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UNIVERSITY OF NEBRASKA COLLEGE OF AGRICULTURE
AGRICULTURAL EXPERIMENT STATION

W. V. LAMBERT, Director E. F. FROLIK, Associate Director

Research Bulletin 180

False Smut of Buffalograss

JOHN L. WEIHING

Department of Plant Pathology

LINCOLN, NEBRASKA
MARCH, 1956

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SUMMARY

Studies on false smut of buffalograss were conducted at the Nebraska Agricultural Experiment Station from 1950 to 1954.

Under natural conditions, false smut (caused by *Cercospora seminalis* Ell. & Ev.) of buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) causes the formation of black spheres 1 to 3 mm. in diameter at the top of the pistillate inflorescences. The spheres consist of indeterminate conidiophores borne on black flask-shaped stromata that are confined within the ovary cavities of spikes in place of the caryopses. Abundant conidia are formed singly and terminally in layers. Beneath each layer and just below the points of spore abscission, branches arise that in turn bear conidia at another level. This process is repeated several times on a sphere. Numerous chlamydospores are formed in the hyphae of the stromata.

The pathogen was easily isolated and grown on such media as potato dextrose agar, but it did not sporulate. At the surface of a nutritive agar medium, the pathogen produced a leathery, sometimes wrinkled, black mat from which whitish or greyish aerial hyphae frequently developed. Chlamydospores, which lived for at least four years, were formed abundantly throughout the mycelium. The rate of growth of the fungus was relatively slow, requiring about six weeks for a colony to cover the surface of a 90-mm. petri plate. Optimum growth was obtained between pH 5 and 6 at temperatures of 25° to 30° C.

Variations in lighting, hydrogen-ion concentration, temperature, organic and inorganic media, and even injury to the mycelium did not stimulate cultures to sporulate. Cultures sporulated only after (1) growing the fungus on red clover stems, soybean stems, or soybean pods, (2) permitting these cultures to dry out for at least three months, and (3) then submerging the colonies in potato-dextrose agar. From within the colonies which grew from these dried cultures, mycelial columns arose at random and produced typical conidia.

False smut occurred only in non-pollinated ovaries of buffalograss; fertilization completely inhibited its development. Microscopic examination disclosed that the initial entry point of the pathogen was generally in the stigmas. Following entrance, the fungus grew intercellularly down the stigma and style and into the ovary. During the first few days following infection, the pathogen grew throughout the ovary wall and ovule but did not attack the funiculus, rudimentary anthers, or accessory floral structures. It did eventually displace the entire ovary. The mycelium grew upward past the summit of the palea, and immediately upon passing to the outside became a mass of indeterminate branching conidiophores forming a spherical head that was first noticeable two to three weeks following infection.

After infected spikes mature, they break free and fall to the ground where the floral accessories eventually decompose, releasing the stromata. As the stromata disintegrate, numerous viable chlamydospores undoubtedly are released onto the soil. The inoculum for spring infection comes from soil infested with both chlamydospores and conidia.

The discovery that syngamy completely inhibited infection by *Cercospora seminalis* Ell. & Ev. led to the study of the application of growth regulators for the control of false smut. In both greenhouse and field trials, 2,4-dichlophenoxyacetic acid (2,4-D) gave good control. The growth regulator did not seriously interfere with subsequent fertilization of the egg, formation of seed, or its subsequent germination. The effect of the 2,4-D on control of the parasite was not that of toxic action but rather it induced a change that caused the host to become incompatible with the fungus.

False Smut of Buffalograss

JOHN L. WEIHING[1]

DESCRIPTION OF THE DISEASE AND CHARACTERISTICS OF THE CAUSAL ORGANISM IN CULTURE[2]

FALSE SMUT of buffalograss, *Buchloe dactyloides* (Nutt.) Engelm., caused by *Cercospora seminalis* Ell. & Ev. is a disease that destroys the unfertilized ovary. The disease was first reported by J. B. Ellis and B. M. Everhart in 1888 *(1)*. They gave only a brief description of the disease and named the causal fungus *Cercospora seminalis*. There are no etiological studies of false smut recorded in the literature. It has been referred to in the Plant Disease Reporter *(7)*, and a brief description is given by Sprague *(6)*. The origin of the term "false smut" is unknown to the author, but it so descriptively fits the general appearances of the disease that one readily understands its usage. The following studies were conducted from 1950 to 1954.

The Host

Buffalograss is an important, low-growing, stoloniferous, dioecious (occasionally monoecious) grass indigenous to the semiarid plains region of the United States. The pistillate plants produce spikelets in clusters of three to five in a short spike or head. There are usually one to three spikes to an inflorescence (figure 1). Each spikelet has an outer thick, rigid, rounded glume and usually a narrow, thin inner glume. The lemma and palea are thin, membraneous, three-veined structures, broad at the base and narrow at their summits. The palea envelopes the unfertilized ovary or caryopsis. The ovary is a small spherical or somewhat oblong body 0.8 to 1.0 mm. in diameter. It supports two elongated styles bearing relatively large, much-branched, purple stigmas (figure 3).

Description of the Disease

The first macroscopic evidence of false smut is the appearance of a black spherical structure atop the female spike (figure 2). The ball-like structure varies in size but usually is one third to one half as large

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[1] Extension Plant Pathologist, University of Nebraska.

[2] A portion of a thesis presented to the Graduate College of the University of Nebraska in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The author wishes to express his appreciation to Dr. J. E. Livingston under whose supervision this work was done.

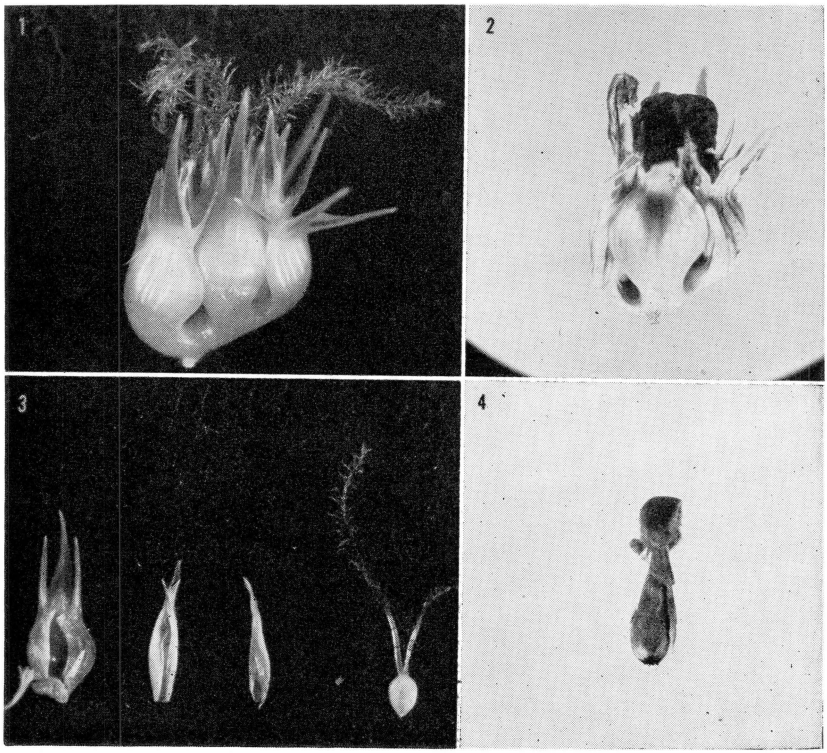


FIGURE 1.—A single female buffalograss spike showing three spikelets borne on a short, thick rachis. The long hairy structures are purple stigmas.

