initiation dates, and fungicides were evaluated. Disease incidence for the untreated control was severe (62%) while disease severity was low (2%). All fungicides, except zoxamide alone, reduced the incidence of Cladosporium leaf spot to 17-23%. There were no differences in the incidence Cladosporium leaf spot among early, middle, or late initiation dates. However, the calendar programs provided significantly lower levels of disease incidence compared to the advisory programs. Results indicated that delaying fungicide applications through 7 days after the first true leaf stage consistently provided disease control similar to a full-season program.

INTRODUCTION

White rust, caused by *Albugo occidentalis* Wilson, is an economically important foliar disease in all spinach production areas of the United States east of the Rocky Mountains [6]. Symptoms begin as small chlorotic lesions on the upper leaf surface.

Sporangia develop as small, glassy, white pustules (sori) in the chlorotic areas on the underside of the leaves and occasionally on the upper leaf surface. Lesions often coalesce, and the pustules can cover the entire leaf surface. Dark oospores may be produced as the lesions mature, giving the leaf a grainy appearance prior to necrosis [6, 23, 24].

Many factors are involved in determining economic thresholds for disease levels in spinach produced for the processing market. In general, processors follow federal guidelines developed by the United States Department of Agriculture that establish three grades for processing spinach; U.S Grade A or U.S. Fancy, U.S. Grade B or U.S. Extra Standard, and Substandard [2]. Grades are based on requirements for product characteristics with respect to quality factors such as flavor and odor, color, stem material, damage, and harmless extraneous material [2]. Processors in the Arkansas River Valley region report that older necrotic lesions and pustules that develop on the upper and lower leaf surface have a more adverse affect on quality than younger, chlorotic lesions that develop on only one side of the leaf surface. While damage is the main concern in quality reduction due to white rust, other factors such as insect feeding, mechanical damage, and foliar diseases other than white rust can also cause damage to the product. Producers must minimize the effect of these factors in order to produce a product that will satisfy the quality demands of the market

Depending on market processing demands, U.S. Grade A quality spinach may be valued at $140.00 per ton, with a 40% reduction for U.S. Grade B, and substandard grades may be rejected. Losses due to a reduction in the quality and marketability of fresh and processing spinach can be 30 to 100% [3, 8].

Management of spinach white rust has included integrated practices, such as crop rotation, the use of partially resistant cultivars, and the use of fungicides [3]. Partially resistant cultivars can provide acceptable levels of disease control when disease incidence is low [7]. However, this resistance can be overcome under favorable environmental conditions [6, 7]. Resistance is lacking in long-standing spinach cultivars useful for the overwinter and spring production seasons. As a result, fungicides are an important component of spinach white rust management.

Historically, chemical control of white rust was based on preventive programs with the EDBCs maneb and zineb. However, their registration was revoked in 1992 [4, 8, 21]. Currently, only copper-based fungicides, metalaxyl, fosetyl-Al, and azoxystrobin are registered for use on spinach. Azoxystrobin is the primary fungicide used by growers in Oklahoma for control of spinach white rust. The current cost of $49.72 per hectare per application [27] and concerns about the development of resistance to the strobulurin fungicides have increased the need for judicious application timings. Azoxystrobin has been shown to provide almost complete control of spinach white rust with an average of 0.2% disease severity over six trials with no phytotoxic effects [9, 13, 14, 16-18]. In artificial inoculations, Sullivan [25] demonstrated complete white rust control when azoxystrobin was applied one day after inoculation and continued post-infection activity when applied up to three days after inoculation. Efforts to delay the development of resistance to strobilurin fungicides have lead to label restrictions that require no more than two sequential applications of azoxystrobin before alternating to a non-strobilurin fungicide and no more than 6 applications per crop per year.

Dainello [8] demonstrated that a reduction in fungicide applications compared to a calendar schedule could be achieved without impacting white rust control by spraying immediately following 12 continuous hours of leaf wetness. Sullivan [26] developed a similar weather-based advisory program based on the observed disease response to temperature (T) and wetness duration (relative humidity ≥ 95%, (W)). Beginning when the first true leaves were fully expanded, wetness durations were weighted for temperature (T*W), accumulated over time, and sprays were applied when T*W values ranged from 3 to 36 hrs. T*W programs of 3, 6, and 12 hr reduced the number of sprays by 1.4, 2.7, and 3 sprays per season, respectively, while providing disease control that was similar to the 7-day calendar schedule.

Determining the most appropriate time to initiate a weather-based spray advisory program may provide further improvement in the efficiency of fungicide programs for white rust. Raabe [23] found young leaves to be very resistant to infection. However, when plants became systemically infected, even the youngest leaves showed symptoms. Thus, the resistance of young leaves appears not to be against fungal growth, but rather to penetration of the leaf by the fungus. This might be attributed to the stomata remaining closed until the leaves are fully developed [23]. Because white rust has often been observed to develop to severe levels late in the season, it may be possible to omit one or more fungicide applications early in the season without sacrificing disease control. The objectives of this study were to determine the optimum time to initiate fungicide programs for control of spinach white rust and to evaluate the efficacy of various fungicides within a calendar-based and a weather-based spray program.

MATERIALS AND METHODS

The effects of fungicides, spray programs, and timing the initiation of the spray program for the control of spinach white rust were evaluated in four field trials. The trials were carried out in the spring and fall of 2001, and spring of 2002 at the Plant Pathology Research Farm, Oklahoma State University, in Stillwater (trials 1 and 2), the Vegetable Research Station, Oklahoma State University, in Bixby (trial 3), and in a commercial field near Hydro OK (trial 4). The susceptible cultivar "Melody" was used in trials 1, 2, and 3 and the partially resistant cultivar "Fall Green" was used in trial 4. Planting dates for trials 1-4 were 08 Mar 2001, 13 Sept 2001, 15 Mar 2002 and 28 Feb 2001, respectively. For all trials, granular fertilizer (23-0-0 kg/ha N-P-K) was broadcast and incorporated prior to direct seeding. Metolachlor (Dual 8E) at 2.24 kg/ha was broadcast immediately after planting. Additional fertilizer at 23-0-0 kg/ha N-P-K was broadcast three weeks after emergence. The fields received sprinkler irrigation as necessary to prevent moisture stress.

In trials 1, 2, and 4 the fungicides zoxamide (Zoxium 80W Dow Agrosciences) at 0.34 kg a.i./ha, pyraclostrobin (Cabrio 20WG, BASF Corp.) at 0.17 kg a.i./ha, azoxystrobin (Quadris 2.08F, Syngenta Crop Protection) at 0.17 kg a.i./ha were applied as foliar sprays. In addition, azoxystrobin at 0.17 kg a.i./ha was alternated with zoxamide at 0.34 kg a.i./ha as a resistance management strategy. In trial 3, azoxystrobin at 0.17 kg a.i./ha was alternated with fosetyl-aluminum (Aliette 80WG, Bayer CropScience) 2.69 kg a.i./ha. Two sequential applications of azoxystrobin were made before alternating with one application of fosetyl-aluminum in order to meet resistance management guidelines specified on the azoxystrobin label. Sprays were broadcast to all four rows of a plot with

a wheelbarrow sprayer equipped with three 8003vk flat-fan nozzles spaced 46 cm apart. The sprayer was calibrated to deliver from 402 to 430 l / ha at 290 kPa.

Fungicides were applied either according to a 7-day calendar program or a weather-based advisory program. The advisory program accumulated hourly periods of favorable weather for infection, herein called T*W hours, that consisted of wetness (W; RH≥90%) while temperature (T) was from 6 to 26º C according to the methods of Sullivan et al [26]. Hours of RH≥90% were used to insure that all periods of favorable wetness were included because the RH sensors (HMP-35B, Vaisala) are less accurate above 95% RH compared to lower RH values. Each hour of W was weighted by a factor that accounted for the effect of temperature. At optimum temperatures of 12 to 18º C, each hour of W was counted as one T*W hour. At sub-optimal temperatures of 10 to 11º C and 19 to 21º C, each hour of W was multiplied by 0.75; while at 6 to 9º C and 22 to 26º C, each hour of W was multiplied by 0.50. Wetness periods at 26º C and >26º C were not included because little or no disease develops at these temperatures. A spray threshold of 12 T*W hours was used in all trials.

Early, middle, and late initiation dates for each the advisory program and the calendar program was compared with an unsprayed control. For the calendar program, the first application was made when the first true leaves were fully expanded (first-true-leaf-stage (early calendar)), one week after the first-true-leaf-stage (middle calendar), and two weeks after the first-true-leaf stage (late calendar). For the advisory program, T*W durations were accumulated beginning at the first-true-leaf-stage (early advisory) one week after the first-true-leaf-stage (middle advisory) and two weeks after the first-true-leaf stage (late advisory). When 12 T*W hours accumulated, a fungicide application was

made within two days. A fungicide application was assumed to provide a 7-day protection period. Therefore, T*W hours were reset to zero following an application and were again accumulated 7 days after the previous spray. The first applications for the early calendar program were made on 8 Mar 01, 13 Sept 01, 11 Apr 02, and 6 Mar 01 for trials 1 to 4, respectively. All spray programs were maintained until 7 days before anticipated harvest.

T*W hours were monitored continuously via the OKLAHOMA MESONET, a network of automated, computer-linked weather stations. A station was within 0.5 km of test sites for trials 1, 2, and 3; and within 5 km of the test site for trial 4. Readings of T and RH were taken every 5 minutes, and the data were processed with a Microsoft Excel spreadsheet that calculated the number of T*W hours for a 24-hour period beginning at 1200 CST.

Treatments in each trial were arranged as a randomized complete-block design with four replicates. Plots consisted of four 6.7-m-long rows spaced 38 cm apart. Plots were evaluated for disease incidence, the percentage of leaves with symptoms, and disease severity, the percentage of leaf area with symptoms, at the end of the cropping season on 24 May 2001, 19 Nov 2001, 21 May 2001, and Apr 16 2001 for trials 1, 2, 3, and 4 respectively. Six, 0.31-m row segments were harvested arbitrarily from the middle two rows of each plot. The harvested leaves were bulked, mixed, and 30 leaves were blindly sampled. The percentage of leaf area covered with white rust was visually estimated on each sampled leaf.

Analysis of the incidence and severity data was performed on the mean of the 30 subsamples per plot. The effects of spray programs, fungicide treatments, and initiation

dates on disease incidence and severity were evaluated by analyses of variance using the SAS Mixed procedure (version 8.2 SAS Institute, Cary NC). All treatments were significantly different from the untreated control. Because the mixed procedure in SAS compares the least squared means of all treatments, the non-treated control was dropped from the analysis so comparisons of fungicides, spray programs and initiation dates were not influenced by the high levels of disease found in the control. The main effects of fungicide, initiation date, spray program, and their interactions were tested for trials 1, 2, and 4. The main effects of spray program and initiation date, and the interaction of spray program x initiation date were tested for trial 3. Means were separated by comparing differences in least squared means. Unless otherwise indicated, only significant ($P \leq 0.05$) differences between means are described below.

