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A BACTERIAL DISEASE OF ALFALFA

BY

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A BACTERIAL DISEASE OF ALFALFA.

By WALTER G. SACKETT.

HISTORY AND DISTRIBUTION.

In May of 1904, Hon. J. L. Chatfield, who resides at Gypsum, Eagle County, Colorado, observed that while the stand of alfalfa on his ranch was good, much of it was shorter than it should be at that time of the year, and that here and there plants were dying. He reported this condition to the Experiment Station at Fort Collins, and in response to his request, Professor Paddock and Professor Gillette visited his fields. They examined a number of plants, but they were unable to give any decisive answer as to the exact cause. Occasionally, worms were found in the crowns and in the roots, and by splitting the latter lengthwise, numerous dark streaks could be traced through the tissue. A few crowns were blackened, as well as some of the stems, but this discoloration was looked upon as due, possibly, to insect work, although no specific insects could be found at that time.

The following year, there was practically none of the trouble to be seen in the whole valley, which has an area of at least five thousand acres, more than one half of which is in alfalfa.

The next year, however, 1906, the conditions were worse than ever before and the universal complaint among the farmers was that there was "something wrong with the alfalfa." Professor Paddock again visited Gypsum, and at this time the blackened stems were very abundant and much more conspicuous than when he was there before. He brought back specimens of this material to the college and a microscopic examination satisfied him that, in all probability, the trouble was of bacterial origin. As a result of these findings, in November, 1906. Professor Paddock (1) called attention to a new alfalfa disease occurring in certain parts of Colorado, which was different from any previously described malady and which, from all appearances, was not related to either leaf spot or mildew.

The disease has spread with increasing severity until at the present time it is a very difficult matter to find one acre of alfalfa land in the whole valley which is entirely free from the trouble. The loss in tonnage for the first cutting is estimated at eighty per cent, or the crop is only one-fifth of what it was in former years. The disease became so serious in 1907 that it was thought advisable to make the study of this malady a theme for special research. Accordingly, May 1, 1908, the writer began an intensive investigation of the trouble and during the past year has been occupied with laboratory, greenhouse and field experiments bearing upon the cause and possible remedies for the disease. The results of this

(1) Press Bulletin No. 28, Colo. Exp. Sta.

work are given in the pages which follow.

DISTRIBUTION.

Within the state of Colorado, the disease occurs generally throughout the Gypsum Valley in Eagle county, and to a somewhat less extent in Garfield county, and at Rocky Ford in the Arkansas Valley. Prof. W. Paddock has noted it in the Plateau Valley, Mesa county, and between Hotchkiss and Paonia in Delta county.

So far as our present knowledge goes, it has not been seen in the San Luis Valley, or in the Boulder, Longmont, Loveland, Fort Collins and Greeley districts.

In our neighboring states, what appears to be a similar bacterial disease has been observed by Professor Northrop in Utah, by Professor Wooten in New Mexico, by Professor Wilcox in Nebraska, and by Professor Roberts in Kansas. Its occurrence is reported as negative by Professor Nelson for Wyoming, by Professor Kennedy for Nevada, by Professor Lewis for Oklahoma, and by Professor Ball for Texas.

DESCRIPTION OF THE DISEASE.

When a field suffering with this bacterial trouble is viewed as a whole, about the only comment which could be made is that the growth is short and the alfalfa is a little off color. The rich, dark green color in the leaves is absent and the juicy, succulent appearance of the stem, so characteristic of a thrifty stand, is wanting. The plants tend to grow more spindling; the leaves often appear dwarfed, narrow, light green and have a tendency all along the stem and in the growing tip to remain partly closed just as they do in cold or dry weather.

The disease is primarily a stem infection and it is here that we find the most valuable characters for diagnosis. In the earliest stages, the stem has a watery, semi-transparent, yellowish to olive green appearance along one side. This extends down the stem from below the point of attachment of a leaf for one to three internodes. Again, on another side of the stem, the infection may cover two or three different internodes or parts of the same ones. Most commonly the first three to five internodes are the worst infected. Such stems are usually healthy and normal below the ground. Soon after they take on this dark, olive green, watery appearance, there oozes out from the diseased tissue a thick, clear, viscid liquid which spreads over the stem and collects here and there in little bead-like droplets. This exudate dries in a short time with a glistening finish, and gives the stem very much the appearance of having been varnished, and where the liquid has collected in little amber colored scales and has hardened, it looks as if the varnish had run and dried. Stems in this condition have a dry, slightly rough feel to

the touch. The exudate also dries uniformly over the surface or just beneath it, and there produces a dark brown, resinous surface which blackens with age. Such stems are very brittle and easily broken, which fact makes it almost impossible to handle the crop without an immense amount of shattering.

If the epidermis is scraped from an infected stem, the tissue underneath has the same yellowish, watery appearance. This pathological condition extends to the center of the stem and if it is split lengthwise, the interior cavity presents a brownish, mealy aspect. Such stems will collapse much more readily when pressed between the fingers than healthy ones. A shoot in this condition is virtually girdled; its circulation is impaired and its food supply is practically cut off as is evident from the poor growth it makes. Some stems remain in this inactive state and struggle along until the mowing machine puts an end to their existence; others turn black, shrivel and die six weeks before time for the first cutting. During the past season, the disease appeared about May 15th, and up until June 10, twenty-six days later, there were no blackened stems to be found. During this period, the trouble was manifested by the characteristic yellowish green, watery look.

The leaves attached to the diseased part of the stem usually show a watery, pale yellow color at the base, along the mid rib of the leaflets, and especially in the tiny petioles. Those on the parts of the stem which are blackened are always dried up, yellow and extremely brittle. The stipules at the base of the petioles are yellow and brittle and usually show the disease before their corresponding leaves.

Sometimes the leaves exhibit the infection independently of the stem. In this case the petioles become watery, pale yellow and droop. The malady may be confined to the petiole and base of the leaflet or it may involve the whole of the blade. Occasionally leaves are found where the inoculation has been made, apparently, in the margin of the leaflet, and the infection has proceeded toward the middle. In such instances, the tender tissue has a watery look, as if it had been bruised. These leaf infections have been observed to occur a little earlier than the stem troubles, although it may be merely a matter of being able to detect the pathological condition there first.

One year old plants may exhibit blackened areas in the crown, and black streaks which run down into the tap root. As the plant grows older, this blackening increases until the whole crown becomes involved and either the crown buds are destroyed or the root is no longer able to perform its functions, and the plant dies.

So far as our present observations go, the disease appears to run its course with the first cutting, and those plants which have

sufficient vitality, throw out a good growth for the second and third cuttings. Strange as it may seem, there is little or no trace of the blight during the remainder of the season, but in the following spring, a renewed outbreak may be looked for. The severity of the attack seems to vary from season to season. As has been noted before, the trouble was moderate in 1904; in 1905 it was practically unseen; in 1906 and 1907 it was extremely bad; in 1908 the attack was mild and during the past year there was but little to be found. This season the crop was the best that has been harvested for four years. This variation in the degree of the attack would seem to indicate that there may be some relation between the prevalence of the disease and the weather conditions, especially late frosts and late freezing, intermingled with warm, pleasant days as compared with a late, cold spring. Not many plants are killed the first year, but they begin to die after the blight has been prevalent more than one season, and after three or four years so many of them may be missing that the stand is practically worthless.

CAUSE OF THE DISEASE.

If a small piece of the yellowish green, watery tissue from a diseased plant, it matters not whether it be stem or leaf, is placed in a drop of clean water on a glass slide, there will appear on all sides of it, after half a minute, a dense, milky cloud, which can be seen readily with the naked eye, and which slowly diffuses out into the drop. When this preparation is examined under the low power of the microscope (Leitz Objective No. 3, Eye Piece IV.) this milky zone easily resolves itself into swarms of bacteria, which under the high power (Leitz Objective No. 7, Eye Piece IV.) can be distinguished as actively motile rods, relatively short and thick, with rounded ends and occurring for the most part singly and in twos.

If the surface tissue is removed and a portion of the deeper layers is examined, identically the same results will be obtained. If a fragment of the dried exudate is likewise placed in a drop of water, the whole gradually disintegrates and becomes a milky cloud, which under the microscope is a mass of motile bacteria.

Now, if stained films are made from the milky cloud of any of the above preparations, using aqueous fuchsin, one invariably finds a practically pure culture of a short, medium thick bacillus with rounded ends, and with a tendency to stain darker at the poles than in the middle.

Nutrient agar plates, prepared from any of this diseased tissue or from the dried exudate, and incubated for 72 hours at 28° C., will give, almost invariably, a pure culture of a smooth, glistening, grayish white colony, slightly raised, round, margins entire or undulating, and concentrically ringed.

Out of twenty-one infected stems examined and plated at different stages of the disease, thirteen gave pure cultures of this colony in the Petri dishes. In seventeen plates, it was the dominant colony. Platings from five different leaves gave pure cultures in three cases and in four out of five the above colony was the most abundant. In other words, pure cultures were obtained in 62 per cent of the original isolations and in 81 per cent this white colony was dominant. In two out of twenty-six isolations or in seven per cent, it was absent. Plates made from the moist or freshly dried exudate, as a rule, gave pure cultures of the same organism.

An examination of the following notes, made in connection with some of the isolations, may be of interest in showing with what degree of purity the organism can be isolated from field material.

Plates made from material collected at Gypsum, Colorado, May 25, 1909. Plated in Nutrient Agar May 27, 1909.