FIGURE 2.—An infected spike showing the black smut-like sphere that is so characteristic of the disease.

FIGURE 3.—Parts of a single female spikelet: (left to right) thick, heavy outer glume with paper-thin inner glume hanging downward from lower left corner; the lemma; the palea; and the ovary, which is supporting two elongated styles bearing long hairy stigmas.

FIGURE 4.—A black stroma of *C. seminalis* dissected from an infected spikelet. Note its characteristic flask shape. The sphere atop the stroma is composed entirely of indeterminate conidiophores whereas the stroma has many chlamydospores. All figures magnified X 10.

as the spike. The sphere is not hard but rather spongy and velvety to the touch. Upon dissection of the spikelets, one or more of the ovary cavities are found to be completely filled with a black, flask-shaped spongy fungus mass that becomes hard and brittle as the tissues of the spike mature (figure 4). The neck of the fungal mass protrudes through the small orifice at the summit of the palea and supports the bulbous structure.

Anatomical Features of the Fungus Mass

Microscopic examination of the black fungus mass revealed the bulbous top to be an asexual spore-producing structure borne upon a stroma. The crushing of a stroma, or any portion of it, for detailed microscopic examination proved unsatisfactory because the tissues and hyphae became obscured by liquid globules that were released upon the disruption of hyphal cells. Free-hand sectioning of a stroma could not be made because of its extreme hardness and brittleness.

Histological methods

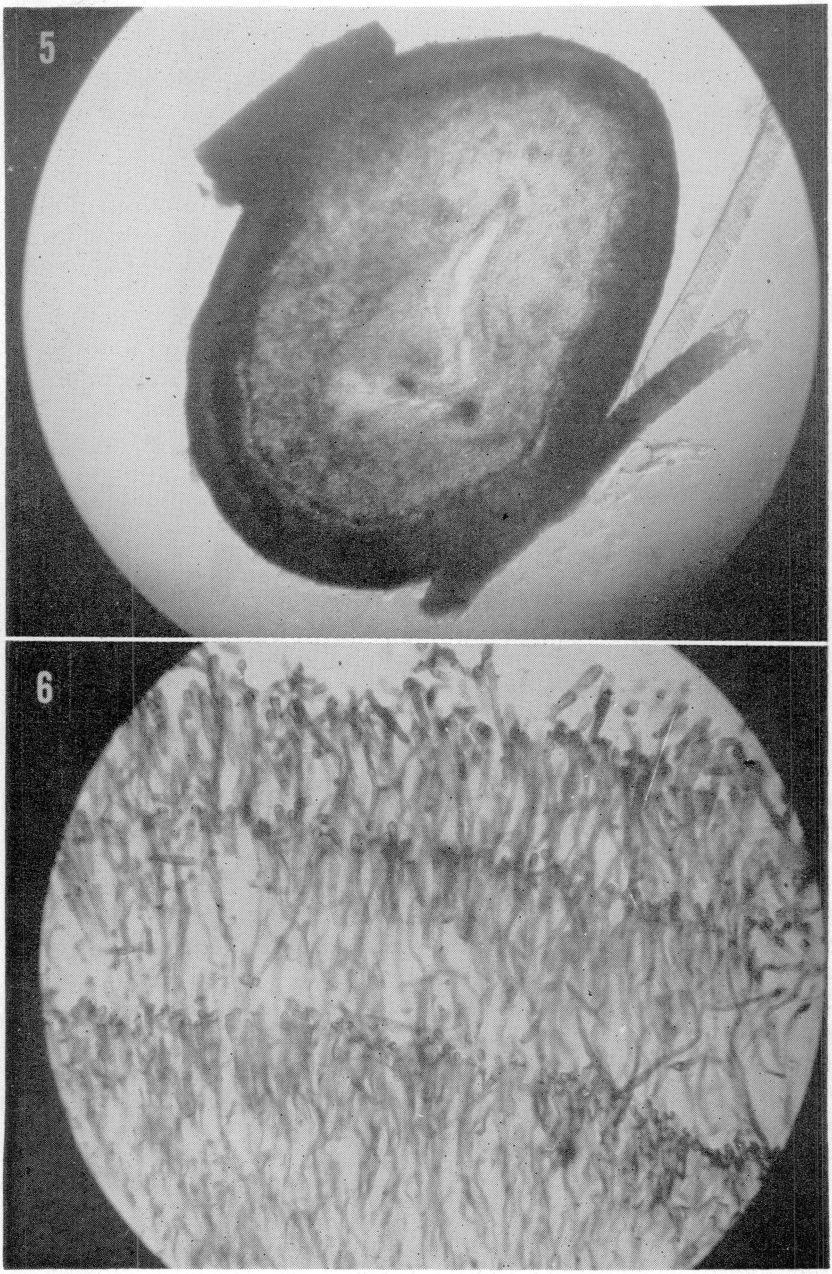
Thin cross sections of stromata for microscopic observations were obtained by first permitting the stromata to soak 24 to 48 hours in water. After soaking, they were placed in melted paraffin which was quickly cooled in ice water. The embedded stromata were then sectioned at 25 μ. The sections were subsequently passed through an alcohol series and mounted in Canada balsam. Such a procedure produced very clear sections in which the elements retained their natural size and color.

Mounting fresh sections directly in lacto-phenol containing methylene blue was a satisfactory temporary stain for protoplasm. Permanently stained mounts were prepared by passing the sectioned tissues through an alcohol series, staining with fast green in absolute alcohol for 15 to 20 seconds, washing thoroughly with xylene, and mounting in Canada balsam. The protoplasm and hyphal walls which had only a slight amount of a natural brown pigment, stained green. Dark brown hyphal walls were not affected by the stain.

Microscopic observations

The mycelium of the stroma is very compact, is highly septate, and has brown to very dark brown walls. A cross section through the center of a stroma revealed two distinct layers of mycelium; (1) an outer layer in which the hyphae were, in general, oriented vertically and (2) an inner layer in which direction of hyphal growth was not definitely oriented and the hyphae were greatly entwined. The positions of the previously existing ovary structures, the nucellus, integuments and ovary wall were easily discernible in cross section. As will be shown and discussed later, initial mycelial growth in the ovary is associated with the cell walls. As a consequence of this characteristic, the positions of the initial ovary parts are well outlined in cross section (figures 5 and 7).