RESULTS

Weather conditions that favored development of white rust were recorded during each trial. Rainfall from planting to harvest totaled 12 cm in trial 1, 11 cm in trial 2, 23 cm in trial 3, and 3 cm in trial 4. Trials 1 and 2 received two supplemental 2.5 cm irrigations and trial 3 received three supplemental 1 cm irrigations. T*W hours totaled 119 in trial 1, 140 in trial 2, 135.2 in trial 3, and 125.5 in trial 4.

White rust levels in the non-treated control plots were moderate to severe in trials 1, 2, and 3. Disease incidence for the control in trial 1, was 75% and disease severity was 11%. Disease levels in trials 2 and 3 were greater than for trial 1. For trials 2 and 3, over 70% of the leaves had symptoms and disease severity averaged over 25%. No white rust developed in trial 4; however, Cladosporium leaf spot caused by *Cladosporium spp.*

developed and effects of initiation date, spray programs, and fungicides were evaluated for that disease.

For each initiation date, the advisory program resulted in significantly fewer fungicide applications per trial compared to the 7-day program in trials 1, 2, and 3 (Fig. 1). Compared to the early calendar program, fungicide applications were reduced by 17.7%, 33.3%, 50%, 61.2%, and 72.2% for mid calendar, late calendar, early advisory, mid advisory, and late advisory, respectively.

For trials 1 and 2, the main effects of fungicide, spray program, and initiation date were significant ($P < 0.01$) for disease incidence and severity of white rust. In trial 3 when only one fungicide treatment was used only the effects of spray program were significant ($P < 0.01$) for disease incidence and for disease severity. There were no significant interactions between the effects in trials 1, 2, or 3.

Control of white rust varied depending on fungicide in trial 1 and 2. Averaged over the spray programs and initiation dates, pyraclostrobin had the lowest disease incidence (Table 1) and severity (Table 2). Disease incidence and severity did not differ among the fungicides zoxamide, azoxystrobin, and azoxystrobin alternated with zoxamide.

Control of white rust also varied among the calendar and advisory programs, and between spray program initiation dates in trials 1 and 2. Within the calendar program, early and middle initiation dates provided lower disease incidence (Table 1) and severity (Table 2) compared to the late initiation date. Levels of white rust did not differ among initiation dates within the advisory program. In trial 3, there were no differences in disease incidence among initiation dates of spray programs (Table 3). However, the early

and middle initiation dates had lower levels of disease severity control compared to the late initiation date.

Averaged over fungicides and initiation dates, levels of white rust for the advisory and calendar programs were reduced compared to the control. For the calendar program in trials 1 and 2, disease incidence was reduced by 70.6% and disease severity was reduced by 20.5%. The advisory program resulted in a reduction of disease incidence of 55.6% and disease severity was reduced by 19.6%. While both programs significantly reduced disease levels, the calendar program generally had significantly less disease incidence and severity compared to the advisory programs. However, disease severity for the early and middle advisory programs did not differ from the early and middle calendar programs. Results for the effects of spray programs in trial 3 (Table 3) were similar to those in trials 1 and 2 except that disease levels were higher for each initiation date of the advisory program compared to respective calendar programs.

In trial 4, incidence of Cladosporium leaf spot was 62% while disease severity was only 2% in the non-treated control plots. Because disease severity was minimal, only data on disease incidence is presented. Because the fungicide zoxamide did not differ from the control, and the analysis compares all treatments, the affect of the lack of efficacy for zoxamide was eliminated by leaving it out of the analysis. The main effects of fungicide and initiation dates were not significant and there were no significant interactions between the main effects. For the spray programs, the calendar treatments reduced disease incidence by 25% compared to the advisory treatments (Table 4).

DISCUSSION

Weather-based advisory programs, based on the development of white rust in response to temperature and duration of wetness under controlled conditions [27], have been shown to improve the efficiency of fungicide programs for control of white rust [26]. The efficiency of a fungicide program is defined as the maintenance of disease levels equivalent to a seven-day program with a reduced number of fungicide applications. Using the first true leaf stage as a standard initiation date, delaying the initiation date by 1 week resulted in a reduced number of applications for both the advisory program and the 7-day calendar program, without an increase in disease severity in 2 of 3 trials where white rust developed. Greenhouse studies have shown [23] young spinach leaves to display a resistance reaction to white rust. This may explain why a one-week delay in fungicide applications did not increase disease levels in this study. Delaying the initiation date by two weeks resulted in a further reduction in the number of fungicide applications. However, disease levels increased significantly for 2 of the 3 trials where white rust developed.

Disease levels were reduced by both the calendar and advisory spray programs compared to the control. However, disease levels for the advisory programs were significantly greater compared to the calendar programs when averaged across fungicides and initiation dates. Therefore, across all fungicides and initiation dates the advisory program cannot be considered as efficient as the 7-day calendar programs. While disease incidence within the advisory programs was always greater for all initiation dates compared to the calendar programs, there were no differences in disease severity for early and middle advisory programs compared to early and middle calendar programs in trials

1 and 2. In trial 3 disease severity was significantly greater for the early and middle advisory programs compared to the early and middle calendar programs but the difference was only 0.5% and 0.6%, respectively. Disease severity may be a better indicator of crop damage than disease incidence as severity is a measurement of the amount leaf area affected while incidence only indicates the percentage of leaves with symptoms. Using only disease severity as an evaluation for efficiency of fungicide programs, early and middle advisory program may be considered more efficient than the early and middle 7-day calendar program.

Azoxystrobin is the primary fungicide in use by growers in Oklahoma for control of white rust. The mode of action for azoxystrobin involves the inhibition of mitochondrial respiration. This mode of action has already been overcome by resistance in the powdery mildew fungus, the gummy stem blight fungus, and the downy mildew fungus in cucurbits.

Sullivan [25] demonstrated that azoxystrobin has post-infection activity against spinach white rust and superior efficacy compared to the protectant EDBC fungicides when used in an advisory program [26]. Pyraclostrobin, an experimental fungicide not registered for use on spinach was the most effective fungicide used in this study. Concerns about the development of resistance to fungicides that are single-site inhibitors of fungal metabolism like the strobilurin fungicides have led to label restrictions for azoxystrobin. For spinach, the label for azoxystrobin permits a maximum of two consecutive applications before switching to a fungicide with a different mode of action. Zoxamide is a new fungicide being developed for foliar use on potatoes, grapes, and vegetables to control Oomycete fungi [30]. The fungitoxic mechanism of zoxamide

involves inhibition of nuclear division as the result of covalent binding to β-tubulin and disruption of the microtubule cytoskeleton [30]. Although, the mode of action for zoxamide is different from the strobulurin fungicides, it is not unlike the mode of action for the benximidaxoles which have had resistance problems [31]. In this study, zoxamide alone and zoxamide alternated with azoxystrobin were similar in efficacy to azoxystrobin alone. Therefore zoxamide could be used in alternation with azoxystrobin for white rust control. Fosetyl-Al is a unique fungicide that enhances plant responses normally associated with disease resistance [22]. It is systemic in both basipetal and acropetal direction [5] and may also have a direct fungicidal mode of action [19, 20]. In previous field trials, efficacy of fosetyl-Al was similar to that for the EDBCs [9-12], but significantly lower when compared to azoxystrobin, pyraclostrobin, or zoxamide [15]. Fosetyl-Al is currently registered for use in spinach and provided good disease control when used in alternation with azoxystrobin in trial 3. Therefore fosetyl-Al may be used in a control program for spinach white rust with azoxystrobin to satisfy resistance management requirements.

The foliar disease Cladosporium leaf spot was the only disease encountered in trial 4. White rust did not develop despite the 125.5 T*W hours that accumulated during the trial, which was similar to the average of 131.3 T*W hours for the other three trials. The advisory programs recommended 3, 1, and 1 sprays in trial 4 for early, middle, and late initiation dates, respectively. Other weather conditions during trial 4 may have resulted in the lack of white rust infection as rainfall totaled only 3 cm through out the trial. It has been speculated that the primary inoculum for spinach white rust consists of oospores that have developed from previous spinach crops and survive in the soil to re-

initiate the disease in the next cropping season [6, 28]. Oospores are known to be the primary inoculum for diseases caused by other members of the Peronosporales. In downy mildew of grape caused by *Plasmopara viticola,* the oospores survive in dead leaf lesions and shoots [1]. The oospores germinate during rainy periods in the spring and produce sporangia and/or its zoospores that are disseminated by wind or water to wet leaves, which they infect through the stomata. Oospores of *Albugo candida,* the causal agent for white rust of Brassica spp. are known to germinate following washing on a rotary shaker or allowing water to slowly drip onto a filter where oospores have been distributed [29]. This suggests that germination in nature is influenced by the leaching action from water. Therefore, it is possible that conditions that favor primary infection did not occur during trial 4. It is also possible that oospores were not present in the field. The time when favorable environmental conditions for sporangial germination and infection occurred may have also had an effect on white rust development. In trial 4, 98 of the 125.5 T*W hours occurred after 31 Mar, late in the season and within 17 days of harvest.

The fungicides azoxystrobin, pyraclostrobin, or azoxystrobin alternated with zoxamide provided good control of Cladosporium leaf spot. The efficacy of the fungicide zoxamide is apparently limited to the control of Oomycete fungi, and therefore zoxamide alone was not effective against Cladosporium leaf spot under any spray program or initiation date. The advisory programs had only limited efficacy while all calendar programs provided good disease control. The late calendar program was the most efficient. Because Cladosporium leaf spot is known to develop late in the spring

season, temperature requirements for disease development may be higher than that for spinach white rust.

The cost of spinach production in Oklahoma, including azoxystrobin has been estimated to be $1161/ha. With each application of azoxystrobin costing over $60/ha, delaying the initiation of a calendar-based fungicide program by one week can significantly reduce the cost of spinach production without sacrificing disease control. Sullivan [26] demonstrated that the advisory program could reduce the number of fungicide applications without sacrificing disease control. In this study the advisory programs significantly reduced the number of fungicide applications per season compared to the calendar programs. However, there was generally a small but significant overall increase in disease levels. The middle advisory program was shown to be more efficient than the early advisory program by providing an equitant level of disease control with fewer sprays per season. Also, the middle advisory program was more efficient than the early and middle calendar programs at reducing disease severity in trials 1 and 2 with only a slight increase in trial 3. However, an increase in disease incidence was observed. Since the registration of azoxystrobin for use on spinach in 1999, no fields in Oklahoma have been rejected for harvest due to white rust. It has not been possible to determine the actual level of white rust that is acceptable by processors. Therefore, it is unclear whether the increased disease incidence level for the early and middle advisory programs would have led to a reduction in grade or the rejection of the crop.