I. Petiole.—Culture pure; growth after 72 hours; culture picked up 6-1-09. Colony round, smooth, grayish white, glistening, slightly raised. Stained film preparation from four day old colony; short rods, medium thick, mostly single, occasionally in twos, slightly curved and wedge shaped; stain readily with ordinary aqueous fuchsin.

II. Leaf.—Culture pure; characteristic white colony; growth after 72 hours; culture picked up 6-1-09. Colony and stained film same as Petiole above.

III. Stem Tissue—New Infection.—Culture pure; characteristic white colony; growth after 72 hours; culture picked up 6-1-09. Colony and stained film same as Petiole above.

IV. Stem Tissue.—Culture pure; characteristic white colony; growth after 72 hours; culture picked up 6-1-09. Colony and stained film same as Petiole above. Orange colonies appeared on June 5th; picked these up.

V. Stem Tissue—Black.—Culture mixed; growth after 48 hours; cultures picked up 6-1-09. Yellow colonies dominant; few white colonies which resemble those described from petiole.

VI. Exudate Scale No. 1.—Culture pure; growth after 72 hours; culture picked up 6-1-09. Characteristic white colonies same as petiole but germs themselves seem shorter, less curved and wedge shaped. Later observations have shown this germ to be the same as the petiole culture.

VII. Exudate Scale No. 2.—Culture pure; growth after 72 hours; culture picked up 6-1-09. Characteristic white colonies; germ same as that described from Exudate No. 1.

In all probability, all of the above characteristic white colonies were the same. Slight differences in staining seemed to make some difference in the size and shape of the organism itself. In every case the germ is a short, medium thick, motile rod with rounded ends and with a tendency to produce slightly curved forms which show granules on staining.

Plates made from material collected from the Experimental Plats at Gypsum, Colorado, June 11, 1909, and plated June 14, in nutrient agar. Notes taken June 19, 1909.

I. Stem Yellow—Typical.—Plate pure; growth just visible after 48 hours, good after 72 hours; culture picked up 6-19-09. Colony round, smooth, grayish white, glistening, slightly raised, margin entire to undulate. Stain: rods, short, moderately thick, rounded ends, many wedge shape and curved.

II. Stem Advanced.—Plate pure; white colony same as "stem yellow" above. Growth just visible after 48 hours, good after 72 hours. Stain: same as "stem yellow." Culture picked up 6-19-09.

III. Stem Brown.—Plate pure; white colony same as above; growth and stain same; culture picked up 6-19-09.

IV. Stem Green.—Plate contained the same white colony, but an orange colored colony dominated; this orange colony (1) previously shown not to be the cause of the trouble. No cultures picked up.

V. Stem Yellow No. 2.—Plate mixed; the same white colony dominated; there was also the above mentioned orange colony and a large yellow one found once before in earlier plates (2). No cultures picked up.

VI. Stem Watery.—Plate pure; white colony same as above; growth visible after 48 hours, good after 72 hours. Stain: same as "stem yellow" above; short, curved rods, wedge shape and irregular. Cultures picked up 6-19-09.

VII. Single Stoma—Old Infection.—Plate pure; white colony and stain same as "stem yellow" above. Growth visible after 48 hours, good after 72 hours. Culture picked up 6-19-09.

(1) Stem tissue—plates of May 27, 1909.

(2) Stem tissue black—plates of May 27, 1909.

VIII. Stomata—New Infection.—Plate pure; white colony and stain same as "stem yellow" above. Culture picked up 6-19-09.

IX. Stomata—Black.—Plate pure; white colony and stain same as "stem yellow" above. Culture picked up 6-19-09.

All of the cultures picked up from the dominant white colonies, present in all of the plates, have been shown to be one and the same organism and when inoculated onto alfalfa plants, all are capable of producing the disease.

Inoculations have been made upon alfalfa plants, grown under greenhouse conditions, with the three different cultures which have been isolated during this investigation, namely, the characteristic white one, the yellow and the orange, and the only one which has produced typical symptoms of the disease, in fact the only one which has produced any pathological condition whatever, is the dominant white colony referred to so frequently above. Cultures obtained from stem, leaf, petiole, or exudate, were equally pathogenic. Detailed descriptions of this part of the work are given on page 24 under "Greenhouse Experiments."

In order to establish further the fact, that this germ was the unmistakable cause of the trouble, an alfalfa plant was inoculated June 7, 1909 with our present stock culture of the causal organism, to which the name *Pseudomonas medicaginis*, n. sp. has been given, which was isolated from an infected stem May 27, 1909. By June 19, typical symptoms had developed, and plates were made from the yellowish green, watery tissue. On June 21, the Petri dishes showed a pure culture of the same white colony and the organism


5
4
3
2
1
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was reisolated on an agar slant. When compared with the original culture, the recovered organism was identical both in the hanging drop and when stained with aqueous fuchsin. The reisolated culture was again inoculated, June 25, by needle pricks into three different stems, and all of the inoculations gave positive results; the needle pricks showed a yellow, watery zone around the point of infection after nine days, and later turned black. When material from these diseased areas was examined microscopically, August 16, the same milky cloud appeared in the mount as has been described for field material, and swarms of motile rods were visible.

In all, one hundred and two inoculations have been made with this culture, introduced either by scraping the stem or by needle pricks, and positive results have been secured with one hundred per cent of the infections. Control inoculations with a sterile needle have been carried along with all of the experiments, and in no case have any of the check plants developed symptoms of the disease.

METHOD OF INFECTION.

In an effort to secure a satisfactory explanation of the method of infection, the writer spent over a month in the field where the disease is most prevalent. As a result of the daily observations and the gross and microscopic examination of more than three hundred plants, collected at all stages of the disease, before it made its appearance and until it was flourishing, he believes the following to be the most tenable and satisfactory solution of the question.

This phase of the investigation was carried on at Gypsum, Colorado, where our first observations were taken May 4, 1909; this was early in the spring for this locality and altitude (over 6,000 feet above sea level). The season was considered cold and backward, and a moderately heavy snow had fallen one week before; traces of this were still to be seen in the valleys, and the surrounding hills and mountains were heavily covered.

The alfalfa was just beginning to grow, the average height in the diseased fields being from 1½ to 2 inches. Most of the plants had a yellowish green color due, presumably, to the cold. An examination of the young, succulent shoots showed that the epidermis of practically every one of them was wrinkled just below the point of attachment of the first four or five leaves, and often this wrinkling extended half way to the next leaf below. The epidermis was loose from the tissue beneath and appeared to be too large for the stem. When this was peeled off, the underlying tissue had a yellowish, green color much like the diseased plants, but a microscopic examination of such material failed to show any micro-organisms present. In those parts of the stem where this wrinkling

was absent, the tissue beneath the epidermis was not yellow, but a whitish green. In many plants, the loosened epidermis had the appearance of partially collapsed blisters, while around and underneath these blisters the tissue seemed darker than normal and watery, with a suggestion of its having been frozen. Numerous lenticular breaks occurred in the epidermis of some stems. These might have been due to insect work although rather early in the season for this. Occasionally stems were found where the wrinkled epidermis had split open for a distance of one centimeter, exposing the moist pyrenchyma beneath.

Ten days later, May 14, the epidermis of practically every stem in the field was split wide open from node to node over the first six internodes, the third to the fifth internodes being the most common. This splitting appeared to have begun with the wrinkled epidermis mentioned above, and had extended the whole length of the internode thereby exposing the succulent, moist tissue beneath to infection. It is the concensus of opinion of those who have observed this phenomenon, that both the breaking away of the epidermis from the underlying tissue and the wrinkling and subsequent splitting are caused by freezing. A similar trouble ascribed to freezing has been observed in cherry trees and less frequently in the apple. Here the bark cracks and later splits open, exposing the green wood beneath just as in the alfalfa stems.

Anyone who has ever lived in Colorado is familiar with the soil carrying capacity of our winds, and with this in mind, it is easy to understand how germ laden soil might be blown into these cracks, where it would adhere to the moist, exposed surface, and bring about a fatal inoculation. As a matter of fact, soil was always found adhering to these surfaces, and, already, typical cases of the disease were developing. A microscopic examination of the tissues from the injured areas usually showed the germs to be present in great numbers, while ten days previous, that is before the splitting had occurred, there was no indication of any infection. Invariably, the disease appeared first and was worst on those parts of the stem where the epidermis had split and where soil had been caught in the open wound. A striking example of this was seen in an alfalfa field adjacent to a field which had been cultivated recently and sown to oats. The oat field was on the windward side, and with every gust, quantities of fine soil were carried over into the alfalfa. All along this side of the field, there was an aggravated attack of the disease, extending twenty to thirty feet into the alfalfa and the whole length of the field. Every plant was gray with soil and it is only reasonable to suppose that the disease was more severe here than in the more remote parts of the field because of the heavier soil inoculation.

This explanation satisfies one of the most difficult questions which has arisen in connection with the problem, namely, why does the first cutting, alone, suffer from the attack? There are never any frosts after the first crop is out of the way, and consequently there are no split stems in which to start the infection.

In a preliminary report (1), the writer has suggested that possibly the constant tramping of cattle and horses, pastured on the alfalfa fields during the winter, might have split open the crowns and bruised the young, tender shoots so that during the first irrigation, soil containing the disease germs was washed into the injured tissue and started the trouble on the first cutting. Our observations during the past season do not warrant such a conclusion in the majority of cases at least, since, in the first place, the disease was active at least two weeks before the first irrigation, and in the second place, our experimental plats, to which stock had no access, suffered just as severely as the fields which were pastured.