The outer layer was composed of hyphae that had developed between the ovary and the enclosing palea. These hyphal walls were a deeper brown color than those that had developed in the tissues of the ovary. The hyphae in the former area of the ovary were greatly entwined because of having first followed the cell walls and then later



FIGURES 5 and 6.

FIGURE 5.—Photomicrograph of a cross section of a stroma of *C. seminalis*. X 75.

FIGURE 6.—Longitudinal section of the smut-like ball that forms atop the pistillate inflorescence infected with *C. seminalis*. X 300. The entire head is a mass of indeterminate conidiophores. The dark bands are the result of contiguous conidial production and simultaneous spore abscission.

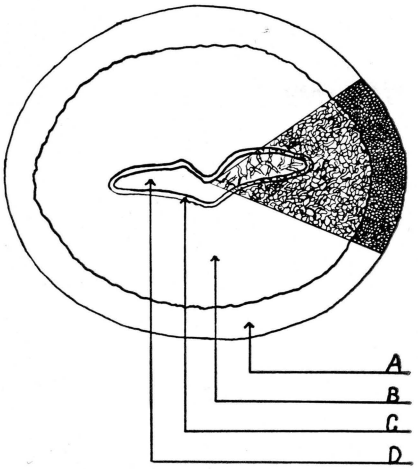


FIGURE 7.—Diagrammatic illustration of a cross section of a stroma. (A) Originally the area between ovary wall and palea; the hyphae are generally oriented parallel with the long axis of the ovary and their walls are a very dark brown. (B) Originally the area of the ovary wall; the mycelium is compact and twisted, has numerous chlamydospores, and its cell walls are a brown color. (C) Originally the area of the integuments; mycelial growth is comparatively sparse. (D) Originally the area of the nucellus; the hyphae are rather sparse and their walls generally are light brown.

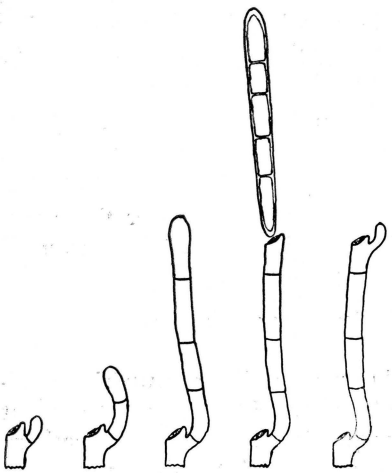


FIGURE 8.—Illustration of the process by which a single conidiophore of *C. seminalis* is capable of producing several conidia.

having traversed the cell cavities. Chlamydospores were abundant in the hyphae of the stroma.

The mycelial strands in the neck of the stroma were all oriented parallel to the long axis of the stroma. The bulbous structure was composed entirely of indeterminate conidiophores that had grown outward radially. In either cross or longitudinal section, several uniformly dark bands appeared where spore abscission had occured simultaneously and at definite intervals on the hyphae (figure 6). Immediately below each spore abscission scar of the individual conidiophore, a branch arose which in turn produced a spore (figure 8). This process was repeated several times so that as many as six dark bands often were seen in cross section of a bulbous head. The conidiophores did, however, produce other branches as well as the one which always arose below the point of spore abscission (figure 9-A). The number of spore-bearing layers varied with the size of the heads, the larger ones having a greater number. Very rarely were spores seen to break free from the conidiophores, since they very readily abscissed at maturity. The spore abscission wall characteristically formed at an angle to the long axis of the conidiophores. Although the over-all mass of conidiophores appears black to the naked eye, actually the walls of the individual hyphae are brown.

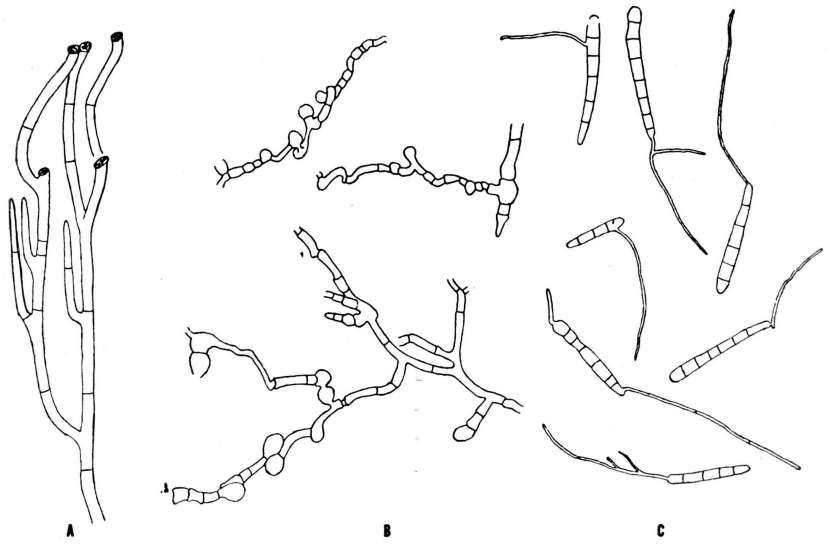


FIGURE 9.—Camera lucida drawings of *Cercospora seminalis*. (A) A single, branched conidiophore. The darkened circular areas represent points of spore abscission. Note that spore abscission occurs only at certain levels. (B) Mature mycelium; the larger, spherical cells are chlamydospores. (C) Germinating conidia; the drawings were made eight hours after the spores had been seeded on water agar and kept at 25° C.

Mature conidia were elongate and slightly obclavate, averaging about 95 μ in length by 5 μ in width. The range in length of conidia based on 100 measurements of random spores was from 47 to 140 μ. Width of spore varied from 3.4 to 6.8 μ, with shorter spores being wider than the longer ones. The average number of septations per spore was four with the range being from 1 to 8.

Isolation of the Fungus in Pure Culture

Cercospora seminalis was easily isolated in pure culture by surface sterilizing stromata in 70 per cent ethanol for one minute and placing them on 3 per cent water agar. Five to seven days later there was adequate mycelial growth, so that hyphal tips could be transferred free of contaminants to a nutritive agar medium.

Cultural Characteristics of Cercospora Seminalis

Colony characteristics

Cercospora seminalis grows readily on an ordinary nutritive medium such as 2 per cent potato-dextrose agar (PDA), but it does not produce conidia. As the colony enlarges, it forms a black, leathery circular mat on the surface of the agar medium. The black mat often buckles and twists, causing the surface to have a crumpled appearance. Frequently, tufts of whitish mycelium rise above the mat, and

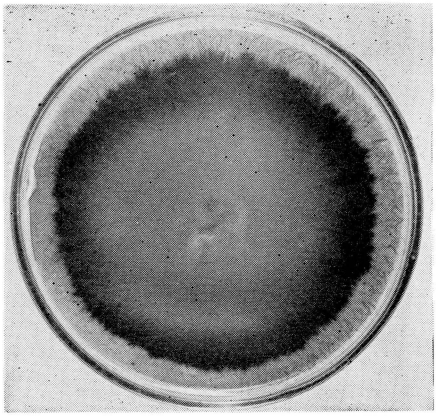


FIGURE 10.—A two-month-old colony of *C. seminalis* growing on potato-dextrose agar. The mycelium at the surface is black whereas the aerial hyphae are white to gray.

occasionally the entire surface is covered with a downy, white or grey mycelium (figure 10). The rate of growth of *C. seminalis* is rather slow. At 25° C. it takes approximately six weeks for a colony to grow to a diameter of 10 cm.