The use of the advisory program can result in a 60% reduction in the number of fungicide application and the use of partially resistant cultivars has shown to delay the development of white rust. In addition, acceptable tolerances for white rust

contamination in spinach for the processing market are not defined. Further studies to determine the value of partially resistant cultivars in a weather-based advisory program and the establishment an economic threshold for disease levels in spinach are needed.

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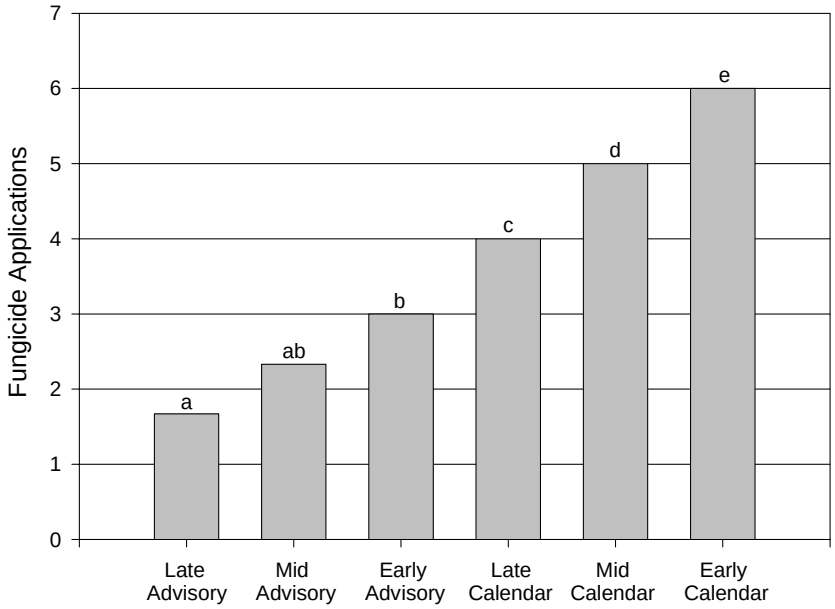

Figure 1. Number of fungicide applications for control of spinach white rust made according to a calendar-based schedule and weather-based advisory programs over trials 1, 2, and 3 during 2001 and 2002

Table 1. Effects of fungicides, spray program, and spray program initiation date on incidence of spinach white rust[1], trials 1 & 2

	Calendar[4]			Advisory[5]			Fungicide
Fungicide	Early	Middle	Late	Early	Middle	Late	mean[6]
Zoxamide	13.3	9.2	21.7	15.4	33.7	30.0	20.5 b[2]
Pyraclostrobin	0.4	0.4	1.7	8.3	5.4	14.5	5.1 a
Azoxystrobin	1.2	10.8	17.1	17.5	26.2	30.8	17.3 b
Azoxystrobin alt./w zoxamide	1.0	4.6	26.7	24.1	29.2	18.7	18.9 b
Initiation date mean[7]	6.2 a[3]	6.2 a	16.8 b	16.3 b	23.6 b	23.5 b	
Spray program mean[8]		9.8 a				22.2 b	

[1]Percentage of leaves with symptoms. Values are the mean of 30 leaves per plot and 4 replicate plots per treatment for each of 2 trials.

[2]Least squared means within a column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by least significant difference (LSD) test.

[3]Least squared means within a row followed by the same letter are not significantly different ($P \leq 0.05$)

[4]Calendar spray programs were 7-day schedules beginning when the first true leaves were fully expanded (early-calendar), one week after the first true leaves were fully expanded (middle-calendar), and two weeks after the first true leaves were fully expanded (late-calendar).

[5]Applications made according to the weather-based advisory spray programs were made as soon as indicated by the advisory program beginning when the first true leaves were fully expanded (early advisory), one week after the first true leaves were fully expanded (middle-advisory), and two weeks after the first true leaves were fully expanded (late-advisory).

[6]Values are the mean of fungicides across spray program and initiation dates.

[7]Values are the mean of initiation dates across fungicides.

[8]Values are the mean of spray programs across initiation dates and fungicides.

Table 2. Effects of fungicides, spray program, and spray program initiation date on severity of spinach white rust[1], trials 1 & 2.

	Calendar[4]			Advisory[5]			Fungicide
Fungicide	Early	Middle	Late	Early	Middle	Late	mean[6]
Zoxamide	1.58	1.24	3.86	1.22	3.93	7.59	3.24 b[2]
Pyraclostrobin	0.01	0.04	0.23	0.98	0.14	1.16	0.43 a
Azoxystrobin	0.05	1.18	2.54	2.49	3.53	2.98	2.13 b
Azoxystrobin alt./w zoxamide	1.07	0.4	4.53	3.67	2.62	3.32	2.60 b
Initiation date mean[7]	0.68 a[3]	0.75 a	2.79 b	2.09 ab	2.26 ab	3.76 b	
Spray program mean[8]		1.39 a			2.80 b		

[1]Percentage of leaf area with symptoms. Values are the mean of 30 leaves per plot and 4 replicate plots per treatment over 2 trials.

[2]Least squared means within a column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by least significant difference (LSD) test.

[3]Least squared means within a row followed by the same letter are not significantly different ($P \leq 0.05$) as determined by least significant difference (LSD) test.

[4]Calendar spray programs were 7-day schedules beginning when the first true leaves were fully expanded (early-calendar), one week after the first true leaves were fully expanded (middle-calendar), and two weeks after the first true leaves were fully expanded (late-calendar).

[5]Applications made according to the weather-based advisory spray programs were made as soon as indicated by the advisory program beginning when the first true leaves were fully expanded (early advisory), one week after the first true leaves were fully expanded (middle-advisory), and two weeks after the first true leaves were fully expanded (late-advisory).

[6]Values are the mean of fungicides across spray program and initiation dates.

[7]Values are the mean of initiation dates across fungicides.

[8]Values are the mean of spray programs across initiation dates and fungicides.

Table 3. Effects of spray programs and spray program initiation date using azoxystrobin alternated with fosetyl-aluminumon on incidence[1] and severity[2] of spinach white rust, trial 3.

	Early		Middle		Late		Spray program means[3]	
Spray Program	Incidence	Severity	Incidence	Severity	Incidence	Severity	Incidence	Severity
Calendar[6]	3.2	0.1	4.2	0.1	2.0	0.2	3.1 a	0.1 a
Advisory[7]	12.5	0.5	13.3	0.6	28.3	1.9	18.1 b	1.0 b
Initiation date mean incidence[8]	7.9 a[5]		8.8 a		15.2 a			
Initiation date mean severity [8]	0.3 a[5]		0.4 ab		1.0 b			

[1]Percentage of leaves with symptoms. Values are the mean of 30 leaves per plot and 4 replicate plots per treatment.

[2]Percentage of leaf area with symptoms. Values are the mean of 30 leaves per plot and 4 replicate plots per treatment.

[3]Values are the mean of spray programs across initiation dates and fungicides.

[4]Least squared means within a column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by least significant difference (LSD) test.

[5]Least squared means within a row followed by the same letter are not significantly different ($P \leq 0.05$) as determined by least significant difference (LSD) test.

[6]Calendar spray programs were 7-day schedules beginning when the first true leaves were fully expanded (early-calendar), one week after the first true leaves were fully expanded (middle-calendar), and two weeks after the first true leaves were fully expanded (late-calendar).

[7]Applications made according to the weather-based advisory spray programs were made as soon as indicated by the advisory program beginning when the first true leaves were fully expanded (early advisory), one week after the first true leaves were fully expanded (middle-advisory), and two weeks after the first true leaves were fully expanded (late-advisory).

[8]Values are the mean of spray program initiation dates across spray programs.

Table 4. Evaluation of fungicides, spray program, and spray program initiation date on incidence of Cladosporium leaf spot[1], trial 4

	Calendar[4]			Advisory[5]			Fungicide
Fungicide	Early	Middle	Late	Early	Middle	Late	mean[6]
Pyraclostrobin	5	1.2	3.3	28.3	42.5	36.7	19.5 a[2]
Azoxystrobin	8.9	3.3	11.7	22.5	23.4	32.5	17.0 a
Azoxystrobin alt./w zoxamide	9.2	16.8	9.2	43.3	34.2	27.5	23.3 a
Spray program initiation mean[7]	7.6 a[3]	7.1 a	8.1 a	31.4 b	33.4 b	32.2 b	
Spray program mean[8]		7.6a[3]			32.3b		

[1]Percentage of leaves with symptoms. Values are the mean of 30 leaves per plot and 4 replicate plots per treatment.

[2]Least squared means within a column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by least significant difference (LSD) test.

[3]Least squared means within a row followed by the same letter are not significantly different ($P \leq 0.05$) as determined by least significant difference (LSD) test.

[4]Calendar spray programs were 7-day schedules beginning when the first true leaves were fully expanded (early-calendar), one week after the first true leaves were fully expanded (middle-calendar), and two weeks after the first true leaves were fully expanded (late-calendar).

[5]Applications made according to the weather-based advisory spray programs were made as soon as indicated by the advisory program beginning when the first true leaves were fully expanded (early advisory), one week after the first true leaves were fully expanded (middle-advisory), and two weeks after the first true leaves were fully expanded (late-advisory).

[6]Values are the means for fungicides across spray program and initiation dates.

[7]Values are the mean of spray program initiation dates across fungicides.

[8]Values are the mean of spray programs across spray program initiation dates and fungicides.

CHAPTER III

Biology of Oospores of *Albugo occidentalis*

ABSTRACT

The primary inoculum that initiates the white rust disease of spinach is uncertain. There are no known alternate hosts in many spinach-producing areas and there have been no descriptions of germination of the oospores of *Albugo occidentalis.* Oospores were extracted from leaves and agitated in water on a rotary shaker at 23º C. After 7 days agitation, oospore suspensions were spray-inoculated onto spinach plants and incubated under 100% humidity at 12º C for 72-hours. After 10 days further incubation in the greenhouse 44% of leaves and 10% of the leaf area had symptoms of white rust. Therefore, the effects of agitation temperature (17, 23, or 29º C), additive (soil, root and leaf extracts), chilling in still culture (9, 13 and 17ºC) after agitation and agitation on germination were evaluated. Although no germination was visually observed, some oospore suspensions produced copious amounts of zoospores (up to 2 x 10^5/ml). Zoospores were separated from the oospore culture by sieving through a 20 µm sieve, adjusted to 1 x 10^5/ml, inoculated to spinach plants and incubated as described above. In one inoculation trial, 12.2% of the inoculated leaves were infected with 2.4% of the leaf area became symptomatic. Symptoms did not develop on control plants inoculated with water. In the other trial, only 2 of the 62 leaves inoculated became diseased. DNA was isolated from separate suspensions of oospores and zoospores and amplified in a seminested polymerase chain reaction (PCR) using primers DC6 and ITS4 in the first round followed by a second round of PCR with primers ITS6 and ITS4. Automated

sequencing of the two amplification products revealed an identical sequence confirming that the isolated zoospore and oospores were *A. occidentalis*. Results of this study indicate that oospores produced by *A. occidentalis* are a source of primary inoculum that can initiate the spinach white rust.