Not infrequently, we find the disease at work on stems where there has been no apparent previous injury to the epidermis; sometimes this assumes the form of a continuous, unbroken infection of the whole internode, and again it occurs as separate, punctiform lesions giving the stem a speckled appearance. This last condition would seem to indicate an infection through the stomata, and inasmuch as we have been able to secure successful inoculations in the greenhouse by applying the culture to the unbroken epidermis, it is altogether possible that stomatal infections take place under field conditions. The leaflets often exhibit yellowish, watery areas along the margin and the larger veins when there is no evidence of the trouble in any other part of the plant: again, the tiny petioles succumb to the disease independently of either the stem or the attached leaflets. Water pore and stomatal infection similar to that described for the black rot of cabbage may explain these cases.

There are doubtless other ways in which infection can take place, but the methods described above, especially the inoculation through the split epidermis, seem to be the most common. It is possible that added observations of another season will give us more light upon this point and so rather than draw any final conclusion as to the *one way* in which inoculation takes place in the field, we prefer to leave the question open.

DESCRIPTION OF THE CAUSAL ORGANISM.

Pseudomonas medicaginis, n. sp. (Sackett.)

I. MORPHOLOGY

1. Vegetative Cells.

When grown upon nutrient agar for 24 hours at 28° C., and

(1) Bulletin 138, Colo. Exp. Sta., Jan. 1909.

stained with aqueous fuchsin, the organism is found to be a short rod with rounded ends, for the most part single, more rarely in short chains of four to six elements, and occasionally in long filaments. Many of the rods appear slightly curved and wedge shaped; the rods taken directly from diseased stems are usually shorter, and when stained with aqueous fuchsin, appear to take the stain more deeply at the poles.

The individual rods usually measure about 1.2 to 2.4μ x .5 to .8μ; the majority are about 2.1μ x .7μ; the filaments vary in length from 20.2μ to 37.2μ.

2. Sporangia.

No sporangia have been observed.

3. Endospores.

No endospores have been observed in agar cultures four months old.

4. Flagella.

When stained by Loeffler's (1) method, it was possible to demonstrate from 1 to 4 flagella attached to each pole. Following Migula's classification, this bi-polar attachment of the flagella places the organism in the genus **Pseudomonas.** A 24 hour agar culture, examined in the hanging drop, exhibits very actively motile rods with rounded ends, from two to four times as long as broad, and which occur, for the most part, singly and occasionally in short chains and filaments.

(1) Centralbl. f. Bakt., 1889, 6, p. 209; 1890, 7, p. 625.

5. Capsules.

No capsules have been observed when stained by Welch's method. (2)

6. Zoogloea.

No zoogloea have been observed.

7. Involution Forms.

Films made from an agar culture four months old and stained with carbol fuchsin show many degenerative forms. Long irregular rods which stain unevenly, giving a granular appearance, and usually darker at the poles, are among the most common. No unusual or strikingly characteristic forms have been observed.

8. Staining Reactions.

The organism stains readily with the ordinary aqueous stains, anilin gentian violet, and carbol fuchsin.

Gram's Stain.

The results from repeated efforts at staining by Gram's method were not sufficiently sharp and decisive to warrant any positive conclusions; the stain was neither entirely lost nor was it retained in its original depth and brilliancy. Both the anilin gentian violet and the iodine solution were used for one and one half minutes and the preparations were left in absolute alcohol for five minutes. When examined, the germs were distinctly stained, but not as intensely as those which had not been treated with the alcohol and for this reason, alone, the organism is here reported as Gram negative, while in reality it is neither positive nor negative.

Loeffler's Methylene Blue.

Stained with Loeffler's methylene blue, the protoplasm of the organism appears very coarsely granular, so much so, in fact, as to give the rods almost a striated appearance.

Neisser's Stain.

Neisser's stain brings out a more or less granular structure to

(2) Bull. Johns Hopkins Hosp., 1892, 3, p. 128.

the protoplasm, but no definite meta-chromatic or polar granules are demonstrable.

II. CULTURAL FEATURES

1. Agar Stroke. Nutrient Agar.

GROWTH, at 27°-28° C., moderate in 24 hours, abundant after 72 hours; no growth at 37° C.
FORM OF GROWTH, filiform becoming more or less echinulate with age.
ELEVATION OF GROWTH, slightly convex.
LUSTER, glistening.
TOPOGRAPHY, smooth.
OPTICAL CHARACTERS, translucent.
CHROMOGENESIS, § grayish white, Engine color No. 12.
ODOR, absent.
CONSISTENCY, butyrous.
MEDIUM, light fluorescent green on the third day.

2. Potato.

GROWTH, scanty in 48 hours, moderate after 6 days.
FORM OF GROWTH, filiform becoming more or less echinulate with age.
ELEVATION OF GROWTH, slightly convex.
LUSTER, glistening.
TOPOGRAPHY, smooth.
CHROMOGENESIS, cream; best described as the color of light manilla paper; color lies between Orange Yellow Tint No. 2 and Engine Color No. 1. After 8 days, the color deepens to Orange Yellow Tint No. 2, and after 12 days, resembles most nearly Engine Color No. 7.
ODOR, absent.
CONSISTENCY, butyrous and slightly viscid.
MEDIUM, grayed and darkened.

3. Loeffler's Blood Serum.

STROKE, moderate in 3 days at 28° C.
FORM OF GROWTH, filiform.
ELEVATION, slightly convex.
LUSTER, glistening.
TOPOGRAPHY, smooth.
CHROMOGENESIS, grayish white.

4. Agar Stab.

GROWTH, best at top; surface growth moderate in 48 hours, abundant after 4 days; surface growth restricted.
LINE OF PUNCTURE, papillate; no liquefaction.
CHROMOGENESIS, grayish white, Engine Color No. 12.
MEDIUM, fluorescent at the surface on the third day.

5. Gelatin Stab.

GROWTH, best at top, no growth in the lower half of the stab.
LINE OF PUNCTURE, filiform to papillate.
LIQUEFACTION, none.
MEDIUM, unchanged.

6. Nutrient Broth.

SURFACE GROWTH, pellicle on the third day.
CLOUDING, slight in 24 hours, moderate in 48 hours, never strong; persistent; fluid slightly turbid.
ODOR, absent.
SEDIMENT, compact, slightly viscid on agitation, scant.

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§ Color terms refer to Standard Colored Papers made by Milton Bradley Co., Springfield, Mass.

7. Plain Milk.

No visible change takes place in the milk in 30 days; there is no peptonization, no coagulation; the reaction becomes neutral to phenolphthalein after 40 days. Reaction after 1 day, +12, after 2 days, +10, after 4 days, +8, after 10 days, +5, after 20 days, +2. The medium is unchanged in color; there is no curd formation and no peptonization at the expiration of 30 days.

8. Litmus Milk.

No change observed until the sixth day when the litmus appears somewhat lighter in color; by the seventh day it becomes bluer and by the fourteenth day it is intense blue. At the end of twenty days, it has the same deep blue color; there is no curd formation and no peptonization at the expiration of 30 days. There is no reduction of the litmus at any time.

9. Gelatin Colonies. Eight days old.

GROWTH, slow, 20° C.
FORM, round.
ELEVATION, slightly convex.
EDGE, undulating.
LIQUEFACTION, none after twenty days.

10. Agar Colonies. Five days old.

GROWTH, slow at 25° C. Fine white colonies visible to the eye after 48 hours.
FORM, round.
SURFACE, smooth, glistening, concentrically ringed.
ELEVATION, convex.
EDGE, entire to undulate; flattened sides.
INTERNAL STRUCTURE, more coarsely granular and denser at the center becoming more finely granular and less dense at the margin.
CHROMOGENESIS, grayish white, Engine Color No. 12.
SIZE, medium, average 1.5 m.m.

11. Glycerine Agar. Agar stroke.

GROWTH, at 27°-28° C., slight in 24 hours; moderate in 48 hours.
FORM OF GROWTH, filiform becoming more or less papillate with age.
ELEVATION OF GROWTH, slightly convex.
LUSTER, glistening.
TOPOGRAPHY, smooth.
OPTICAL CHARACTERS, translucent.
CHROMOGENESIS, grayish white, Engine Color No. 12.
ODOR, none.
CONSISTENCY, butyrous.
MEDIUM, very light fluorescent green on the third day; not as deep a green as nutrient agar.

12. Alfalfa Agar. Agar stroke.

GROWTH, slight growth in water of condensation at base of stroke atfer 5 days; slight along line of inoculation in 12 days.
FORM OF GROWTH, filiform.
ELEVATION OF GROWTH, slightly convex.
LUSTER, glistening.
TOPOGRAPHY, smooth.
OPTICAL CHARACTERS, nearly opaque.
CHROMOGENESIS, Green Yellow Tint No. 2. Growth studded with fine black particles as if sprinkled with pepper.

ODOR, none.
CONSISTENCY, butyrous.
MEDIUM, surface becomes clouded as if a very fine precipitate had been formed in the medium as a result of the growth.

13. Synthetic Agar Low in Nitrogen. Agar stroke.

GROWTH, at 27°-28° C., moderate in 24 hours; very abundant after 3 days.
FORM OF GROWTH, filiform.
ELEVATION OF GROWTH, decidedly convex.
LUSTER, glistening, watery.
TOPOGRAPHY, smooth.
OPTICAL CHARACTERS, transparent; resembles boiled starch.
CHROMOGENESIS, watery white.
ODOR, none.
CONSISTENCY, butyrous or more exactly the consistency of starch jelly.
MEDIUM, no change.

14. Cohn's Solution, 28° C.

GROWTH, absent.
FLUID, no growth.

15. Uschinsky's Solution, 28° C.

GROWTH, slight in 24 hours, moderate in 3 days, abundant in 10 days.
FLUID, viscid sediment after 4 days; clouding, strong, persistent, fluid turbid. Light green after 21 days.