Mutations occasionally arose in culture. The most obvious variants were those that had an increased growth rate or loss of color. They were not tested for pathogenicity.

Mycelial characteristics

The very young hyphae are hyaline, septate, and branching and have a rather granular cytoplasm. As the hyphae age, they become highly septate and larger, and their walls begin to show a brownish pigmentation. The pigment continues to darken with age, causing the colony to appear black. Numerous spherical to cylindrical chlamydospores are formed singly or in chains throughout the mycelium (figure 9-B). The average diameter of the spherical chlamydospores is approximately 10 μ, whereas the dimensions of the cylindrical type are 10 X 20 μ. The chlamydospores have relatively thick walls compared to the thin walls of the mother hyphae, which are 2 to 4 mm. in diameter.

Longevity in culture

A remarkable feature of *C. seminalis* is its ability to remain alive for years in a dried state. At the outset of this study, several petri dish cultures of an original isolate growing on PDA were set aside in the laboratory at 25° C. In time they dried down to a thin, tough, brittle sheet on the bottom of the petri dish. Approximately every six months, over a period of four years, the cultures were tested for viability by transferring a small piece of the dried mycelium to fresh PDA. Each time growth readily occurred.

Conidia and chlamydospore germination

Conidia germinated readily either in tap water or on water agar. Spores collected from naturally infected buffalograss flowers began to germinate three hours after being placed either in water or on water agar, and in six hours approximately 90 per cent of them had germinated. A spore produced one, two or even three germ tubes (figure 9-C).

Six hours following germination the germ tubes were 250 to 300 μ in length, contained several septa, and were approximately one μ in diameter. After 24 hours, a highly septate and branched mycelium had formed.

Conidia from naturally infected plants stored at 25° C. in the laboratory began to lose their ability to germinate after several months. In six months, germination was less than 50 per cent, and it was completely absent after 12 months. On the other hand, conidia that were on naturally infected material outdoors remained viable for a longer period of time. More than 90 per cent of the spores from collections made as late as December 28 germinated. Unfortunately, a check on spore viability could not be made throughout the winter, since the spore-producing heads atop spikes deteriorated so badly that spores could not be found.

The chlamydospores produced on artificial media evidently must either dry and/or age before they will germinate. Those from cultures in which the agar medium still had sufficient moisture to support growth would not germinate; however, chlamydospores from colonies that had stopped growing because the agar medium was too dry started germinating in 8 to 10 hours after being placed on water agar. In 24 hours, the chlamydospores produced highly septate hyphal strands several hundred μ in length.

Effect of various hydrogen-ion concentrations

Two tests were conducted on the reaction of *C. seminalis* to various H-ion concentrations, one on a solid nutritive medium devoid of any buffers and the other in an aerated broth that contained phosphate buffers. Growth on the solid medium was measured by increase in colony diameter and that on the broth as dry weight of the mycelium. The results of both tests showed that hydrogen-ion concentration for optimal growth is between pH 5.0 and 6.0. A second, but lesser, optimum was reached on the alkaline side at pH 8.0.

Effect of temperature on growth

The organism was grown on PDA at temperatures 5, 10, 15, 20, 25, 30, and 35° C. Best growth was obtained at 25° C., but since growth at 30° C. nearly equaled that at 25° C., the optimum is probably between these two temperatures.

Inducing Sporulation in Culture

Cercospora seminalis does not sporulate readily on a common nutritive medium such as PDA. Manipulation of temperature, nutrition, light, moisture, and hydrogen-ion concentration were ineffective in the induction of sporulation.

During the course of trials, the fungus was grown on natural media such as stems of red clover and stems and pods of soybeans. The cul-

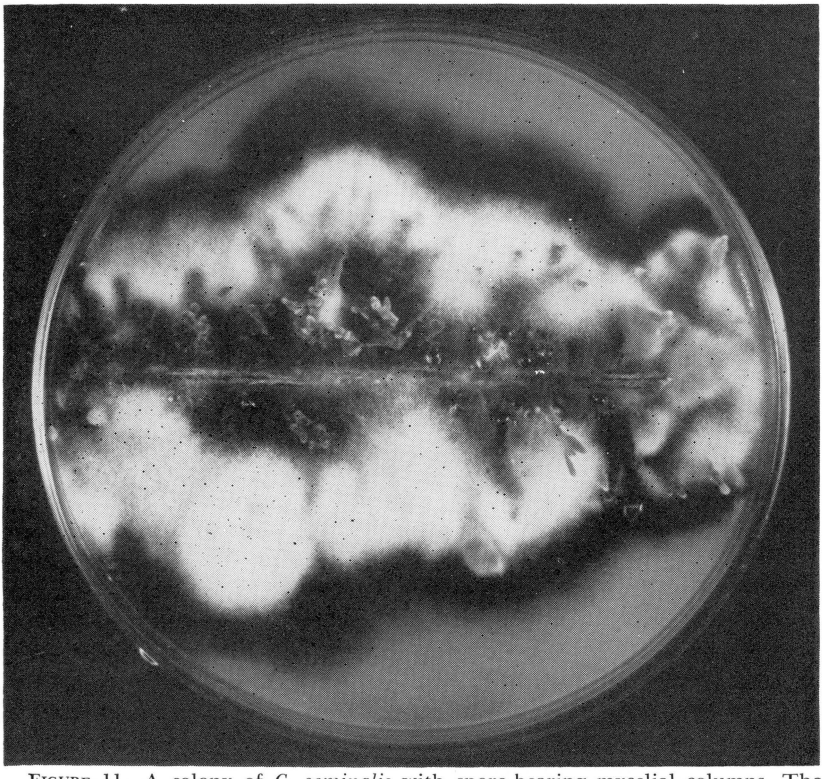


FIGURE 11.—A colony of *C. seminalis* with spore-bearing mycelial columns. The originally inoculated red clover stem can still be seen.

tures were not discarded following the observational period for spore production, but instead they were permitted to dry under laboratory conditions. One year later, the remains of six old, dried, colony-bearing stems of sweetclover, red clover, and soybeans were submerged in about 30 ml. of melted PDA in petri dishes and subsequently stored at 25° C. All cultures were viable and showed growth in two days. In approximately seven days, upright columns of mycelium appeared in the revived cultures. The columns continued to elongate with their final height frequently limited by the petri dish cover. They appeared slightly knobby, were dark grey to black in color, and arose at random over the surface of the colony except near the edges (figure 11). Examination revealed these structures to be bearing a few spores. The spores were typical in size and shape of these produced under natural conditions on buffalograss spikes.