INTRODUCTION

White rust (*Albugo occidentalis* Wilson) is the most economically important foliar disease of spinach in Oklahoma,Texas, and Arkansas [2, 4, 13]. Primary infections are found on lower leaves in direct contact with, or close proximity to the soil surface. Oospores that formed on previous spinach crops are considered to be the primary inoculum, as *Chenopodium capitatum* (L.), the only known alternate host for the pathogen is not found in Texas, Oklahoma, or Arkansas. Intensive cropping of spinach has led to the deposit of large numbers of oospores in commercial fields [10, 13]. Sporangia, released from pustules on diseased plants are responsible for the polycyclic phase of the disease. Environmental factors affecting sporangial germination and infection have been described and are important for secondary increase of the disease [3, 11, 12]. No studies have reported on the germinability of the oospores of *A. occidentalis* [2].

Though the biology of *A. occidentalis* is not completely understood, it is thought to resemble that of *Albugo candida* (Pers.) Kunze, the more thoroughly studied white rust pathogen of the Brassica spp. [2]. In 1866, de Bary [5] reported germination of *A. candida* oospores by the development of a sessile vesicle. In this method, the epispore ruptures and the protoplasm moves out of the endospore as a sessile vesicle in which

zoospores are formed. The zoospores then escape by rupturing the vesicle membrane [5]. In 1959, Vanterpool [14] confirmed this germination mechanism and described a second mode of germination by means of a discharge tube and terminal vesicle. Vanterpool also observed zoospores swimming inside intact oospores of *A. candida*. Germination was described as inconsistent and irregular, with a maximum rate of only 4%. In 1975, Verma and Petrie [15] improved germination rates of *A. candida* by agitating a suspension of extracted oospores in water on a rotary shaker for 3 to 4 days followed by a day in still culture at 13ºC. This method provided better germination at a maximum rate of 67%. Verma and Petrie confirmed the earlier report of germination by sessile and terminal vesicles. and described a third mode of germination by a germ tube. Vanterpool and Verma both found germination by a sessile vesicle to be the most common type of germination. Confirming Verma and Petries' agitation method of germination of *A. candida* oospores, Liu and Rimmer [7] determined that chilling at 13 ºC after agititation to be of primary importance for increased germination and showed that a mixture of β-glucuronidase and aryl sulfatase enhanced the germination of immature oospores.

A. occidentalis is a close relative of *A. candida*, and both cause white rust diseases that affect cool season crops. While oospores of many species in the Peronosporales are notoriously difficult to germinate [14], the objectives of this study were to induce oospore germination of *A. occidentalis*, to demonstrate their pathogenicity, and examine factors which may influence their germination rate.

MATERIALS AND METHODS

Oospore production. Spinach seedlings were grown in a greenhouse at 20 to 30ºC in plastic pots (10-cm diameter) containing a soiless-growing medium (65% peat moss, 20% vermiculite, 10% perlite, and 5% hort sand) for 40 to 60 days. Plants were watered as needed and nutrients were supplied by applying soluble fertilizer (0.2, 0.08, and 0.03 g/L N/P/K, respectively) weekly.

An isolate of *A. occidentalis* was obtained from diseased plants collected in field plots at the Oklahoma State University Vegetable Research Station in Bixby. The isolate was maintained on plants in the greenhouse as previously described. A sporangial suspension was prepared by agitating leaves with mature pustules in distilled water. The suspension was sieved through a 177 μm sieve and adjusted to $1x10^5$ sporangia/ml with a hemacytometer. The suspension was sprayed to run-off onto the upper and lower leaf surface of the plants using a hand-held spray bottle. Inoculated plants were incubated in a dew chamber (Model I-60DL, Percival, Boone, IA) at 13º C for 48 hours at 100% humidity. Plants were removed from the dew chamber and further incubated in a greenhouse that maintained a minimum temperature 30º C until 80% of oospores were dark brown to black, about 25 days after inoculation (Fig. 1).

Oospore extraction and pathogenicity test. Leaves containing oospores were dried on the laboratory bench and stored at room temperature for at least 2 weeks. To determine whether the sporangia were no longer viable, the desiccated leaves were mixed and a sample of 10 leaves was blindly drawn. The sampled leaves were agitated in water to dislodge the sporangia the suspension was sieved through a 20 μm sieve which retained the oospores. The sporangial suspension was then subjected to a temperature of

12ºC for up to 20 hours. Sporangial germinationas evidenced by the presence of zoospores was not observed. Leaf pieces were ground to a fine powder in a household blender and screened through a 75 µm sieve. The material remaining on the sieve was again ground in the blender and sieved. The grinding and sieving process was repeated 3 times, resulting in a greenish-brown powder consisting of about 50% oospores and 50% leaf debris. Oospores were further separated from the leaf tissues by stirring 10 g of the oospore-containing powder in 300 ml distilled water on a stir-plate for 5 minutes, allowing the suspension to stand until the oospores had settled, then decanting off the supernatant. This process was repeated 3 times. The suspension was then sonicated twice for 5 minutes, and decanted after each sonication. After washing the suspension 3 times in sterile distilled water by centrifugation at 600 g for 5 minutes, it was practically free of plant material. The suspension was adjusted to 100 oospores/25 µl with sterile distilled water. Ten aliquots of 30 ml in 125 ml Erlenmeyer flasks were incubated on a rotary shaker at 200 rpm for 7 days at 23º C. Two plants at the eight-true-leaf stage were inoculated with the oospore suspension and two plants eight-true-leaf stage were inoculated with sterile distilled water and incubated as described above. The experiment was repeated once. The true leaves from each plant were evaluated for disease incidence, the percentage of leaves with symptoms, and severity, and the percentage of leaf area with symptoms 16 to 21 days after inoculation.

Oospore germination and zoospore pathogenicity test. Oospores were extracted from leaves as described above. Leaf extract, root extract, and soil extract were added to agitation water. Treatments consisting of surface sterilization, agitation temperature (17, 23, 28ºC), chilling in still culture (9, 13 and 17ºC) after agitation and

soaking in still culture with no agitation were applied in an attempt to induce germination and increase germination rate. Leaf and root extracts were prepared by macerating 5 g of the respective parts of spinach plant material with a mortar and pestle in 50 ml of distilled water. The suspension was centrifuged for 5 minutes at 3200 g, the supernatant was decanted and filter sterilized using a 0.45μm syringe filter. For the soil extract, 100 g of soil, was taken from a field previously cropped to spinach, mixed with 100 ml of distilled water and allowed to stand for 3 hours. The supernatant was decanted, centrifuged and filtered-sterilized as described above. Extracts were added at 1.5 ml/28.5 ml oospore suspension to make a 5% solution. For surface sterilization, extracted oospores were suspended in a 1% sodium hypoclorite solution for 2 minutes, the solution was removed by filtration through #1 Whatman filter paper, and oospores were rinsed twice for 2 minutes in sterile distilled water as described above. Oospores were resuspended in sterile distilled water and the various extract solutions.

Oospore suspensions were incubated on a rotary shaker as described above at 17, 23, and 28º C; and at 23º C with no agitation. After 72, 96, and 120 hours of agitation three aliquots of 3ml from each treatment were incubated in still culture at 9, 13, and 17º C for up to 48 hours in 35 mm plastic petri dishes. Agitation suspensions were checked for signs of germination (zoospore production or formation of vesicles) twice daily for 7 days and the still culture suspensions were examined for germination twice daily for 2 days following chilling in still culture. The experiment was conducted in a completely randomized design with 3 replicates. A replicate consisted of one, 125 ml flask while on the rotary shaker and one, 35 ml petri dish while in still culture. The experiment was repeated once.

Suspensions that produced zoospores were combined and sieved through a 20 μm sieve, to separate the zoospores from the oospores. The filtrate containing only zoospores was adjusted to 1 x 10^5/ml with sterile distilled water and inoculated onto three plants as described above and three plants that were inoculated with water served as controls. Following inoculation, plants were incubated at 13 and 21º C in the dew chamber at 100% humidity for 48 hours. The plants were then placed in the greenhouse for a 14 day incubation period. All leaves from each plant were evaluated for disease incidence and severity as described above. The experiment was repeated once.

Isolation of Oospores and Zoospores for DNA Analysis. Oospore suspensions from the oospore germination trial that produced zoospores were combined, separated from the oospores as described above, and pelleted by centrifugation at 6750 g for 5 minutes. Microscopic examination of the zoospore pellet revealed a few oospores that were manually removed using a dental pick. DNA from oospores and zoospores was extracted using the protocol of Murray and Thompson [8] with slight modifications. Spores from 1.5 ml of each of the spore suspensions were pelleted by centrifugation and the supernatant was discarded. The spores were resuspended in 300 μl CTAB buffer (2% (w/v) cetyl-trimethyl-ammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA), ground in a microcentrifuge tube with a micropestle (Brinkman, Westbury, NY), and incubated for 10 min at 65°C. A chloroform extraction was performed after the suspension had cooled to room temperature. The aqueous upper phase was transferred to a new microcentrifuge tube and the DNA was precipitated and washed with absolute ethanol and 70% ethanol, respectively.

DNA was amplified using the universal primers ITS4 and ITS6 in combination with DC6 in a seminested polymerace chain reaction (PCR) using primers DC6 and ITS4 in the first round followed by a second round of PCR with primers ITS6 and ITS4 [1]. In combination with ITS4, DC6 selectively amplifies the internal transcribed spacer (ITS) regions of members of the Peronosporales [1]. PCRs consisted of 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, and a final cycle of 72°C for 5 min.

After gel electrophoresis on agarose, amplification products were purified with a QIAqick gel extraction kit (Qiagen Inc., Valencia, CA) and their concentrations were estimated by comparison with calf thymus DNA (BD Bioscience Clontech, Palo Alto, CA). Direct sequencing of PCR products was performed by the OSU Recombinant DNA/Protein Resource Facility, using dye-terminated thermal cycle sequencing and an Applied Biosystems/PerkinElmer 373 sequencer (Perkin Elmer Inc., Wellesley, MA).