16. Dunham's Solution, 28° C.

GROWTH, moderate.
CLOUDING, moderate, persistent, fluid slightly turbid.

17. Asparagin Solution, 28° C.

GROWTH, abundant.
CLOUDING, moderate, persistent, fluid turbid.

18. Nitrate Broth, 28° C.

GROWTH, moderate.
CLOUDING, moderate, persistent, fluid slightly turbid.

19. Dextrose Bouillon, 28° C.

GROWTH, abundant.
CLOUDING, strong, persistent, fluid turbid.

20. Saccharose Bouillon, 28° C.

GROWTH, abundant.
CLOUDING, strong, persistent, fluid turbid.

21. Lactose Bouillon, 28° C.

GROWTH, abundant.
CLOUDING, strong, persistent, fluid turbid.

22. Maltose Bouillon, 28° C.

GROWTH, abundant.
CLOUDING, strong, persistent, fluid turbid.

23. Glycerine Bouillon, 28° C.

GROWTH, abundant.
CLOUDING, strong, persistent, fluid turbid.

24. Mannite Bouillon, 28° C.

GROWTH, abundant.
CLOUDING, strong, persistent, fluid turbid.

25. Growth in Bouillon Over Chloroform.

GROWTH, absent. 1 c.c. of chloroform was added with a sterile pipette to 10 c.c. of sterile bouillon in a test tube and after the chloroform had collected at the bottom of the tube, the broth was inoculated with two loops of a 24 hour broth culture.

26. Sodium Chloride in Bouillon.

The effect of sodium chloride upon growth has been determined by inoculating tubes of nutrient broth (+15° Fuller's Scale), containing different amount of chemically pure sodium chloride, with two loopfuls of a 24 hour broth culture. A preliminary test in which the broth contained from 0 to 12 per cent of salt, each tube differing by 1%, showed that the toxic strength lay somewhere between 3 and 4 per cent. In the next determinations, the tubes contained 3.0, 3.25, 3.5, 3.75 and 4 per cent of NaCl respectively. This same series was repeated three times with the result that growth took place in the presence of 3.75 per cent of NaCl, but **4 per cent was sufficient to inhibit it.**

27. Nitrogen.

Nitrogen has been obtained from **peptone, asparagin, glycerine, and beef broth,** but not from ammonium tartrate. No determinations have been made with other ammonium salts, urea or free nitrogen. An abundant growth has been obtained on a synthetic agar low in nitrogen, such as is used for growing the nodule forming bacteria; this would seem to indicate the possibility of the organism being able to utilize atmospheric nitrogen. Determinations of this particular feature are in progress at the present time.

28. Best Media for Long Continued Growth.

Standard nutrient agar with a reaction of +15° Fuller's scale has proved entirely satisfactory.

29. Quick Tests for Differential Purposes.

Fluorescent green color produced in the nutrient agar streak; no growth at 37½° C.; non liquefaction of gelatin; surface pellicle on nutrient broth; luxurient and characteristic growth on synthetic agar low in nitrogen (q. v.).

III. PHYSICAL AND BIOCHEMICAL FEATURES

1. Gas Production.

Four fermentation tubes, each, of dextrose, saccharose, lactose, maltose, glycerine and mannite broth were inoculated with a 48 hour culture; two of each were kept at 28° C. and two at 37½° C. Those held at 28° C. gave growth in the open arm after 24 hours, but none in the closed arm and no gas; those at 37½° C. showed no growth whatever. The turbidity in the open arm of those kept at 28° C. increased with age, extending over half way into the U, but at the end of ten days there was **no growth in the closed arm** and **no gas.** There was a well defined line between the growth in the bulb and the clear liquid in the closed arm, which seemed to point strongly toward the aerobic nature of the organism.

2. Production of Acid and Alkali.

60 c.c. portions of dextrose broth, saccharose broth, lactose broth, maltose broth, glycerine broth and mannite broth in 200 c.c. Erlenmeyer flasks were inoculated with two loopfuls of a 24 hour broth culture of **Ps. medicaginis, n. sp.** Five cubic centimeters were taken from each flask for titration every twenty-four hours for a period of ten days. This was diluted with 45 c.c. of distilled water in a porcelain evaporating dish and titrated cold with N/10 NaOH or N/10 HCl as the case required. Phenolphthalein was used as the indicator. An examination of Table No. 1 will show that in no instance has there been any acid produced, while on the other hand, after the third day, there has been a gradual production of alkali until after

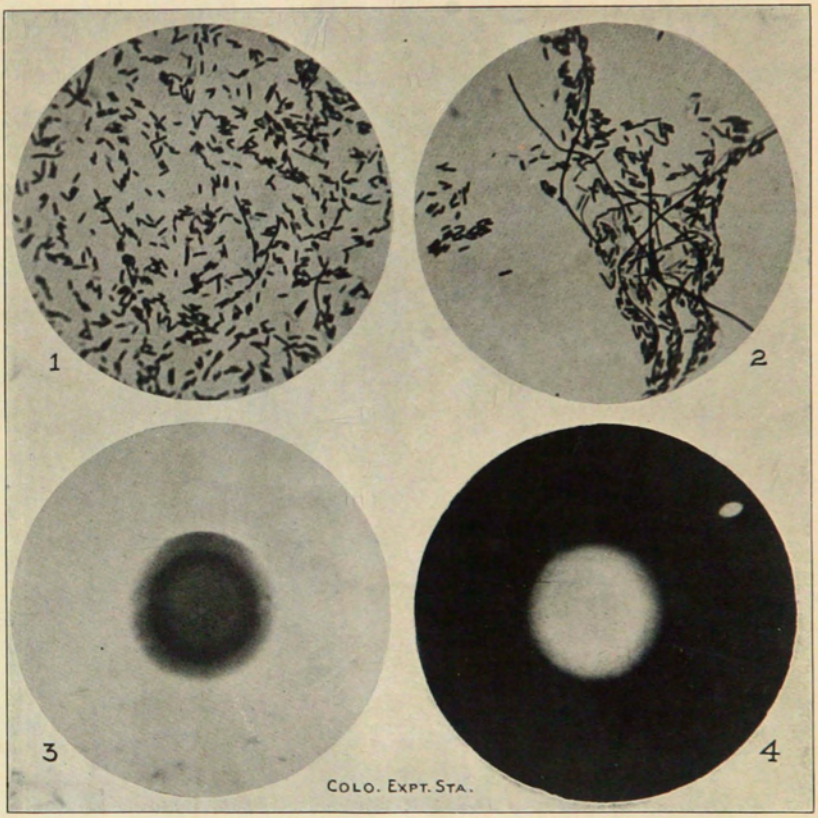

PLATE I.

ten days growth the cultures were decidedly alkaline. The dextrose broth fell from +10 to —3, the saccharose broth from +8 to —5, the lactose broth from +8 to —6, the maltose broth from +8 to —5, the glycerine broth from +8 to —4, the mannite broth from +8 to —7. The results of the daily titrations are given in Table No. 1.

TABLE NO. 1—PRODUCTION OF ACID AND ALKALI.

Sugar Free Broth plus 1%	Degree of Reaction After									
	No ds	1 d	2 ds	3 ds	4 ds	5 ds	6 ds	7 ds	8 ds	9 ds	10 ds
Dextrose	10	10	10	10	9	6	4	2	1	0	—3
Saccharose	8	8	8	8	8	6	3	1	0	—4	—5
Lactose	8	8	8	8	6	4	2	0	—1	—4	—5
Maltose	8	8	8	8	7	7	5	1	0	—4	—5
Glycerine	8	8	8	8	7	5	2	1	0	—3	—4
Mannite	8	8	8	8	7	4	2	1	—1	—5	—7

3. Production of Ammonia.

The production of ammonia has been determined in nutrient broth, Dunham's peptone solution, Jordan's asparagin solution and nitrate broth. 100 c.c. portions of nutrient broth and Dunham's solution were placed in 500 c.c. Erlenmeyer flasks, sterilized, and inoculated with a 48 hour agar culture. Similar portions of each of these media were kept as sterile controls. After ten days, both the cultures and the uninoculated checks were analyzed for ammonia by distillation with magnesium oxid. The distillates were collected in N/10 H_2SO_4 and subsequently titrated with N/10 NaOH. Ammonia was produced by the culture both in the nutrient broth and in the peptone solution, the larger amount being present in the former. The results are given in Table No. 2 below.

TABLE NO. 2—PRODUCTION OF AMMONIA.

Medium	Milligrams NH_3 in 100 c. c. of culture	Milligrams NH_3 in 100 c. c. uninoculated control	Milligrams NH_3 produced by the bacteria
Nutrient Broth	21.63318	18.39672	3.23646
Dunham's Solution	4.00279	2.81060	1.19219

Large quantities of ammonia were produced in the asparagin solution after ten days. This was easily demonstrated by adding one cubic centimeter of Nessler's solution to inoculated tubes of this medium.

No ammonia was produced in nitrate broth by the reduction of the nitrates.

4. Nitrates in Nitrate Broth.

Nitrates are not reduced. Tubes of nitrate broth inoculated with **Ps. medicaginis, n. sp.** were tested after five days and ten days for nitrites and ammonia, and both were found absent. A subsequent test for nitrates showed the original nitrate to be present as such.

5. Indol Production.

There is no indol produced in Dunham's peptone solution.

6. Relation to Acid and Alkali.

The organism exhibits much more latitude in the reaction of its food stuffs on the acid side of the neutral point than on

the alkaline, the limits of growth being from —4° to +32° Fuller's scale. The optimum reaction appears to be between +15 and +18. Growth is retarded by a neutral reaction, on the one side, and by +32 on the other, and is prevented entirely by —6 and +34.