The importance of drying to subsequent spore production was studied by the following procedure. Two pieces of red clover or soybean stems or soybean pods were placed in test tubes containing 5 ml. of sterile water. This material was sterilized by autoclaving and subse-

quently seeded with the pathogen. After three weeks, the sterile tissues were covered with mycelium. At this time, one of the two pieces of plant material was removed from each of the tubes and submerged in PDA. No upright columns of mycelium developed and consequently no spores. The rest of the material in the test tubes was permitted to dry. Three months following the time of inoculation, these dried pieces were transferred to PDA in petri dishes. In five to six days, mycelial columns began to appear, and in two weeks, numerous columns had developed and were producing spores. Spore production was sparse, however. This test thus indicated that for spore production, either aging or drying, or both, of the inoculated plant tissues is necessary before they are embedded in PDA.

The following test was made to determine if the drying process had influenced sporulation. One-quart glass milk bottles were half filled with either soybean stems or soybean pods. Water was added to these bottles until they were approximately one fourth to one third full. The purpose of the excessive amount of water was to maintain the plant tissues in a moist condition for a period of at least several months. The bottles of tissues were steam-sterilized and subsequently seeded with the pathogen. Mycelium developed very well on the plant tissues above water but did not grow below the surface of the water. A mat of mycelium formed over the water's surface, thereby greatly reducing water loss through evaporation. Six months following inoculation, at least half of the original amount of water still remained even though the cultures had been subjected to three months of hot summer weather. At this time, six pieces each of soybean stems and pods were taken from the bottles and implanted in PDA. The resultant growth did not produce any mycelial columns, thus indicating that drying out of the inoculated plant tissues is a contributing factor to the stimulation of spore-producing columns.

Structure of the Spore-Producing Columns

Small blocks of mycelium about 10 mm. square containing mycelial columns were embedded in paraffin, serially sectioned, and stained with orange G.

In longitudinal section, the mycelial column appeared as an extension of the aerial hyphae immediately above the black mycelial mat. The hyphae composing the column generally grew parallel with the column. At various levels, however, hyphae at the edge of the column fanned out slightly. The hyphal walls were typically brown to dark brown in color. Spores were found adjacent to the fanned-out areas, and some spore abscission scars were noted on the mycelium. Possibly, the principal spore production occurs at these areas. Numerous chlamydospores were present in the older mycelium but none in the young hyphae at the tip of the column.

ETIOLOGY AND PATHOGENESIS

Inoculation Studies

Successful inoculations were made with mycelium, conidia, or chlamydospores of *Cercospora seminalis* Ell. & Ev. In each trial, the inoculum was placed on the stigmas of buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.) and the inoculated plants placed at 21° C. for 48 hours. The source of each type of inoculum was as follows: mycelium, a petri-dish colony shredded in a Waring Blendor with 100 ml. of water; conidia, an aqueous suspension of spores obtained from infected spikes; chlamydospores, stromata crushed to free the chlamydospores and water added to make a suspension. Two to three weeks after inoculation, it was noted that infected ovaries were replaced by the dark mycelia of *C. seminalis*. It was necessary to remove the ovaries from the floral parts to reveal infection, since in the greenhouse the disease failed to produce a dark-colored sphere atop the spikelet. Approximately 50 per cent of the ovaries became infected with each type of inoculum.

Method of Overwintering and Dissemination of the Pathogen

Viability of the pathogen throughout the winter and spring

Mature, naturally infected spikes showing infection were collected in December, February, March, and April. From each collection, a stroma was dissected from each of five spikes and tested for viability. The stromata from all collections were viable, thus indicating that the pathogen is not harmed by cold weather.

Soil infestation by the pathogen

Soil was tested for the presence of the pathogen. In the spring, ½ inch of the topsoil was obtained from the buffalograss plots. The soil was pulverized and the dust passed over four flowering buffalograss plants until the stigmas were well covered with soil. Two of the plants were placed in a humidity chamber for 48 hours, while the other two were left in the greenhouse which had a very low relative humidity. Two other plants that did not receive the dust treatment were placed in the humidity chamber as checks. After three weeks, the ovaries were examined for infection. Twenty-five per cent of the ovaries whose

TABLE 1.—Amount of false smut obtained when soil was taken from buffalograss plots in early spring and dusted onto buffalograss stigmas.

Treatment	Number of ovaries	Placed in humidity chamber for 48 hours	Percentage infection
Soil dusted onto stigmas	24	Yes	25
Soil dusted onto stigmas	25	No	0
No dust	21	Yes	0

stigmas were dusted with soil and placed in the humidity chamber for 48 hours became infected (table 1). The checks, i.e., the flowers that were dusted but not placed in the humidity chamber, were not infected. These results showed that the pathogen is capable of surviving on or in the soil during the winter and that moisture is necessary for infection.

Effect of Pollination on Infection

During some of the preliminary studies of the pathogenesis of the disease, infected spikes were collected from a stand of buffalograss containing both male and female plants. It was noticed that some of the spikes contained healthy seed in one spikelet and an infected ovary in the adjacent spikelet (figure 12). There was no apparent reason why such seed did not become infected, particularly since the spore-bearing mycelial heads of adjacent infected ovaries were shedding numerous spores directly above them. Because of this phenomenon, the effect of pollination on infection by *C. seminalis* was investigated.

The inoculum employed was an aqueous suspension of shredded mycelium of *C. seminalis*. The stigmas of six spikes containing 32 spikelets were dusted with pollen. Eleven hours later these pollinated

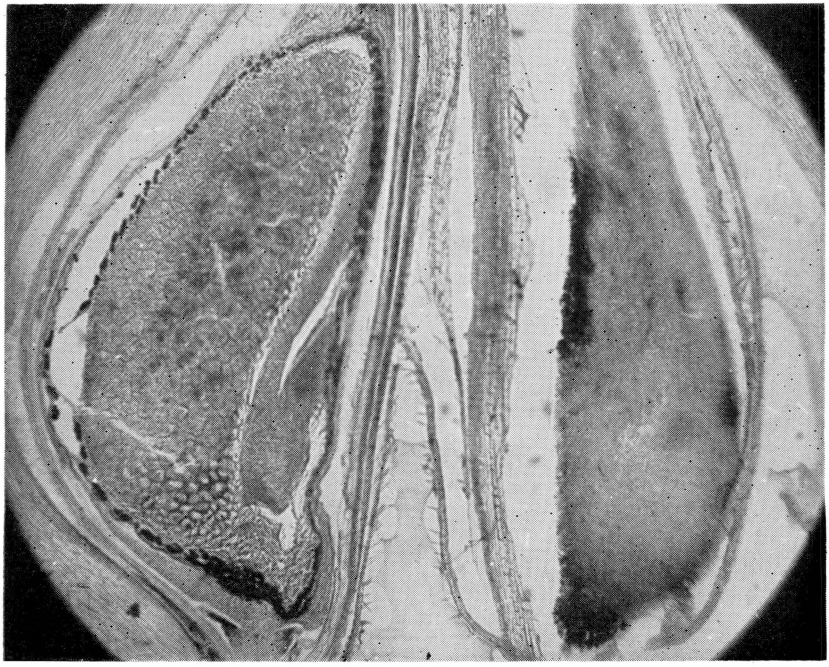


FIGURE 12.—Photomicrograph of a cross section of a spike of buffalograss showing one spikelet (left) with a normal, healthy caryopsis, and another (right) containing a mass of mycelium of *C. seminalis* that has replaced the ovary. X 75.

flowers, as well as four non-pollinated spikes containing 20 spikelets were inoculated with the mycelial suspension by means of a camel's-hair brush. All inoculated spikes were placed at 100 per cent relative humidity for 48 hours and observed 16 days later for infection.