Sequence analysis. The resulting sequence was compared with published sequences of other oomycetes (Table1) using the ClustalW software program. A phylogenetic tree was compiled with PHYLIP programs [6]. A distance matrix was calculated with DNADIST and used to construct a tree by neighbor joining as implemented in NEIGHBOR. The phylogenetic tree was displayed using TreeView [9]. The alignment was bootstrapped 100 times using SEQBOOT, and bootstrap values were added to the branch points of the distance tree.

RESULTS

Oospore pathogenicity. In both trials, white rust developed in inoculated plants, but not in non-inoculated plants. In the first trial, disease incidence averaged 50% and disease severity 5% over two plants with 8 leaves each (Fig.2). In the second trial, disease incidence was 37.5% and disease severity was 15.7%. Disease did not develop in the non-inoculated control plants. No vesicles or oospore germination was observed during periodic examinations prior to inoculation. However, after 72 hours of agitation numerous biflagellate zoospores measuring 3.6 x 6.9µm and 4.3µm (Table 2) when encysted (Fig.5) were observed in 3 of the 10 incubation flasks in the first trial and 1 of the 10 flasks in the second trial.

Oospore germination and zoospore pathogenicity. Oospore cultures produced small vesicles with a mean diameter of 27.6µm (Table 1), after 36 hours agitation. The vesicles contained protoplasm, which cleaved into 2 or 4 (usually 4) hyaline organisms that were reniform in shape and measured 14 x 25µm (Table 1). After 60 to 90 minutes, the vesicles ruptured and the mobile reniforms were released (Fig 3). These organisms occurred in greatest numbers in the leaf and root extract cultures, but were observed in all treatments except for the 17° C agitation treatment and all surface sterilization treatments. DNA sequencing revealed these organisms to be a common free living protozoan, a ciliate of the genus Colpoda.

Occasionally, oospores were observed releasing their protoplasmic contents (Fig.4). The protoplasmic material, some of which was contained inside one or more small vesicle-like membranes that were of the same size and morphology as the

protozoan vesicles, would oscillate for up to 45 minutes and become still. No zoospores were observed forming within these vesicles. No other vesicles were observed at any temperature under any treatments.

Zoospores, identical to those observed in the oospore pathogenicity trials were produced in some incubation treatments after 72 hours of agitation. The 17 ° C agitation temperature did not produce zoospores (Table 4). Zoospores were produced for some of the treatments at the 23 and 29 ° C, but production was inconsistent. Attempts to increase the number of flasks that produced zoospore with additives of leaf extract, root extract, and soil extract to the agitation water were not successful. Oospores soaked in water with no agitation also produced zoospores, however the incubation time required for zoospore production increased to 120 hours. Chilling in still culture at various temperatures after various periods of agitation did not lead to better zoospore production.

White rust developed in the zoospore pathogenicity trial when the plants were inoculated with suspensions that contained zoospores and incubated under 100% humidity at 13° C for 48 hours. In one trial disease incidence was 12.2% and disease severity was 2.4%, while in the second trial only 2 of the 62 inoculated leaves became diseased. Disease did not develop when the inoculated plants were incubated at the 21° C temperature under the conditions described above or on the control plants sprayed with water and exposed to the same conditions.

Sequence analysis. DNA was extracted from each of the separated suspensions of oospores and zoospores and subjected to PCR amplification of a region comprising partial ITS1, 5.8S rRNA gene, and partial ITS2. Automated sequencing of the two amplification products revealed an identical sequence confirming that the isolated

zoospores and oospores were of the same organism. The aforementioned sequence of the causal agent of spinach white rust, *A. occidentalis*, was submitted to GenBank (accession number AJ553900).

Homologous DNA sequences from other oomycetes were aligned with that of *A. occidentalis* and a phylogenetic distance tree was generated (Fig. 6) to place the organism based on molecular data. *A. occidentalis* falls into the same group as *A. candida* which is supported by a bootstrap value of 98%.

DISCUSSION

The mode of germination for *A. occidentalis* oospores could not be determined in this study. Vanterpool, [14] observed zoospores swimming inside the intact oospores of *A. candida,* and speculated that the oospores may have the ability to germinate by the complete maturation of the zoospores within the oospore, followed by direct escape through the ruptured epispore. In this study, the observation of the discharge of the oospore contents through a ruptured epispore may have indicated a premature release of contents before the zoospores matured. All observations of this discharge were made while observing the oospores under a cover slip. It is possible that heat from the microscope light may have caused the premature discharge. The discharge of the contents of the oospore was completed within 6 seconds, leaving the opening in the epispore as the only evidence of the release. Some of the oospore contents were contained inside membranes the same size and shape as the cyst of the Colpoda organisms. It could be speculated that these organisms had parasitized the oospores.

However, it is known that the Colpoda are free-living organisms able to survive and reproduce on the nutrients available in rain or dew droplets.

Because the origin of the zoospores could not be determined through microscopic observation, DNA analysis was preformed to verify their identity. To my knowledge there are no published descriptions of the genetic sequence of *A. occidentalis*. Therefore, the ITS regions of the oospores were compared with that of the zoospores in question. This comparison confirmed that the oospores isolated from spinach leaves and the zoospores produced by agitating the oospores in water were the same organism. The sequence was then compared with that of other Oomycetes to determine the molecular relationship of the oospores used in this study to related Oomycetes and therefore, *A. occidentalis*. The phylogenetic analysis confirmed the traditional taxonomy using morphology by placing *A. occidentalis* into a group homologous with *A. candida*.

Oospores agitated in water on the rotary shaker for 72 hours produced zoospores. Zoospores were also produced from non-agitated oospores. However, the amount of time for zoospore production was increased to 120 hours. The washing action of the rotary shaker may simulate the leaching action of water in soil. In the field, suspected primary infections of white rust have been observed to occur after heavy rains or in low areas of the field.

The production of biflagellate zoospores with the ability to cause white rust symptoms when inoculated to spinach plants and the molecular evidence associating the zoospore with the oospores of *A. occidentalis* strengthens the case for oospores serving as primary inoculum for the white rust disease of spinach.

Many factors influencing the germination of these oospores are still unknown. More detailed studies of zoospore production should be made to determine the mode of action for germination and to define optimum conditions for germination of oospores of *A. occidentalis*. Efforts to control this disease through a reduction or elimination of soilborne oospores might be productive. However, methods to determine the viability of oospores in soil will be needed to evaluate the effectiveness of control strategies.

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Figure 1. Grey leaves in the lower rosette are a result of oospore development in spinach plants infected with *A. occidentalis*.

Figure 2. Chlorotic lesions resulting from inoculation of spinach plants with oospores from *A. occidentalis*.

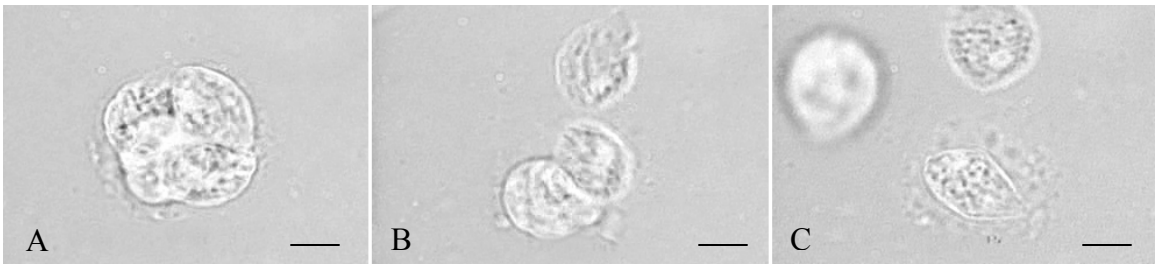

Figure 3. A. Four *Colpoda spp.* inside transparent vesicle. B. *Colpoda spp.* escaping vesicle. C. Swimming *Colpoda spp*. with one remaining trapped inside the remains of the vesicle. Bars = 10 µm.

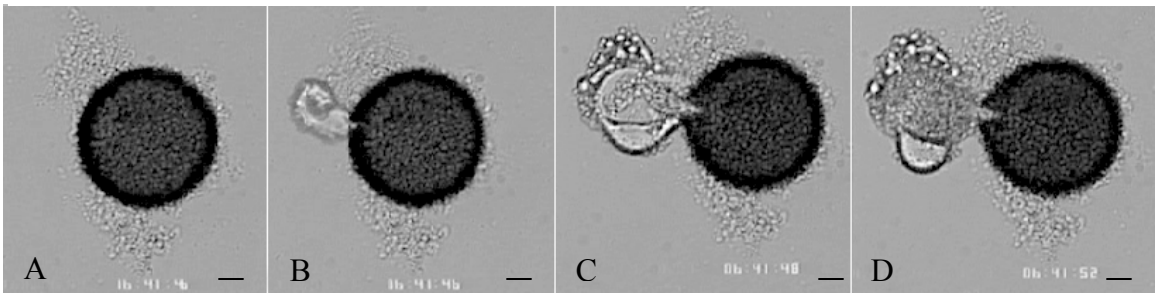

Figure 4. A. Oospore of *A. occidentalis.* B. Rupturing epispore. C. Contents being discharged. D. Discharge complete. Bars = 10 µm.

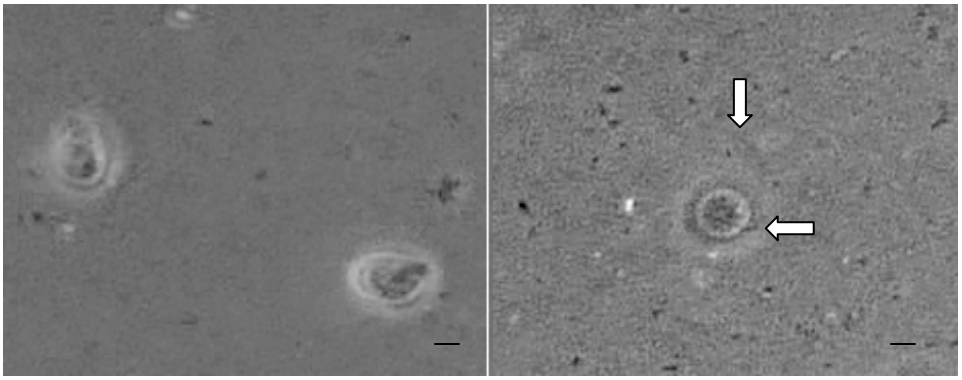

Figure 5. (A) Swimming zoospores of *A. occidentalis*. (B) Encysted zoospore with flagella (arrows). Bars = 2 µm.