To tubes containing exactly 5 c.c. of sterile broth, sufficient standard NaOH or HCl was added, with aseptic precautions, to give them reactions ranging from —40 to +40, each tube differing by 2° from the others, thus: —40°....—8°, —6°, —4°, —2°, 0°, +2°, +4°, +6°, +8°.....+40°. Six tubes were prepared for each reaction, one of which was titrated to verify the degree of acidity or alkalinity. Without further sterilization, these tubes were inoculated with two loopfuls of a 48 hour broth culture and allowed to develop at 28° C. We found by trial that if the broth was sterilized after the acid or alkali had been added, the reaction might change considerably, especially in the case of the alkali, due, presumably, to certain chemical combinations which took place between the NaOH and the broth constituents at the sterilizing temperature (95.5° C. Altitude 4,981 feet above sea level). This was borne out by the fact that if the medium was not sterilized after adding the NaOH, —6° was sufficient to inhibit growth, but if sterilized, broth reacting —10° before being heated was required to accomplish the same result.

Detailed results of the experiment are given in Tables Nos. 3 and 4.

TABLE NO. 3—RELATION TO ACID.

Degree of Acid	Growth Resulting After Given Number of Days									
	1 d.	2 d.	3 d.	4 d.	5 d.	6 d.	7 d.	8 d.	9 d.	10 d.
0	—	—	—	+	+	+	+	+	+	+
2	—	+	+	+	+	+	+	+	+	+
4	—	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+
14	(+)	+	+	+	+	+	+	+	+	+
16	(+)	+	+	+	+	+	+	+	+	+
18	(+)	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+
24	—	+	+	+	+	+	+	+	+	+
26	—	+	+	+	+	+	+	+	+	+
28	—	+	+	+	+	+	+	+	+	+
30	—	+	+	+	+	+	+	+	+	+
32	—	—	—	—	—	+	+	+	+	+
34	—	—	—	—	—	—	—	—	—	—
36	—	—	—	—	—	—	—	—	—	—
38	—	—	—	—	—	—	—	—	—	—

TABLE NO. 4—RELATION TO ALKALI.

Degree of Alkali	Growth Resulting After Given Number of Days									
	1 d.	2 d.	3 d.	4 d.	5 d.	6 d.	7 d.	8 d.	9 d.	10 d.
0	−	−	−	+	+	+	+	+	+	+
−2	−	−	−	−	+	+	+	+	+	+
−4	−	−	−	−	−	+	+	+	+	+
−6	−	−	−	−	−	−	−	−	−	−
−8	−	−	−	−	−	−	−	−	−	−
−10	−	−	−	−	−	−	−	−	−	−
−12	−	−	−	−	−	−	−	−	−	−
−14	−	−	−	−	−	−	−	−	−	−
−16	−	−	−	−	−	−	−	−	−	−
−18	−	−	−	−	−	−	−	−	−	−
−20	−	−	−	−	−	−	−	−	−	−
−22	−	−	−	−	−	−	−	−	−	−
−24	−	−	−	−	−	−	−	−	−	−
−26	−	−	−	−	−	−	−	−	−	−
−28	−	−	−	−	−	−	−	−	−	−
−30	−	−	−	−	−	−	−	−	−	−
−32	−	−	−	−	−	−	−	−	−	−
−34	−	−	−	−	−	−	−	−	−	−
−36	−	−	−	−	−	−	−	−	−	−
−38	−	−	−	−	−	−	−	−	−	−

(+) Best growth.

7. Optimum Reaction.

The optimum reaction for growth in bouillon is +15° to +18° Fuller's scale.

8. Vitality on Culture Media.

Vitality on culture media is only moderate. Transfers made from a stab culture in nutrient agar which was four months old failed to give any growth; however, growth has been obtained from cultures on agar which were six weeks old. No difficulty has been experienced in carrying the culture upon nutrient agar if it transferred as often as once in ten days.

9. Temperature Relations.

THERMAL DEATH-POINT.—The thermal death-point lies between 49° and 50° C. 10 c.c. portions of nutrient broth, +15 Fuller's scale, in thin walled test tubes of resistance glass, having a uniform diameter of 16 to 17 m.m., were inoculated with two loopfuls of a 24 hour broth culture grown at 28° C. As soon as inoculated, the tubes were plunged into water of the desired temperature up to the cotton plugs, and kept there for exactly ten minutes. In order to insure a uniform temperature, the water in the water bath in which the exposures were made, was kept in constant motion by a horizontal paddle attached to a stirring machine and operated by a water motor. The different temperatures were maintained by hand regulations with a Bunsen burner. At the end of the ten minutes exposure, the tubes were immediately cooled in cold water and subsequently allowed to develop at 28° C. Twelve tubes of broth were used for each temperature and determinations were made for each degree of temperature from 40° to 60° C. If turbidity appeared in any of the tubes within ten days, this was taken as an indication that the temperature to which the tubes were exposed was not fatal.

OPTIMUM TEMPERATURE.—The optimum temperature lies between 28° and 30° C.

37½° C., no growth occurs on nutrient agar, in nutrient broth or in the various sugar broths.

28° C., abundant growth on agar and moderate in broth after 48 hours.

20° C., moderate growth on agar and slight in broth after 48 hours.

10° C., slight growth on agar and none in broth in 48 hours.

6.5° C., slight growth on agar and none in broth in 48 hours; moderate growth on agar and slight in broth in 6 days.

10. Relation to Oxygen.

Ps. medicaginis, n. sp. appears to be an obligative aerobe. Stab cultures were made in glucose litmus gelatin and placed in an atmosphere of hydrogen in a Novy jar. No growth whatever took place in 120 days. Again, in our studies upon gas production from the different sugars, glycerine and mannite, we have observed that no growth occurred in the closed arm of the fermentation tubes and that there was a sharp line of demarcation between the growth in the open arm and the clear liquid in the closed arm. This would seem to point strongly toward the aerobic nature of the germ. The total absence of any deep growth in the gelatin stab serves to emphasize further this feature.

11. Effect of Dessication.

The organism is relatively resistant to drying, being able to withstand dessication for eleven days when spread on cover glasses and dried in sterile Petri dishes. A suspension of the 24 hour growth on agar was made in sterile water, and one loopful of this was spread over each of a number of one-half cover glasses, sterilized by flaming. These smears were placed in sterile Petri dishes at once and allowed to dry. Every twenty-four hours for a period of twenty-two days, two of these inoculated cover glasses were taken out and dropped into tubes of sterile broth; if growth resulted, this was taken as evidence that the germs had not been killed by drying. Detailed observations are given in Table No. 5 below.

TABLE NO. 5—EFFECT OF DESSICATION.

Number of Days Dried	Growth Resulting in Broth After Given Number of Days									
	1 d.	2 d.	3 d.	4 d.	5 d.	6 d.	7 d.	8 d.	9 d.	10 d.
1	0	0	+	+	+	+	+	+	+	+
2	0	0	+	+	+	+	+	+	+	+
3	0	0	+	+	+	+	+	+	+	+
4	0	0	+	+	+	+	+	+	+	+
5	0	0	0	0	+	+	+	+	+	+
6	0	0	0	0	0	0	0	0	0	0
7	0	0	+	+	+	+	+	+	+	+
8	0	0	0	+	+	+	+	+	+	+
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	+	+	+
12	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0
0	0	0	+	+	+	+	+	+	+	+

12. Relation to Freezing.

1/100 c.c. and 1/10,000 c.c. portions of a 24 hour broth culture grown at 28° C., were plated in nutrient agar as controls. This culture was then frozen in a mixture of snow and salt, and kept in this condition for 24 hours, at the end of which time it was thawed out by placing it in an incubator at 28° C. As soon as melted, dilution plates were made using the same amounts as before freezing. After four days, colony counts were made which showed the culture to have contained 470,000 germs per c.c. before freezing and 5,100 after freezing, or 98.9 % had been killed.

13. Relation to Light.

DIRECT SUNLIGHT.—Three tubes of liquefied agar were sown thinly with different amounts of a 24 hour broth culture and poured into sterile Petri dishes. As soon as the agar had solidified, one half of each plate was covered with one thickness of heavy, black, glazed paper. They were then placed on a bed of firmly packed snow in a large crystalizing dish and the whole was exposed for fifteen minutes to the bright sunshine of Fort Collins, Colorado (altitude 4,981 feet above sea level), Nov. 20, 1909, 11:30-11:45 A. M.

100 % of the germs were killed in the exposed portions of the plates, while 25, 9, and 5 colonies, respectively, developed in the protected parts.

DIFFUSED LIGHT.—Our cultures have been kept in diffused light in a culture room supplied with light from a north window, which was perhaps eight feet from the table on which the cultures were kept. We have never noticed any detrimental effect from this light upon either the vigor or the virulence of the cultures. Other cultures have been kept next to the window for a week at a time, and when subcultures were made from these, no difficulty has been experienced in getting good growths.

14. Production of Hydrogen Sulphide.

Nutrient broth in tubes was inoculated with a 48 hour agar culture, and at the same time narrow strips of filter paper, moistened with lead acetate, were suspended in the upper part of the tubes and held in place by the cotton plugs. These strips of paper were remoistened with lead acetate every twenty-four hours for a period of ten days but there was no blackening to indicate the production of hydrogen sulphide.