No infection was obtained among the ovaries of the pollinated flowers, whereas 75 per cent of the non-pollinated ones became infected. This clearly indicated that infection did not occur following pollination.

Pathogenesis of the Disease

An investigation was made to establish where, in the flower, infection takes place and to determine the progressive development of the disease thereafter. As a basis for the discussion of parasitism that follows, a brief description of the ovary and its accessory parts is given. A more detailed account of the development of the buffalograss ovary is given by Harlan (*3*).

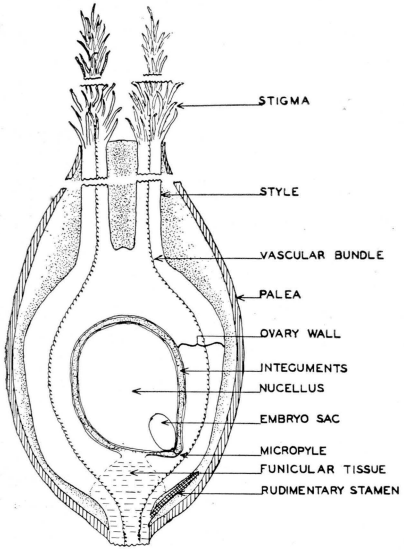


FIGURE 13.—Diagram of a cross section of a buffalograss ovary and accessory parts.

Description of the ovary and its accessory structures

The ovary prior to fertilization is spherical to oblong and about 0.8 to 1.0 mm. in diameter (figure 13). Subtended by the ovary are two elongated styles, which in turn bear large, highly branched, purple stigmas. Three rudimentary stamens are located at the base of the ovary. The ovary wall consists of an outer and inner epidermis and an adjacent layer of parenchyma several cells thick. Passing through the cell wall are two vascular bundles each supplying a style and a stigma. The ovule is covered by two integuments, each one-cell thick. The nucellus is composed of large thin-walled cells. The embryo sac is a turgid, transparent, spherical structure.

Penetration of floral parts and development of pathogen within the ovary

Preliminary histological studies revealed that infection occurred in the stigmas and that the pathogen passed down through the style and into the ovary. The following histological techniques were employed for the examination of internal portions of the ovary.

Methods and materials.—During the course of study it was found that buffalograss flowers remained in good condition and susceptible to *C. seminalis* for 7 to 10 days when detached with approximately 1 inch of their stems and immediately placed in a vial of water. This technique was employed in this experiment since there were too few flowers present at any one time to make an adequate number of inoculations. Over a period of several days, 15 flowers were collected, inoculated, and placed in a common humidity chamber at 25° C. Inoculations were made by holding a naturally infected spike above the flower and sharply striking the spore-head several times with a scalpel, causing the spores to fall upon the stigmas. Three flowers were removed at intervals of 24, 48, 72, 96, and 120 hours following inoculation and placed in Craf III killing and fixing solution. After several weeks the ovaries, with lemma and palea attached, were dissected from the spikes and embedded in paraffin. From each group of ovaries collected at the various time intervals following inoculation, six ovaries were serially sectioned (three transversely and three longitudinally). The sections were affixed to glass microscope slides in series and subjected to the following triple-stain procedure:

1. Graded through alcohols to water.
2. Stained in a saturated aqueous solution of safranin O for 45 minutes.
3. Graded through alcohols to absolute alcohol.
4. Stained in a saturated solution of orange G in absolute alcohol for 1½ to 2 minutes.
5. Rinsed in clear absolute alcohol.
6. Stained in a saturated solution of fast green in absolute alcohol for 2 to 5 seconds.
7. Rinsed in absolute alcohol.
8. Washed in xylene.
9. Mounted.

Safranin O stains the protoplasm of both the fungus and the host red, but when orange G is included the pathogen appears brownish-red in contrast to the pinkish-red of the host. Fast green stains the cell wall. Care must be taken not to over-stain with this dye since it is rapidly acquired by the host's tissues.

Microscopic observations.—*Twenty-four hours following inoculation*—No infection was found in any of the ovaries or their accessory parts.

Forty-eight hours following inoculation.—Hyphae were present in the stigmas of two of the six ovaries observed. The other four ovaries had either escaped infection, or infection was not yet observable. The mycelium was always closely associated with the cell walls and was

intercellular. The mycelium was more abundant in the parenchyma than the vascular tissue, particularly the vessels that pass through the style and stigma. The pathogen did invade the immature vascular elements located at the tips of the stigmas, however. The hyphae growing intercellularly in the stigmas did not cause distortion of the cells. In a cross section of a stigma, from several to possibly a dozen hyphal strands were observed. The hyphae in general were growing parallel with the long axis of the stigma and were just entering the parenchyma of the style.

Seventy-two hours following inoculation.—Once the pathogen entered the style, subsequent parasitism of the ovary evidently occurred rather quickly because 72 hours after inoculation there was an extensive development of the mycelium throughout most of the ovary. The hyphae always grew closely associated with the cell walls of the parenchyma tissues of the style and ovary. Hyphal development was more extensive in the styles than in the stigmas. In portions of the style, the hyphae filled the area formerly occupied by the host cells except the vessels of the vascular elements. There was extensive mycelial development in the tissues of the ovary wall but very little among the cells of the integuments. The parasite was, however, capable of growing through the integuments and parasitizing the nucellus. Hyphal development in the nucellus was not so extensive as in the ovary wall. The pathogen had grown to the funicular tissue but had not entered it. A considerable amount of mycelium passed outside the ovary wall and grew over the surface of the ovary. Such mycelium was generally oriented in a plane parallel to the long axis of the ovary and was a dark brown color.

The protoplasmic contents of the cells disappeared as the cell walls were replaced by the hyphae. Those cells which had only one or two mycelial strands traversing a portion of their walls generally had a considerable amount of cytoplasm remaining, but where the tissues were heavily parasitized the cell cavities were completely void of cytoplasm.

Mycelial development in the stigmas had not proceeded beyond that observed at 48 hours. Since development of the pathogen was not extensive in the stigma, most of the cell walls remained intact; consequently, the physical structure of the stigma was not lost.