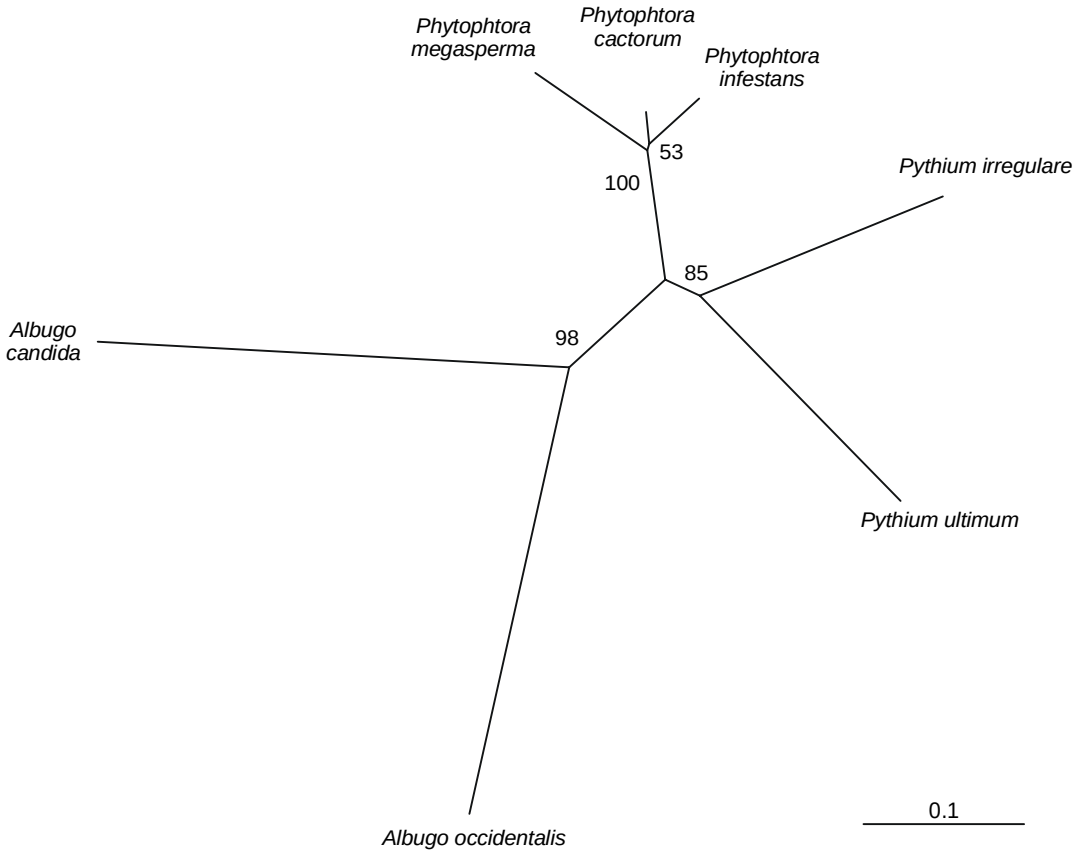

Figure 6. Phylogenetic tree obtained by DNA distance-based analysis of the combined ITS1, 5.8S subunit, and ITS2 region of the genomic ribosomal RNA gene. The numbers at the branch points indicate the percentage of bootstrap values (100 bootstraps).

Table 1. Size of Colpoda spp., observed in oospore suspensions.

	Number	Mean (μm)	Std. Deviation	Range (μm)[1]
Reniform length	50	25.1	3.0	16.3
Reniform width	50	14.0	3.5	13.8
Cyst diameter	50	27.6	1.7	6.25

[1]The difference between the smallest and the largest.

Table 2. Size of zoospores and cyst of *A. occidentalis*, observed in oospore suspensions.

	Number	Mean (μm)	Std. Deviation	Range (μm)[1]
Length	50	6.8	0.86	3.5
Width	50	3.6	0.40	1.0
Cyst	50	4.3	0.42	1.5

[1]The difference between the smallest and the largest.

Table 3. Comparison of homologous sequences of various Oomycete species from GeneBank

Species	GeneBank Accession	Reference
Albugo candida	AF271231	Cooke *et al*., 2000
Phytophthora cactorum	AF266772	Cooke *et al*. 2000
Phytophthora infestans	AF266779	Cooke *et al*. 2000
Phytophthora megasperma	AF266794	Cooke *et al*. 2000
Pythium irregulare	AF271226	Cooke *et al*. 2000
Pythium ultimum	AF271225	Cooke *et al*. 2000

Table 4. Effects of additive, agitation, agitation temperature, and surface sterilization on zoospore production from oospores of *A. occidentalis*[1].

		Agitation temperature			No agitation
Additive	Surface sterilized[1]	17° C	23° C	29° C	23° C
Root extract	Yes	0[2]	1	2	2
Root extract	No	0	0	1	1
Leaf extract	Yes	0	2	2	1
Leaf extract	No	0	1	0	3
Soil extract	Yes	0	1	0	1
Soil extract	No	0	2	2	0
No additive	Yes	0	1	2	2
No additive	No	0	2	1	1

[1]Treatments that were surface sterilized were suspended in a 1% sodium hypochlorite solution for two minutes then rinsed with sterile distilled water prior to addition of the additive.
[2]Values are the number out of six of flasks that produced zoospores over 2 trials with 3 replications.

CHAPTER IV

Post Inoculation Activity of Fungicides against Cercospora Leaf Spot of Spinach

ABSTRACT

The protectant and post-inoculation activity of maneb, azoxystrobin, pyraclostrobin, and zoxamide against Cercospora leaf spot of spinach was determined in a greenhouse study. Fungicide applications were made one day before, immediately before, and one to five days after inoculation. Disease incidence (the percentage of leaves with symptoms) and severity (the percentage of leaf area with symptoms), estimated 14 days after inoculation, were 100% and 6.2% respectively, for the control. For maneb, azoxystrobin, and pyraclostrobin, little or no disease occurred on plants sprayed one day before or immediately before inoculation. Zoxamide had a low level of efficacy at any application timing. When plants were treated with zoxamide one day before inoculation through one day after inoculation, disease incidence averaged 71.9% and disease severity averaged 2.4%, however, these levels were significantly lower than the control. When treated with zoxamide three days after inoculation, disease levels did not differ from the control. No disease developed when pyraclostrobin was applied within two days of inoculation. Incidence and severity for pyraclostrobin at three days after inoculation were 59.4 and 1.1%, respectively, and were significantly lower than the control at four days after inoculation. At five days after inoculation with pyraclostrobin, disease levels were not different from the control. Azoxystrobin also showed post-inoculation activity. Incidence and severity were 3% and 0.09%, respectively, one day after inoculation. However, disease levels increased to 84.4% disease incidence and to

3.49% severity when treated two days after inoculation but remained significantly lower than the control when treated up to four days after inoculation. Maneb showed no post-inoculation activity. Disease levels did not differ from the control when plants were treated one day after inoculation. These results indicate that applications of azoxystrobin up to one day after inoculation and of pyraclostrobin up to two days after inoculation should be effective to control of Cercospora leaf spot of spinach. Fulfillment of Koch's postulates revealed that the *Cercospora spp.* isolated from the diseased plants in this study was the primary cause of the leaf spot observed. DNA analysis verified that the *Cercospora* isolate from this study was in the *Cercospora* group comprising *C. beticola, C. apii, and C. nicotianae.*

INTROCUCION

Spinach (*Spinacia oleraceae L.*) is an economically important leafy vegetable crop throughout the United States [4]. Approximately 18,000 ha are grown annually with a crop value of approximately $185 million for fresh and processed markets [13]. Major production states include California, Texas, Oklahoma, Arkansas, Maryland, Virginia, New Jersey, and Colorado [15].

Major constraints to spinach production include insect pests, weeds, and disease that reduce yield and quality. Spinach white rust, caused by *Albugo occidentalis* Wilson and downy mildew, caused by *Peronospora effusa* (Grev.) Tul. are considered the most economically important foliar diseases of spinach in the U.S. [4]. However, at least five different fungal foliar diseases are responsible for reducing quality and marketability of spinach. Therefore an integrated disease management approach, including the use of

disease-resistant cultivars, crop rotation, and fungicides, is often necessary to produce a high-quality product.

Cercospora leaf spot caused *Cercospora beticola* is one of the most serious and widely distributed foliar diseases of sugar beet worldwide [16] and is considered an economically important disease of spinach [4]. Favorable environmental conditions for infection of Cercospora leaf spot of sugar beet have been determined [21, 22]. Temperatures between 24 and 29º C with relative humidity >90% are optimum for infection. Infection increased sharply when inoculated plants were exposed to these conditions for 48 hours or more. In Texas, Cercospora leaf spot affects approximately 50% of the spinach acreage and a 5% infestation can render the first spinach cutting of fresh market fields in the fall and early winter unmarketable [11]. The disease causes lesions (3 to 5 mm in size) on older spinach leaves (Fig. 1). During periods of warm temperatures and high humidity or leaf wetness, tan necrotic spots on lower leaves turn gray and lower quality or render the leaves unmarketable. Under 200x magnification, dark conidiophores with hyaline conidia can often be observed in lesions that are useful diagnostic characteristics (Fig. 2).

The fungicides maneb and zineb (ethylene bisdithiocarbamates (EDBC)) were used in preventive spray programs to control spinach white rust until their registration for use on spinach was revoked in 1991 [1, 5, 12]. Until the registration of azoxystrobin in the U.S. in 2001, only copper-based fungicides, metalaxyl (or mefenoxam), and fosetyl-Al were registered for use on spinach. Phytotoxicity problems with the copper-based fungicides and fosetyl-Al; the high cost of metalaxyl and label restrictions that limit its efficacy; have limited the use of these fungicides by growers [18].

The fungicide azoxystrobin has systemic activity against several Ascomycete, Basidiomycete, and Oomycete pathogens on various crops [26]. Azoxystrobin has been shown to have post-inoculation activity against spinach white rust [17]. Pyraclostrobin is another strobulurin fungicide registered for control of numerous fungal diseases on a variety of fruit, nut, and vegetable crops. Sullivan demonstrated complete control of spinach white rust with pyraclostrobin when used up to three days after inoculation, and continued post-inoculation activity up to four days after inoculation. Pyraclostrobin is not currently registered for use on spinach.

The primary objectives of this study were: to determine the post-inoculation activity of zoxamide, to confirm the post-inoculation activity of azoxystrobin, pyraclostrobin and maneb, and to determine the activity of pyraclostrobin beyond four days after inoculation against spinach white rust. However, the white rust incidence was < 1% in the non-treated control plants. The inoculum of *A. occidentalis* used in this study was apparently contaminated with spores of *Cercospora spp.* and Cercospora leaf spot developed to severe levels in the non-treated control plants. Therefore the post-inoculation activity of the fungicides was evaluated for this disease. Quantification of the efficacy and post-inoculation activity of these fungicides against Cercospora leaf spot should be beneficial in the development of management programs for foliar diseases of spinach.