A second method of demonstrating H_2S was to make stab cultures in nutrient gelatin to which .5% of iron potassium tartrate had been added. If hydrogen sulphide had been produced, the line of puncture should have been marked by a well defined black line due to the formation of iron sulphide. Tubes of this medium inocualted with **Ps. medicaginis, n. sp.** failed to show the production of any H_2S.

15. Production of Ferments.

Determinations have been made for the presence of diastase, invertase, zymase, rennet, pepsin and glucase. Tests were made for these enzymes after five, seven and thirty days, and the only one which we have been able to demonstrate was invertase. A trace of this was evident on the seventh day, and was abundant at the end of thirty days. A 48 hour agar culture has been employed in all of the inoculations.

DIASTASE.—100 c.c. of sugar free beef broth containing.1% soluble starch were inoculated with the organism under study. After five, seven and thirty days, portions were removed from the flask with a sterile pipette and tested for starch with iodine

solution, and for maltose with Fehling's solution. Starch was present at all times, and at no time was there any reduction of the Fehling's solution; therefore no diastase was formed.

INVERTASE.—Saccharose broth, prepared by adding 1% of saccharose to sugar free broth, was used in the determination of invertase. 100 c.c. of this medium were inoculated and after five, seven and thirty days tests were made for invert sugar with Fehling's solution. There was no reduction at the end of five days, a slight one after seven days and at the end of thirty days, a good test for glucose was obtained from the inoculated flask, while the sterile control gave no reduction of the Fehling's solution; therefore invertase was formed.

ZYMASE.—Glucose broth, prepared by adding 1% of glucose to sugar free broth, was used in our study of zymase. Observations covering a period of thirty days failed to show at any time the evolution of CO_2 gas, or the presence of alcohol in the inoculated tubes, and consequently, we have concluded that there was no zymase produced.

RENNET.—Plain milk was employed as the medium in the study of rennet production. At no time during the thirty day period of examination, did the inoculated milk tubes show any evidence of curd formation, which fact has been interpreted as indicating the absence of rennet.

PEPSIN.—Plain milk and nutrient gelatin, contained in test tubes, were epmloyed as the media in the study of pepsin formation. Stab cultures were made in the gelatin and the milk was inoculated in the usual way. There was neither any liquefaction of the gelatin nor any digestion of the casein of the milk during the period of observation, which was sixty days for the gelatin and forty days for the milk.

GLUCASE.—Beer wort, and sugar free broth to which 1% of maltose was added, furnished the media for the study of glucase, but owing to the difficulty in distinguishing maltose from glucase when present in the same solution, no satisfactory conclusions could be reached regarding the presence of glucase, and hence concerning the production of glucase.

16. Crystals Formed.

No crystals have been observed to form in any of the media employed.

IV. PATHOGENICITY

1. Pathogenic to Animals.

Inasmuch as the organism does not grow at 37½° C. it has been considered unnecessary to carry on any animal inoculations.

2. Pathogenic to Plants.

Pathogenic for alfalfa **(Medicago sativa)**; not pathogenic under field conditions for sweet clover **(Melilotus alba)**, white clover **(Trifolium repens)**, or winter vetch **(Vicia sativa)**.

V. NUMERICAL CLASSIFICATION

According to the numerical system of recording the salient characters of an organism, **Ps. medicaginis, n. sp.** (Sackett) becomes Ps. 212.3332133.

MEDIA EMPLOYED.

The *nutrient broth, nutrient gelatin, nutrient agar* and *sugar free broth,* employed in this work, have been prepared according to the Standard Methods of Water Analysis (1). Reaction +15° Fuller's scale.

Sugar Broths.—These were prepared by adding one per cent of the different sugars, as well as glycerine and mannite, to sugar free broth.

Glycerine Agar.—Prepared by adding four to six per cent glycerine to nutrient agar.

Dunham's Solution.—

Distilled water	1000.0 c. c.
Witte's Peptone	10.0 grams
NaCl	5.0 grams

Nitrate Broth.—

Distilled water	1000.0 c. c.
Witte's Peptone	1.0 gram
KNO_3 (nitrite free)	.2 gram

Asparagin Solution.—(Jordan). (2)

Redistilled water	1000.0 c. c.
Asparagin	2.0 grams
$MgSO_4$	1.0 grams
K_2HPO_4	1.0 grams

(1) Supplement Journal Infect. Diseases, May, 1905, p. 104.
(2) Jordan. Bot. Gaz., 1899, 27, p. 9; Jour. Expt. Med., 1899, 4, p. 627.

Uschinsky's Solution.—(1)

Distilled water	1000.0 c. c.
Glycerine	30–40 grams
NaCl	5– 7 grams
$CaCl_2$	.1 grams
$MgSO_4$	.2–.4 grams
K_2HPO_4	2.–2.5 grams
Ammonium lactate	6 –7 grams
Sodium asparaginate	3 –4 grams

Cohn's Solution.—(2)

Distilled water	1000.0 c. c.
KH_2PO_4	5.0 grams
$MgSO_4$	6.0 grams
Ammonium tartrate	10.0 grams
KCl	.5 grams

Synthetic Agar, Low in Nitrogen.—

Distilled water	1000.0 c. c.
Cane sugar	50.0 grams
KH_2PO_4	1.0 gram
$MgSO_4$	.2 gram
Shredded agar	15.0 grams

Reaction +3° Fuller's scale.

(1) Cent. f. Bakt., Erste Abt., Bd. XIV., 1893, p. 316.
(2) DeBarry, p. 86, Vorles. u. Bact., 2 Auflage.

Potato.—Cylinders of raw potato were washed in running water over night, rinsed in a very dilute solution of NaOH, and sterilized in the autoclave for five minutes at 120° C.

Alfalfa Agar.—Prepared by adding 15 grams of finely shredded agar to 1,000 c.c. alfalfa infusion made by steeping 500 grams finely chopped green alfalfa in 1,000.0 c.c. tap water for one hour. Reaction not changed.

Litmus Milk.—Prepared by adding to plain milk one per cent of a solution of azolitmin made by dissolving 1 gram of azolitmin in 40 c.c. of distilled water and kept at 37.5° C. for 12 to 18 hours.

GREENHOUSE EXPERIMENTS.

The constant occurrence of characteristic white colonies, in such a large percentage of our plates, was sufficient to make us suspicious that the micro-organisms making up such colonies were the immediate cause of the disease. However, the crucial test of a pathogenic organism is its power to reproduce the given disease when introduced in pure culture into its normal host. Accordingly, we have fulfilled this requirement by making a large number of inoculations upon alfalfa plants under greenhouse conditions, and by this means we have been able to establish *Ps. medicaginis, n. sp.* beyond the remotest shade of possible doubt, as the unquestionable cause of the trouble. We have reproduced the infection in from five to seven days with practically its characteristic field symptoms, and we have been able to follow its progress through the different changes up to the blackening and complete destruction of the stem after six weeks.

In the plant inoculations, which are described below, we have employed three different germs. These are the only ones which we have met with in our isolations, and for convenience they are here referred to as "Yellow Colony," "Orange Colony" and "White Colony," the last, *Ps. medicaginis, n. sp.*, having been shown to be alone responsible for the disease.

Successful inoculations have been obtained by scarifying the epidermis of the stems with a sterile scalpel, and then immediately smearing the freshly exposed, moist surface with a 48 to 72 hour agar culture of the organism. Another method has been to prick the stem at intervals of about one centimeter with a needle previously dipped into the growth of a 48 to 72 hour agar streak. Again, we have rubbed the culture over the surface without any previous injury to the epidermis.

No precautions have been taken against the drying out of the inoculated areas other than to spray the plants once just after inoculation, and once a day thereafter for the next three days with sterile water. A water bottle with a fine rose nozzle has been used for this work. The spray would collect in large drops, and ultimately run down the stems, and in this way moisten the inoculated regions. This treatment together with the moist atmosphere of the greenhouse seemed adequate.

Plant Inoculations of June 3, 1909.

Cultures used were isolated June 1, 1909.

Pot No. 1.—The epidermis of each of four stems was scarified for a space of 10 cm. and smeared with a 48 hour agar culture of **Ps. medi-**

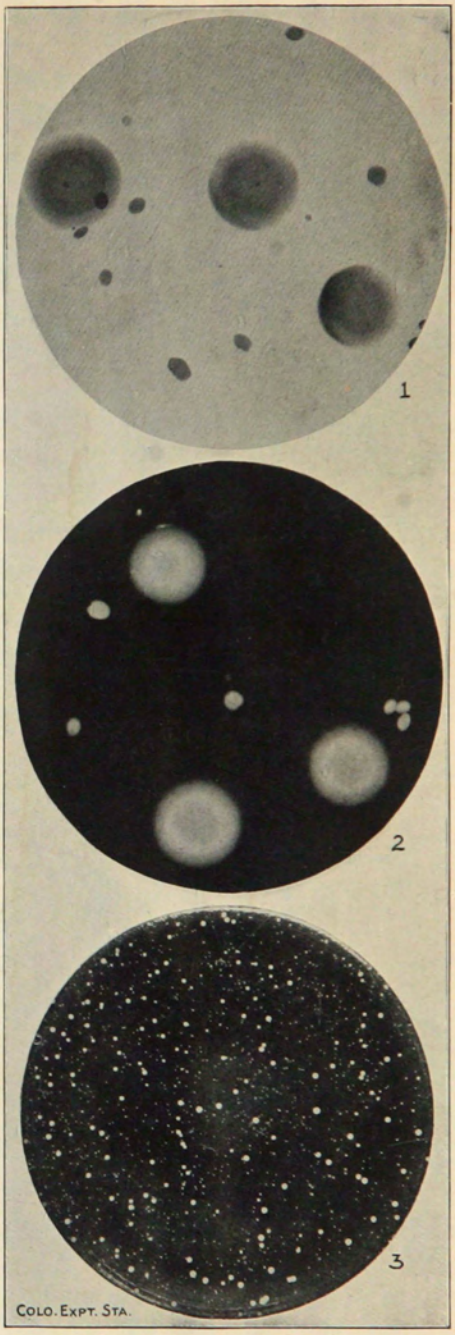

PLATE II.

ginis, n. sp. obtained from an infected stem. For comparison with inoculations not specially protected against dessication, this pot was covered with a bell jar, the opening in the top being stoppered with a cotton plug. Circulation of air was possible yet there was a moist, if not saturated, atmosphere surrounding the plant all the time. By June 18th., the scraped areas were blackened and a microscopic examination of this tissue showed the characteristic milky cloud and swarms of bacteria.