Nintey-six hours following inoculation.—The pathogen had by this time thoroughly permeated all of the ovary tissues except the funiculus and rudimentary anthers, which it failed to invade (figure 14). All of the cytoplasm was gone from the cells of invaded tissues. The styles were entirely filled with dark brown hyphae, and even the vessels were not discernible. Further mycelial development in the stigmas was not apparent, however. A considerable amount of dark brown mycelium had developed outside the ovary wall and had grown down to the base

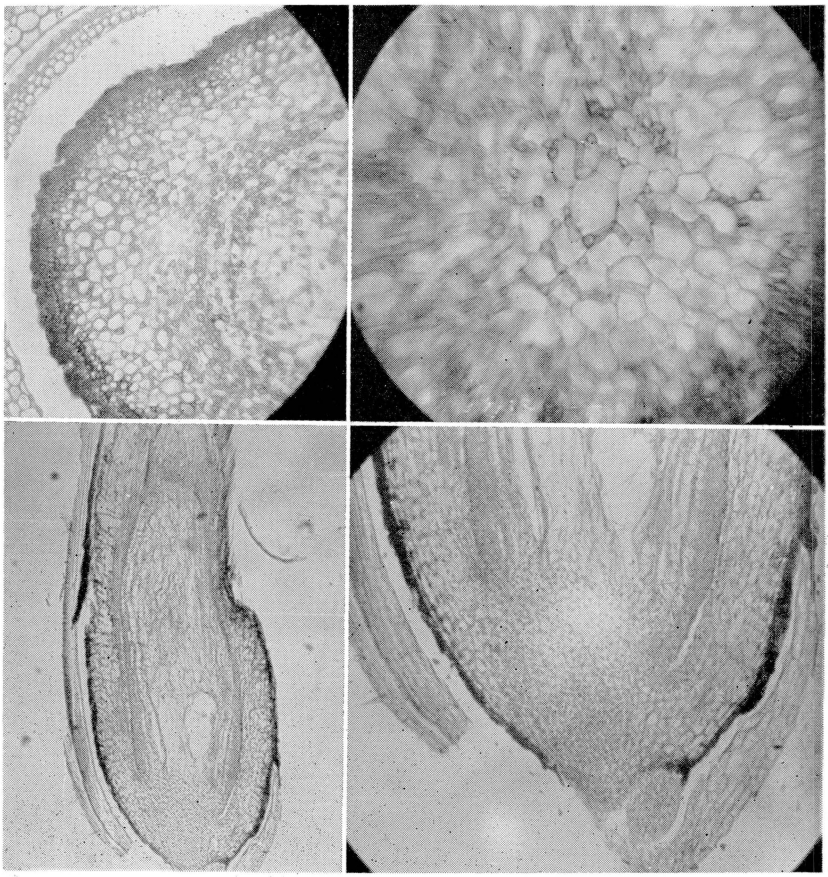


FIGURE 14.—Photomicrographs of buffalograss ovary tissues infected with *C. seminalis*. Upper left: A portion of a cross section of an ovary 120 hours following inoculation. X 200. The tissues at the extreme left are those of the palea and are not infected. Dark colored mycelium adheres closely to the surface of the ovary. Mycelium can be seen following the cell walls within the tissues of the ovary. Upper right: Close-up of a portion of the ovary tissues at left. Note the hyphae are intercellular. X 500. Lower left: Longitudinal section of an ovary 96 hours following inoculation. X 75. The ovary is surrounded with dark brown mycelium. Numerous hyphae can be seen in the ovary wall and ovule, but the pathogen has not penetrated the funiculus (the dense tissues at the base of the ovule) or the rudimentary anther (at the base and to the right of the ovary). Lower right: Base of ovary at lower left more highly magnified. X 200. Mycelial strands can be seen in the ovary wall, nucellus and egg sac and to the outside of the ovary.

of the ovary where it came into intimate contact with the rudimentary anthers, but it failed to invade them. Also, the pathogen came in contact with the palea but did not invade it.

One hundred twenty hours following inoculation.—By this time the pathogen still had not invaded the funicular tissue or rudimentary

anthers. Evidently these structures are not invaded until after they reach senility or die. Observations of infected mature spikes indicated that the funicular tissue evidently became replaced by mycelium that penetrated to the transition zone of the funiculus and the principal vascular element supplying the ovary. The pathogen had continued to permeate more extensively the ovule and ovary wall. In some areas of the ovary wall, the mycelium had completely replaced all of the cells. Mycelial development in the stigmas, however, had progressed but very little, if any.

CONTROL BY A GROWTH REGULATOR

Studies by Gustafson (*2*) indicated that a material change in the hormone concentration of the ovary occurs following fertilization. The fact that pollination inhibited false smut infection (see page 16) suggested that possibly an increase in hormone level could be a contributing factor in the cause of this phenomenon. It was therefore decided to test this hypothesis by supplying known plant growth regulators to the plants and subsequently examining their reaction to false smut.

Effect of Two Plant Growth Regulators on False Smut Infection

Two plant growth regulators, indolebutyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D), were employed in water solutions at concentrations of 0.04 and 0.01 per cent, respectively. The solutions were sprayed onto buffalograss flowers with a DeVilbiss atomizer until the hairs of the stigmas were heavily laden with droplets. The treatment was made at 9:00 A.M., and the plants subsequently were placed in a greenhouse. Twenty-four hours after treatment, half of the flowers were inoculated and the other half pollinated. Seventeen days later the inoculated spikes were harvested and examined for infection. The seeds in the fertilized spikes were harvested 33 days following pollination, and the percentage germination tested on water agar. The germinated seeds were transferred to pots and allowed to grow into plants to check for any abnormal development that might have resulted from the treatment.

The 2,4-D very materially reduced the amount of false smut, whereas the IBA had little or no effect (table 2). Sixty per cent of the

TABLE 2.—False smut in buffalograss ovaries following treatment of stigmas with growth regulators.

Growth regulator	Concentration in water (per cent)	No. ovaries treated	Percentage infection
IBA*	0.04	37	51
2,4-D†	0.01	34	6
Check		27	39

* Indolebutyric acid
† 2,4-Dichlorophenoxyacetic acid

TABLE 3.—Percentage of treated ovaries that produced seed following pollination and the viability of such seed.

Treatments*	No. of ovaries pollinated	Percentage of ovaries that produced seed	Percentage of seeds that germinated and produced plants
2,4-D	32	60	33
IBA	40	35	40
Check	31	97	45

* Treatment preceded pollination by 24 hours.

2,4-D and 35 per cent of the IBA-treated ovaries that were dusted with pollen, but not inoculated, produced normal-appearing seed (table 3) as compared with 97 per cent for the check. All of the germinated seeds produced normal-appearing plants.

Effect of Concentration of 2,4-D and Time-Interval before Inoculation on Amount of False Smut Infection

Methods

Four concentrations of 2,4-D in water solution, 0.005, 0.01, 0.02, and 0.04 per cent, were applied to stigmas and leaves of the plants with a hand atomizer until run-off occurred. Time of application was approximately 8:00 P.M. Immediately after treatment the plants were placed in an unlighted greenhouse that remained dark until 5:00 A.M., at which time artificial lights were turned on. Two days following treatment, half of the flowers were inoculated with minced mycelium from a three-week-old culture, and seven days later the remaining half were inoculated with mycelium from the same culture. Nontreated flowers were inoculated at the same time as the checks.