MATERIALS AND METHODS

"Melody" spinach plants were grown in a greenhouse at 20-30º C in plastic pots containing a soilless growing medium (65% peat moss, 20% vermiculite, 10% perlite,

and 5% hort sand) for 40 to 60 days. Plants were watered as needed and nutrients were supplied by applying liquid fertilizer (0.2, 0.8 and 0.03 g/L N/P/K, respectively) weekly.

Spinach leaves with symptoms of Cercospora leaf spot and white rust, were collected from field plots and stored frozen at -20° C for 10 days. Spore and sporangial suspension was prepared by agitating infected leaves in distilled water. The suspension was sieved through a 177 µm sieve and adjusted to $1x10^5$ sporangia of *A. occidentalis*/ml with a hemacytometer. The suspension was sprayed to runoff onto the upper and lower leaf surface of plants using a hand-held spray bottle and incubated for 24 hours at 100% humidity in a dew chamber (Model I-60DL, Boone IA) at 13ºC.

The fungicides maneb (Maneb 75DF, Cerexagri, inc.) (2.4 kg/ha), azoxystrobin (Quadris 2.08F, Syngenta Crop Protection) (0.83 Kg/ha), pyraclostrobin (Cabrio 20WG, BASF Corp) (0.42 kg/ha), and zoxamide (Zoxium 80W, Dow Agrosciences) (0.42 kg/ha), were added to 0.3 l of water at a rate equivalent to 935 l per ha. Individual plants were sprayed to runoff with maneb (2.4 g a.i./l), azoxystrobin (0.92 g a.i./l), pyraclostrobin (0.45 g a.i./l) and zoxamide (0.45 g a.i./l) using a hand-held spray bottle. The fungicides were applied to plants one day before inoculation; immediately before inoculation; and daily from one to five days after inoculation. For a control individual plants were sprayed to runoff with sterile distilled water one day prior to inoculation. Fungicide treatments were arranged in a completely randomized design with two plants for each combination of fungicide and application timing, and the experiment was repeated.

Fungicide applications made before inoculation were intended to determine the protectant activity of the fungicides. To determine the post inoculation activity of the

fungicides, applications were made each day from one to five days after inoculation. After a 24-hour incubation period in the dew chamber, the plants were returned to the greenhouse. Fourteen days after inoculation, disease incidence (the percentage of leaves with symptoms) and disease severity (the percentage of leaf area with symptoms) was determined. Fully expanded leaves were removed from each plant, mixed and eight leaves were blindly drawn. The percentage of leaf area with symptoms was visually estimated on each sampled leaf.

Isolation, culture, and artificial inoculation. The fungus was isolated from symptomatic leaves by the following procedure. The leaves were rinsed with water and pieces of approximately 5 mm^2 that contained lesions were excised. The pieces were sterilized using a 0.8% sodium hypochlorite solution for 60 s and rinsed with sterile distilled water. The leaf pieces were placed on potato-carrot agar containing 0.03% lactic acid (PCAL). The plates were incubated for 3 days at room temperature. Agar plugs taken from the growing edge of the colonies were transferred to new PCAL plates and incubated for 4 days at room temperature. Pure colonies were smeared over PCAL and V8 agar plates and incubated for 7 days at room temperature. The cultures on V8 agar produced more conidia than the PCAL cultures; therefore the V8 cultures were used for inoculation. Plates were flooded with 15 ml of sterile distilled water spores were suspended by gently rubbing the agar surface and stirring with a 1 cm flat-bristled artists' paintbrush. The resulting suspension was sieved through a 177 micron sieve and adjusted to 5 x 10^3 conidia/ml. Six, "Melody" spinach plants, produced in the greenhouse as described above, were sprayed to runoff with the spore suspension and six plants were sprayed with sterile distilled water using a hand held spray bottle. Three inoculated and

three control plants were incubated at 15º C at 100% humidity for 48 hours and three inoculated and three control plants were incubated at 100% humidity at 24º C for 48 hours. Plants were further incubated in the greenhouse at 21 to 27º C. After 14 days in the greenhouse, disease incidence and severity were evaluated as described above. Isolations were made from lesions that formed on the leaves as described above.

Statistical analysis. Analysis of the disease incidence and severity data from the post-inoculation activity study and the artificial inoculations was performed on the mean values of 8 leaves per plant. Comparisons of the fungicide application timings in the post-inoculation activity study were performed on the incidence and severity data using the SAS Mixed procedure (version 8.2 SAS Institute, Cary NC). The effects of fungicide and application timing, and fungicide x application timing interaction were tested. For the artificial inoculations, a T-test was preformed on the severity data and the effect of incubation temperature was determined. Only significant ($P \leq 0.05$) differences between treatment means are described in the results.

DNA isolation, amplification, and sequencing. Fungal material from a pure culture isolated from diseased plants in the post-inoculation activity study was removed from the agar plates by scraping the surface with a razor blade. DNA was isolated using the DNeasy Plant Mini Kit following the protocol of the manufacturer (Qiagen Inc., Valencia, CA).

The sequence of the 5.8S ribosomal RNA gene and the intergenic spacer regions ITS1 and ITS2 was amplified by polymerase chain reaction (PCR) using the primers ITS6 [3] and ITS4 [23]. PCR was performed applying 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 60 s, and a final cycle of 72°C for 2 min.

After agarose gel electrophoresis, amplification products were purified using QIAqick gel extraction kit (Qiagen Inc., Valencia, CA) and their concentrations were estimated by comparison with calf thymus DNA (BD Bioscience Clontech, Palo Alto, CA). Direct sequencing of PCR products was performed by the OSU Recombinant DNA/Protein Resource Facility, using dye-terminated thermal cycle sequencing and an Applied Biosystems/PerkinElmer 373 sequencer (Perkin Elmer Inc., Wellesley, MA). To determine the relationship of the pathogen used in this study to other Cercospora species the resulting sequence was aligned with published sequences of the *Cercospora* species (Table.5) using ClustalW [20] and a distance matrix was calculated with DNADIST [7].

RESULTS

For the post-inoculation study, heterogeneity of variances between combinations of fungicide and application timing was severe, in the ANOVA. The effect of timing of fungicide applications was significant for disease incidence and disease severity (<0.001). Therefore, only the effect of application timing for each fungicide was considered. For the artificial inoculations the effect of incubation temperature was not significant.

Cercospora leaf spot developed to severe levels in both trials. Disease incidence was 100% and disease severity was 6.25% for the control. Maneb significantly reduced disease incidence and severity at both one-day prior to and at inoculation (Table 1). Preventive treatments of pyraclostrobin (Table 2) and azoxystrobin (Table 3), applied either one-day prior to or immediately before inoculation, resulted in complete disease control. Zoxamide had limited activity when applied preventively however, disease incidence and severity was significantly reduced compared to the control (Table 4).

Pyraclostrobin resulted in the longest period of post-inoculation activity of the four fungicides evaluated. Disease did not develop when pyraclostrobin was applied up to 2 days after inoculation (Table 2). Disease incidence and severity increased to intermediate levels at 3 days and 4 days after inoculation, and not differ from the control by five-days after inoculation.

Azoxystrobin provided a briefer period of post-inoculation activity compared to pyraclostrobin. Disease levels were low when applications of azoxystrobin were made one-day after inoculation (Table 3). Disease incidence and severity sharply increased to intermediate levels between two and four-days after inoculation. Disease levels for applications of azoxystrobin made five days after inoculation did not differ from the control.

Zoxamide had post-inoculation activity up to two days after inoculation, but the degree of disease control was low (Table 4). Disease incidence at two days after inoculation did not differ from the control and disease severity was high at 3.7%. Disease levels for fungicide treatments made 3 to 5 days after inoculation were not different from the control. For maneb all post-inoculation treatments were similar to the control (Table 1). Incidence and severity of white rust were low and inconsistent, (<1%) throughout both trials.

Isolation and artificial inoculations. The fungus was isolated consistently from the diseased plants that were artificially inoculated. The colonies produced a bright pink-red pigment in the culture that diffused into the agar (Fig. 3). Disease developed to severe levels at both the 15º C and 24º C incubation temperatures (100% disease incidence and 35.5% disease severity averaged over the two incubation temperatures).

There were no significant differences in disease incidence and severity between the 15 and 24º C incubation temperatures. Disease did not develop on the non-inoculated controls. The fungus was re-isolated from the leaf lesions that developed following inoculation. The isolates appeared identical to the isolates from the post-inoculation activity study.

Sequencing of amplified DNA from the *Cercospora* isolate, which had been isolated from symptomatic spinach plants in the post-inoculation study, resulted in a 510 bp band from the intergenic spacer regions ITS1 and ITS2 of the 5.8S ribosomal RNA gene. The alignment with homologous sequences of various *Cercospora* species from GeneBank revealed that the sequence of the isolate is identical to those of *C. apii, C. beticola,* and *C. nicotianae* (Table 6).

DISCUSSION

The genetic homology of the fungus isolated from the symptomatic plants to other *Cercospora* isolates, including *C. apii, C. beticola,* and *C. nicotianae* is consistent with the analysis of Goodwin *et al*., 2001 [10], who also found that several isolates of various *Cercospora* species did not differ in their DNA sequence of the aforementioned region. Thus, by DNA analysis based on the 5.8S rRNA gene, ITS1, and ITS2 it is apparent that the *Cercospora* isolate from the post-inoculation study is in the *Cercospora* group comprising the species mentioned above. To my knowledge, *C. apii* and *C. nicotianae* have not been reported to be pathogenic on spinach.

The consistent association of *Cercospora sp.* with leaf spot in the post-inoculation study, and the ability of the fungus to cause the disease in artificially-inoculated spinach

plants, and the recovery of *Cercospora sp*. from the diseased tissue of the artificially inoculated plants fulfills Koch's postulates and indicates that the fungus is the primary cause of the leaf spot disease in the post-inoculation study.

Pyraclostrobin and azoxystrobin provided post-inoculation activity when applied within four days following inoculation. However, the use of pyraclostrobin provided a greater level of disease control compared to azoxystrobin. The efficacy of the fungicide zoxamide may be limited to the control of Oomycete fungi and therefore provided only limited disease control when used either preventively or post-inoculation against Cercospora leaf spot. Maneb preformed as would be expected of a protectant fungicide providing good pre-inoculation protection and little post-inoculation activity.