Pot No. 2.—Each of four stems was inoculated by ten needle pricks with a 48 hour agar culture of **Ps. medicaginis, n. sp.** This pot was also covered with a bell jar as described above. By June 18th, each needle prick showed a dark brown zone 1 mm. wide surrounding the point of infection. A microscopic examination of the tissue from this zone showed clouds of motile bacteria.

Pot No. 3.—Four stems were inoculated in the same manner as those of Pot No. 1, but they were not covered with a bell jar. The plant was sprayed with sterile water once a day for the first three days after inoculation. By June 18th., the stems were blackened and exhibited the characteristic phenomena under the microscope. The diseased condition of the stems was much more typical than in those which were covered with the bell jar.

Pot No. 4.—Four stems were inoculated in the same manner as those of Pot No. 2, but they were not covered with a bell jar. They were sprayed with sterile water once a day for the first three days after inoculation. On June 18th., there was a brown area surrounding each needle prick and a microscopic examination of the discolored tissue showed swarms of motile bacteria. The gross appearance of the diseased parts was more typical than in the plant covered with a bell jar.

Pot No. 5.—Four stems were scarified and inoculated with a 48 hour culture of **Ps. medicaginis, n. sp.** isolated from a diseased leaf. The plant was covered with a bell jar. The stems were blackened by June 18th., but they were not as typical as those which were left uncovered.

Pot No. 6.—Four stems were inoculated by needle pricks with a 48 hour culture of **Ps. medicaginis, n. sp.** isolated from a diseased leaf. The plant was covered with a bell jar. June 18th., the disease was evident from the blackened areas around each needle prick, but it was not as typical as in those stems which were not covered.

Pot No. 7.—Four stems were inoculated in the same manner as Pot No. 5, but were exposed to the air and sprayed with sterile water. By June 18th the disease was apparent by the blackened stems and a microscopic examination showed swarms of bacteria coming from the tissue and a milky cloud surrounding the whole; more typical than stems under bell jar.

Pot No. 8.—Four stems were inoculated in the same manner as Pot No. 6, but were exposed to air and sprayed with sterile water. June 18th. there were dark brown areas surrounding each puncture; very typical; microscopic examinations characteristic.

Plant Inoculations of June 7, 1909.

Cultures used were isolated June 1, 1909.

Pot No. 9.—Each of four stems was scarified and inoculated with a 48 hour culture from a yellow colony isolated from a black stem. By June 18th. the stem was slightly yellow and shiny but probably nothing more than the result of the scraping; nothing typical had developed; there was no sign of any growth of the germ and no evidence of any discolored or diseased tissue. Microscopic examination failed to show the presence of bacteria and consequently this germ was considered as not responsible for the trouble.

Pot No. 10.—Each of four stems was scarified and inoculated with a 48 hour culture from an orange colony, isolated from a diseased stem.

By June 18th. there was no change whatever in the inoculated stems, and on June 25th. they were in all respects the same as the uninoculated controls; therefore, this germ was eliminated as a possible cause of the disease.

Plant Inoculations of June 21, 1909.

The cultures used were isolated June 19, 1909.

Pot No. 11.—Five stems were inoculated by spreading a 48 hour agar culture of **Ps. medicaginis, n. sp.** upon the unbroken epidermis, the object being to determine whether stomatal infection could be produced in this way. After twenty days, isolated brownish spots appeared on the inoculated portions of all the plants, and after thirty days these brownish areas were black. A microscopic examination of the tissue showed the true infection.

The object of the following inoculations was to determine whether **Ps. medicaginis, n. sp.** was equally pathogenic when isolated from different stages in the progress of the disease.

Pot No. 12.—One stem was scarified and smeared while a second one was inoculated by needle pricks with a culture of **Ps. medicaginis, n. sp.** isolated from a stem which had reached the brown stage of the disease. The infection developed in both stems after seven days and by August 1st. both were black.

Pot No 13.—One stem was scarified and smeared while a second one was inoculated by needle pricks with a culture of **Ps. medicaginis, n. sp.** isolated from a stem in the earliest stage of the infection when it showed the light green, yellow, watery tissue. Both of the stems developed the disease in seven days and were black after five weeks.

Pot No. 14.—One stem was scarified and smeared while a second one was inoculated by needle pricks with a culture of **Ps. medicaginis, n. sp.** isolated from a black stem in the advanced stage of the disease. Both stems developed the trouble in five to seven days and were black August 1.

Pot No. 15.—One stem was scarified and smeared while a second one was inoculated by needle pricks with a culture of **Ps. medicaginis, n. sp.** isolated from what appeared to be a very recent stomatal infection on the stem; there was no indication of the epidermis being broken, and the disease occurred in isolated spots around the stomata. Both stems developed typical symptoms in five to seven days and were black by August 1st.

Pot No. 16.—One stem was scarified and smeared while a second one was inoculated by needle pricks with a culture of **Ps. medicaginis, n. sp.** isolated from an advanced, black stomatal infection of the stem. Both stems developed the disease in five days, and the inoculated areas were black August 1st.

Pot No. 17.—One stem was scarified and smeared while another was inoculated by needle pricks with a culture of **Ps. medicaginis, n. sp.** isolated from a single infected stoma of the stem. Both stems developed very good symptoms in four days and were black by August 1st.

Plant Inoculations of August 25, 1909.

Cultures used isolated June 1, 1909.

Pot No. 18.—Stem scraped over the whole internode and smeared with a culture of **Ps. medicaginis, n. sp.** isolated from a diseased stem; Sept. 3rd. inoculated area yellowish green color, watery and darker along the edge; Sept. 7th. dark brown; Sept. 30th. black.

Pot No. 19.—Stem scraped in patches and smeared with a culture of **Ps. medicaginis, n. sp.** isolated from a diseased stem. Sept. 3rd. inoculated spots plainly visible by the watery, yellowish green patches; Sept. 7th. spots dark brown; Sept. 30th. spots black.

Pot No. 20.—Stem inoculated by needle pricks with culture of **Ps. medicaginis, n. sp.** isolated from diseased stem. Sept. 3rd. watery areas for 1 to 2 mm. around each needle prick and yellowish; Sept. 7th. dark brown; Sept. 30th. black.

Pot No. 21.—Controls. One stem scarified with a sterile scalpel and a second pricked with a sterile needle. No change visible at any time in either stem.

Plant Inoculations of November 2, 1909.

Cultures used were isolated June 1, 1909.

Pot No. 22.—Three stems were scarified and smeared with a culture of **Ps. medicaginis, n. sp.** isolated from an infected stem. On Nov. 7th, two of them were watery, shiny and yellow, while a liquid had been oozing from one and had dried on the stem. The third stem showed almost no change from the check. By Nov. 18th. all of the scraped areas were dark, watery green and turning brown; by Nov. 20th, they were a dark brown; a microscopic examination on Nov. 23rd. showed a typical infection. (See colored plate.)

Pot No. 23.—Two stems were inoculated by needle pricks with a culture of **Ps. medicaginis, n. sp.** isolated from a diseased stem; Nov. 7th, yellowish, watery appearance around each one of the stabs; Nov. 18th, needle pricks all taking well; very well defined brownish green areas one to two mm. around each; infection spreads slowly; Nov. 20th. the diseased spots dark brown and slightly sunken; Nov. 23rd. an examination of the diseased tissue gave milky cloud in the mount and swarms of germs under the microscope. (See colored plate.)

Pot No. 24.—Stem inoculated by smearing culture of **Ps. medicaginis, n. sp.** upon the unbroken epidermis. The object, here, was to attempt to secure a successful infection through the stomata. Nov. 10th, two yellowish, watery spots developed on the part of the stem smeared; Nov. 18th. these spots were dark brown and by Nov. 24th. they were almost black. A subsequent microscopic examination showed a true bacterial infection.

In order to eliminate the germicidal action of the direct rays of the sun, the plants were shaded by one thickness of canvas, placed next to the glass roof.

In order to determine whether the infection was communicated to the plants through the roots, twelve pots were prepared with sick soil containing quantities of the diseased stems. Fifteen germinated alfalfa seeds, which had been sterilized previously in a 1-500 mercuric chloride solution, were planted in each of the above pots. A good, vigorous stand was obtained. The possibility of frozen stems was eliminated by growing the alfalfa in the greenhouse and the danger from dust infection was reduced to a minimum by keeping the surface soil in the pots moist. These plants are now sixteen months old and up to the present time not a single stem in any of the twelve pots has shown any sign of the disease. From these results, we can say with a reasonable degree of certainty, that the disease is not, primarily, a root trouble, and if the roots do become diseased, the infection must start from the crown and work downward.

SUMMARY OF GREENHOUSE EXPERIMENTS.