Results

The higher the 2,4-D concentration the more effective was the control of false smut (table 4). A seven-day interval between time of treatment and inoculation did not reduce the effectiveness of the 2,4-D as a control measure. In this experiment, 2,4-D at 0.01 per cent gave a

TABLE 4.—False smut in buffalograss treated with various concentrations of 2,4-D at two intervals prior to inoculation.

Concentration of 2,4-D (per cent)	Inoculation 2 days following treatment		Inoculation 7 days following treatment	
	No. of ovaries	Percentage infection	No. of ovaries	Percentage infection
0.005	75	40	77	23
0.01	85	31	46	30
0.02	78	24	84	24
0.04	70	6	51	0
No treatment	66	65	20	60

much lower degree of control than in the previous test. The time of day at which the plants were treated apparently caused this difference. In this second experiment, the plants were immediately placed in the dark following treatment, whereas in the first test they were exposed to 11 hours of light before darkness. According to Mitchell and Brown (*4*), 2,4-D is not translocated during darkness, and according to Rohrbaugh and Rice (*5*) its ability to move in the plant is associated with sugar translocation. This possibly accounts for the apparent differences in results between the two tests.

Control of False Smut with 2,4-D under Field Conditions

Methods

Five female buffalograss clones[3] growing in long rows about 3 feet in width were each divided into 16 plots 5 feet in length for testing rates, dates, and multiple applications. It was not possible to convert the amount of 2,4-D that had been applied to the greenhouse plants to a rate for field application. Therefore, three arbitrary rates[4] were chosen, 5 ml. (½N), 10 ml. (1N), and 20 ml. (2N), of 2,4-D[5] solution per gallon of water. This amount was applied over 225 square feet with an ordinary 3-gallon knapsack sprayer. To determine the effect of date of application, one plot in each row was sprayed with 1N rate on May 30, another plot on June 10, and a third on June 20. The dates of application were selected on the assumption that flowering would occur about June 10, thereby permitting a check on preflower, in-flower and postflower treatments.

The effects of multiple applications at the various rates of application were tested by spraying one plot in each row on May 30, a second plot on May 30 and June 10, and a third plot on May 30, June 10 and June 20. There were five check plots in each row. The plots were sprayed between 8:00 and 10:00 A.M., in bright sunshine. There was very little air movement and mid-day temperatures rose to between 90° and 100° F.

The amount of false smut in each plot was determined by manually stripping the spikes from the buffalograss. Later, 100 spikes from each plot were examined macroscopically for the smut-like sphere atop the spikelets indicating infection.

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[3] All clonal buffalograss materials and field plots utilized in these studies were provided by Dr. L. C. Newell, Department of Agronomy. The author wishes to extend his appreciation to Dr. Newell for his cooperation in supplying plant materials and valuable suggestions.

[4] A normal rate of application (1N) used for ordinary weed control is 10 ml. per gallon of water; therefore the rates of application were designated accordingly.

[5] The product used in this experiment was the dimethylamine of 2,4-D, and contained 4 pounds of active ingredients per gallon.

TABLE 5.—Degree of false smut control obtained with 2,4-D in the field at various dates and rates of application.

Concentration of 2,4-D	Dates of applications	Percentage infection*	Visible foliage injury†
5 ml/gal. (½N)	May 30	34.0	Yes
	May 30, June 10	3.6	Yes
	May 30, June 10, June 20	1.4	Yes
10 ml/gal. (1N)	May 30	18.6	Yes
	June 10	3.8	Yes
	June 20	25.6	Yes
	May 30, June 10	0.2	No
	May 30, June 10, June 20	0.2	No
20 ml/gal. (2N)	May 30	12.6	No
	May 30, June 10	0.0	No
	May 30, June 10, June 20	0.0	No
No treatment		52.8	No

* The percentages were obtained from examination of 500 spikes from each treatment except the checks in which 2500 spikes were examined.
† Injury was manifested as a "tip burn" of the grass blades.

Results

All treatments gave control of false smut but in varying degrees, depending upon time of application, concentration of 2,4-D, and the number of applications (table 5). Complete control resulted with repeated application of the 2N rate, however, foliage injury occurred at this concentration.

Effect of time of application.—Single applications of 2,4-D at 1N concentration effected best control when applied on June 10 (3.8 per cent infection) when the buffalograss was in flower. Applications made on May 30 or June 20 reduced infection to 18.6 and 25.6 per cent respectively, compared with 52.8 per cent for the checks. Time of application is therefore very important in obtaining effective control of false smut. Unfortunately, the ½N concentration was not included in the date of application tests. It is possible that the low 3.6 per cent infection in the plots sprayed with ½N on May 30 and June 10 was due mainly to the June 10 application.

Effect of repeated applications.—The results indicated that a single 1N application made at the proper time (June 10 in this experiment) was sufficient for adequate control of false smut. Two and three applications further reduced infection but not enough to warrant their use, especially since with every application there was increased danger of injury to the host.

Effect of rate of applications.—The degree of false smut control increased with an increased concentration of 2,4-D. Foliage injury in the form of "die-back" or "tip-burn" of the leaf blades, however, became very apparent at the higher concentration, particularly in those plots receiving several applications.

Relative Nontoxicity of 2,4-D to the Pathogen

The pathogen was grown on potato-dextrose agar containing 0.01 per cent 2,4-D. The growth regulator was passed through a Seitz filter, and the filtrate was then added to a sterile lukewarm solution of potato-dextrose agar. The fungus grew very well on this medium with no noticeable changes in type of colony growth.

DISCUSSION

There are certain features which make the disease false smut of buffalograss particularly well adapted to the area in which it is found. The pathogen forms chlamydospores which are able to live several years under dry conditions. Also, these chlamydospores are unaffected by cold winter temperatures. Both of these facts are significant since extended periods of drought and cold winters are common to the semi-arid plains of central United States, the native habitat of buffalograss. The flower position of buffalograss is also important. Being only an inch or two above the ground, the pathogen, which was shown to be soil-borne, can be easily blown or rain-splashed onto the stigmas where infection takes place if there is a protracted period of very high humidity, dew, or rain. Commonly the stigmas are found completely covered with moist soil after a rain. They do not dry readily since the flowers are located among the wet, dense, grassy foliage.

Excellent control of false smut was obtained with 2,4-D. Both time of application and concentration were shown to be critical. Applying 2,4-D twice at a low concentration gave control equal to a single application of a higher concentration. Which of the two concentrations is the more practical to use is still unanswered since all of the tests were conducted with female plants and therefore it is not known what effect 2,4-D may have on normal pollen production and viability. There may be less likelihood of affecting normal syngamy with repeated applications of lower concentrations since it is obvious that buffalograss is injured at the higher concentrations.

The action of the 2,4-D resembled that of a chemotherapeutant rather than that of a protective fungicide. It appeared that the 2,4-D induced a reaction which altered the host's tissues, making them incompatible with the pathogen.

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