Results on the post-inoculation activity of the fungicides against Cercospora leaf spot in this study are similar to those reported for these fungicides against spinach white rust [17]. Sullivan reported complete control of spinach white rust when azoxystrobin and pyraclostrobin were used up to one and three days after inoculation, respectively. Post-inoculation activity continued against white rust for up to three days after inoculation for azoxystrobin and at least four days after inoculation for pyraclostrobin. For maneb applied one day after inoculation, Sullivan reported white rust severity to be only 20% compared to 83% for the control. This may not be due to post-inoculation activity, but to the 12-hour wet and 12-hour dry cycle used in the study. It is possible that after the first 12-hour favorable period ungerminated sporangia survived the 12-hour unfavorable period to infect during the next 12-hour favorable period. In this study a single 24-hour favorable temperature and wetness period was used, and maneb showed no post-inoculation activity against Cercospora leaf spot.

Numerous foliar diseases are economically important to spinach production. Spinach white rust is the major foliar disease of spinach in Oklahoma [6] and downy mildew (or blue mold) is probably the most widespread and potentially destructive disease of spinach worldwide [4]. Other fungal foliar diseases of economic importance include Anthracnose, caused by *Colletotrichum dematium* (Pers.) Grove f.sp. *spinaciae* (Ellis & Halst.) Arx (= *C.spinaciae* Ellis & Halst.), *Cladosporium macrocarpum* G, Preuss, *Alternaria sp., Stemphyllium sp*., and *Cercospora beticola* Sacc. [4]. Azoxystrobin, pyraclostrobin, and zoxamide have been shown to be effective against spinach white rust (Chapter 2). Results of this and an earlier study (Chapter 2) indicate that zoxamide has only limited efficacy against Cercospora and Cladosporium leaf spots while azoxystrobin and pyraclostrobin provided good control of these pathogens. Zoxamide is active against Oomycete fungi, and is registered for foliar use on potatoes and grapes to control late blight and downy mildew, and has good activity against spinach downy mildew.

A weather-based spray advisory program has been developed to predict outbreaks of spinach white rust [19]. This model uses a 12-hour threshold of favorable temperature and wetness periods to schedule a fungicide application. It is known that most fungal pathogens need periods of free moisture to germinate and cause infection. Therefore the white rust advisory may be effective in predicting outbreaks of foliar diseases other than white rust. In the artificial inoculations in this study, Cercospora leaf spot developed to severe levels at both the 15º C and 24º C incubation temperatures. While 15º C is within the optimum range for spinach white rust development, 24º C is well above that optimum. Therefore, the advisory program may not be effective on Cercospora leaf spot

when temperatures a warm. Because white rust can develop during a 12-hour period of favorable temperature and wetness, and because it may not be possible for a grower to respond immediately to a spray advisory the post-inoculation activity of the fungicides may be important in achieving optimum disease control.

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Table 1. Effects of application timing on control of Cercospora leaf spot with maneb.

Fungicide (Rate)	Timing[1]	Disease incidence (%)[2]	Disease severity (%)[3]
Maneb (2.4 g/L)	-1	9.4 a	0.13 a
	0	0.0 b	0.00 a
	1	96.9 cd	5.49 bc
	2	81.3 c	3.13 b
	3	100.0 d	5.22 bc
	4	87.5 cd	3.78 b
	5	90.6 cd	4.75 bc
No treatment		100.0 d	6.25 c

[1] Maneb was sprayed to runoff one day before (-1), immediately before (0), and one to four (1-4) days after inoculation with conidia of *Cercospora spp.*
[2] The percentage of leaves with symptoms.
[3] The percentage of leaf area with symptoms.
[4] Least square means in a column followed by the same letter are not significantly different (P≤0.05) as determined by Fishers least significant difference (LSD) test.

Table 2. Effects of application timing on control of Cercospora leaf spot with pyraclostrobin.

Fungicide (Rate)	Timing[1]	Disease incidence (%)[2]	Disease severity (%)[3]
Pyraclostrobin (0.46 g/L)	-1	0.0 a	0.00 a
	0	0.0 a	0.00 a
	1	0.0 a	0.00 a
	2	0.0 a	0.00 a
	3	59.4 b	1.13 ab
	4	68.7 b	2.13 b
	5	93.8 c	5.16 c
No treatment		100.0 c	6.25 c

[1] Pyraclostrobin was sprayed to runoff one day before (-1), immediately before (0), and one to four (1-4) days after inoculation with conidia of *Cercospora spp.*
[2] The percentage of leaves with symptoms.
[3] The percentage of leaf area with symptoms.
[4]Least square means in a column followed by the same letter are not significantly different (P≤0.05) as determined by Fishers least significant difference (LSD) test.

Table 3. Effects of application timing on control of Cercospora leaf spot with azoxystrobin.

Fungicide (Rate)	Timing[1]	Disease incidence (%)[2]	Disease severity (%)[3]
Azoxystrobin (0.92 g/L)	-1	0.0 a	0.00 a
	0	0.0 a	0.00 a
	1	3.0 b	0.09 b
	2	84.4 c	3.49 c
	3	81.3 c	2.75 c
	4	81.3 c	4.16 c
	5	93.8 cd	6.03 d
Control		100.0 d	6.25 d

[1] Azoxystrobin was sprayed to runoff one day before (-1), immediately before (0), and one to four (1-4) days after inoculation with conidia of *Cercospora spp.*
[2] The percentage of leaves with symptoms.
[3] The percentage of leaf area with symptoms.
[4] Least square means in a column followed by the same letter are not significantly different (P≤0.05) as determined by Fishers least significant difference (LSD) test.

Table 4. Effects of application timing on control of Cercospora leaf spot with zoxamide.

Fungicide (Rate)	Timing[1]	Disease incidence (%)[2]	Disease severity (%)[3]
Zoxamide (0.46 g/L	-1	65.6 a	2.41 a
	0	75.0 ab	2.56 a
	1	75.0 ab	2.22 a
	2	90.7 bc	3.65 ab
	3	90.7 bc	5.75 c
	4	93.8 c	5.81 c
	5	100.0 c	5.06 bc
Control		100.0 c	6.25 c

[1] Zoxamide was sprayed to runoff one day before (-1), immediately before (0), and one to four (1-4) days after inoculation with conidia of *Cercospora spp.*
[2] The percentage of leaves with symptoms.
[3] The percentage of leaf area with symptoms
[4] Least square means in a column followed by the same letter are not significantly different (P≤0.05) as determined by Fishers least significant difference (LSD) test.

Table 5. Published sequences from the 5.8S ribosomal RNA gene and the intergenic spacer regions ITS1 and ITS2for various *Cercospora* species.

Cercospora species	**Isolate**	**GenBank no.**
Cercospora isolate from spinach	-	-
C. beticola	CB4	AY266165
C. nicotianae	CN17	AY266159
C. canescens	CCA19	AY266164
C. hayi	CH5	AY266163
C. kikuchii	CK35	AY266161
C. sojina	CS13	AY266158
C. asparagi	-	AF297229
C. violae	STE-U 2222	AF362069
C. caricis	CG666	AF284388
C. apii	CA1	AY266168

Table 6. Alignment of homologous sequences from the 5.8S ribosomal RNA gene and the intergenic spacer regions ITS1 and ITS2 for various *Cercospora* species from GeneBank

Cercospora species	**Genetic distance to spinach isolate***
C. apii	0
C. beticola	0
C. nicotianae	0
C. kikuchii	0.0023
C. asparagi	0.0023
C. canescens	0.007
C. hayi	0.007
C. sojina	0.007
C. violae	0.0359
C. caricis	0.0513

* Based on DNA analysis of the 5.8S rRNA gene and the ITS1 and ITS2 region using DNADIST

Figure 1. Symptoms of Cercospora leaf spot following artificial inoculation.

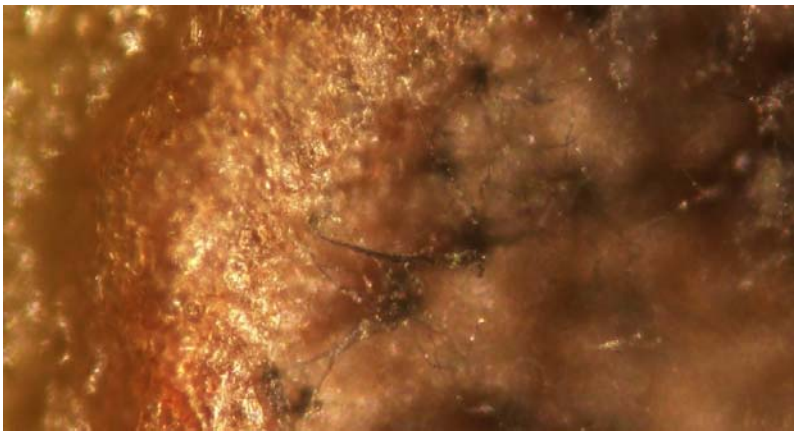

Figure 2. Conidiophores of *Cercospora* spp. (200 x magnification)

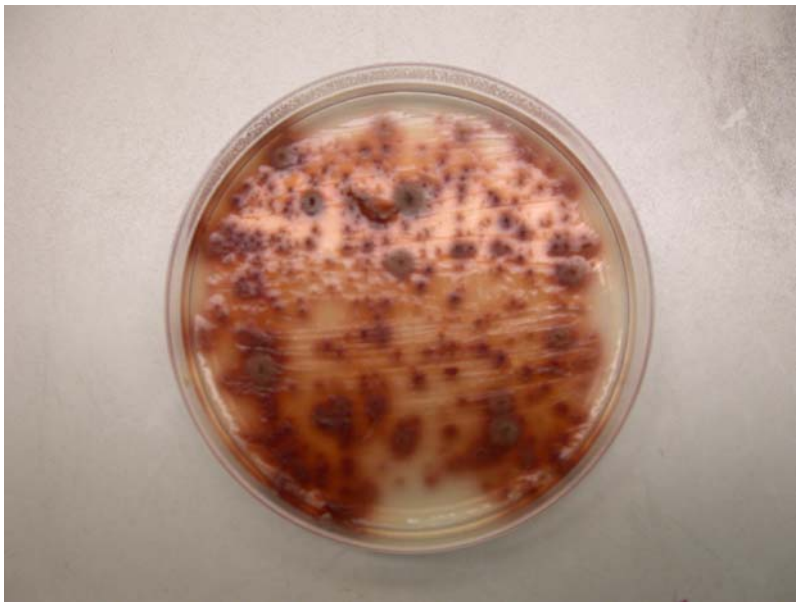

Figure 3. Seven-day-old *Cercospora* spp. in pure culture on PC

VITA

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Master of Science

Thesis: ETIOLOGY AND MANAGEMENT OF SPINACH WHITE RUST

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