From these experiments it will be seen, first, that we have been able to produce the disease successfully in 100 per cent of the

plants inoculated with pure cultures of *Ps. medicaginis, n. sp.*; second, that cultures of this organism produce identically the same symptoms irrespective of their origin, i. e., whether they were originally isolated from stem, leaf or exudate; third, that it has been possible to produce the disease by introducing the germs through needle pricks and by smearing them upon scarified areas, as well as by spreading them upon the unbroken epidermis; however, inoculations by the last method develop more slowly and are not as typical of field lesions; fourth, that after cultivation upon nutrient agar for five months, *Ps. medicaginis, n. sp.* seems to have retained its initial virulence practically undiminished; fifth, that neither of the other two cultures which have been found associated with the trouble are able to produce the disease; sixth, that the infection is not communicated to the plants through the roots.

FIELD EXPERIMENTS.

Inasmuch as the disease seems to be directly tracable to soil infection, and consequently may be considered a soil trouble, the only practical method of controling it is by the introduction of resistant varieties. To this end we have planted twenty-six varieties of alfalfa on sick land with the hope of obtaining one or more blight resistant strains. The seed for this work was procured from the United States Department of Agriculture through Mr. W. J. Brand, and planted April 16 and 17, 1907. The ground upon which the plats are located is owned by Hon. J. L. Chatfield, and had been in alfalfa a number of years, was plowed in the spring of 1905 and planted to oats and potatoes; in 1906 again planted to oats and produced 100 bushels per acre. The rows of the plot are about fifteen rods long and run from east to west. Two rows of each variety were planted through the plot, then the series was repeated in the same order but only one row of a kind was used. The variety designated as Gypsum No. 1 was grown from cuttings which were taken from land that had been in alfalfa, and was plowed up because of the prevalence of the disease, and planted in oats. These plants were very vigorous at the time the cuttings were made. This part of the work was begun by Professor Paddock over two years ago.

The following is a list of the varieties which we have used in our field tests.

No. 9451, Sairam.
No. 11275, first quality commercial.
No. 12398, from Colorado.
No. 12409, Utah, non-irrigated.
No. 12671, from Kansas.
No. 12702, from Sherman, Texas.
No. 12747, from Billings, Mont.
No. 12748, from Germany.
No. 12784, Utah, irrigated.

No. 12801, from Texas Panhandle.
No. 12816, from Chinook, Mont.
No. 12820, from Nebraska.
No. 12846, from Kebilli Oasis, Tunis.
No. 13291, from New York.
No. 13259, from Nebraska.
No. 13857, from Simbirsk, Russia.
No. 17698, from Chinook, Mont.
No. 18751, from Turkestan.
No. 19508, from Kansas.
No. P. L. H. 3251, grown in South Dakota, from Baltic seed.
No. P. L. H. 3252, also grown in South Dakota.
No. 9322, from Touggourt.
No. 12694, from Provence, France.
No. 9453, from Bokhara.
No. 13437, from Arizona.
No. 1, Gypsum, Colorado, from cuttings.

A very satisfactory stand was secured with all varieties except No. 12,846 and No. 9,322. When the plants were one year old, they were examined very carefully for the presence of the disease with the result that all varieties but one, P. L. H. 3,251, were affected to a greater or less degree. While not all of the plants in each variety were suffering, some from each, with the exception of the one mentioned above, were diseased. Table No. 6, below, gives the results of these observations which were made June 20, 1908.

TABLE NO. 6

Showing Condition of the Different Varieties of Alfalfa When One Year Old. June 20, 1908.

Variety	Stand	Vigor	Prevalence of Disease	Size of Plants
9451	Fair	Fair	Present	Medium and small
11275	Good	Good	Present	Variable
12398	Fair	Fair	"	Variable
12409	Good	Good	"	Large
12671	Fair	Good	"	Variable
12702	"	Fair	"	Variable
12747	"	Good	"	Large
12748	"	Very good	"	Large
12801	"	Fair	"	Small
12816	"	Good	"	Variable
12820	"	Good	"	Variable
12846	Very poor	Very poor	"	Almost no plants
13291	Poor	Fair	"	Large
13259	Fair	Fair	"	Small
13857	Poor	Good	"	Large, varieties mixed
17698	Very good	Good	"	Low and large
18751	Good	Good	"	Variable
19508	"	Fair	"	Small
P. L. H. 3251	"	Very good	Absent	Large
P. L. H. 3252	Fair	Fair	Present	Small
12694	"	Poor	"	Frost bitten, varieties mixed
9453	"	Fair	"	Large spreading
13437	Poor	"	"	Small
12784	Fair	"	"	Variable
9322	.....	....	.....	No plants
Gypsum 1	Very good	Very good	Present	Small

Similar observations, made June 11, 1909, when the plants were two years old, showed that all varieties were affected, including P. L. H. No. 3,251, which had promised immunity the previous season. At this time of the year, the alfalfa was from ten to twenty inches high; the majority of it had a good color, and while the blight was present in all varieties, it was not abundant enough to do any serious damage to the crop. Only a few stems of each plant were suffering and only the lower internodes of these. Such stems had the characteristic watery, yellow green color, very little blackening having occurred up to this time. Occasionally plants were found on which the attack had been so acute that they were entirely destroyed. Their location was marked only by patches of dwarfed, shriveled stems, now dried and prostrate. Four of the twenty-six varieties were noticibly freer from the infection than the rest, namely, No. 12,398, No. 12,671, No. 12,784, and P. L. H. 3,251. It is a matter of considerable practical interest, that the first three of these are from seed which we may consider as home grown; the first is from Colorado, the second, Kansas; the third, Utah. If we are so fortunate as to find high resistance in plants from local seed, the question of obtaining resistant varieties will be much more easily solved than if we are compelled to breed up a strain from foreign seed. Table No. 7 gives the detailed observations on the variety plats, made June 11, 1909.

TABLE NO. 7

Showing Condition of the Different Varieties of Alfalfa when Two Years Old. June 11, 1909.

Variety	Vigor	Prevalence of the Disease
9451.....	Fair	Present
11275.....	Fair	Present
12398.....	Good	Present, but not serious
12409.....	Fair	Present
12671.....	Good	Present, but not serious
12702.....	Good	Present
12747.....	Good	Present
12748.....	Fair	Present
12784	Good	Present, but not serious
12801.....	Poor	Present
12816.....	Fair	Present
12820.....	Good	Present
12846.....	No plants	No plants
13291.....	Fair	" "
13259.....	Fair	" "
13857.....	Poor	" "
17698.....	Good	" "
18751.....	Fair	" "
19508	Good	" "
3251.	Very good	Present, but not serious
3252.....	Fair	Present
9322.....	No plants	No plants
12694.	Very good	Present, but not serious
9453.....	Poor	Present
13437.....	Fair	Present, but not serious
GypsumNo.1	Good	Present, but not serious

We shall continue this part of the investigation with the same, as well as additional varieties, since the only practical way of testing out the disease resistance of these different kinds of alfalfa is to grow them under actual field conditions on infected soil, where natural agents are at work. We shall introduce, also, legumes other than alfalfa, in order to determine the susceptibility of these to the disease, so that we may be in a position to recommend other crops as substitutes where the land is so badly infected as to make profitable alfalfa growing no longer possible.

PREVENTION AND TREATMENT.

Where the areas under cultivation reach such tremendous proportions as the alfalfa fields on the mountain ranches, all schemes for soil sterilization are obviously impracticable at the outset. The same may be said of the use of germicides to be applied to the plants either in the form of sprays or otherwise, for even though some such means should be discovered by which the infection could be prevented, the cost would undoubtedly make it prohibitive. Obviously, then, as stated before, the only practical way of combating and controlling the blight is by the introduction of resistant varieties. What is being done in this direction has been mentioned before.

Our field observations during the past year seem to indicate that immunity to the disease is closely related to resistance to late spring freezing. On the one hand, those plants which were severely injured by the late spring frost were, without exception, the first to show the disease and were the worst infected later in the season: on the other hand, those varieties which grew from hardy stock and which suffered only slightly from the frost, were more nearly free from the blight. This coming year we shall endeavor to determine whether the relation between disease resistance and frost resistance is a constant one, and if it proves to be such, then we shall attempt to stamp out the trouble by securing frost resistant varieties.

In the meantime, we recommend, as a means of control, that the frosted alfalfa be clipped as soon as one is reasonably certain that there is no more danger from frost. By this means, the frost split stems, in which the disease appears to originate, will be gotten rid of, thus affording an opportunity for the early growth of a new cutting. Prof. P. K. Blinn, who has charge of the Experiment Station work at Rocky Ford, informs the writer that this practice of early clipping to remove the frost bitten shoots, which retard growth, is rapidly growing in favor among the farmers in his locality.

ACKNOWLEDGMENT.

The writer wishes to thank Prof. B. O. Longyear and Miss M. A. Palmer for the preparation of the colored plate.

EXPLANATION OF PLATES.

PLATE I.—Fig. 1, Ps. medicaginis, n. sp.; 24 hour agar culture stained with aqueous fuchsin; original x 1,000, reduced by engraver 6:5.

Fig. 2, Ps. medicaginis, n. sp.; 48 hour agar culture stained with aqueous fuchsin to show the formation of filaments; original x 1,000, reduced by engarver 6:5.

Fig. 3, Ps. medicaginis, n. sp.; surface agar colony 7 days old, photographed by transmitted light to show concentric rings of growth; original x 20, reduced by engraver 6:5.

Fig. 4, Ps. medicaginis, n. sp.; surface agar colony 7 days old, photographed by reflected light; original x 20, reduced by engraver 6:5.

PLATE II.—Fig. 1, Ps. medicaginis, n. sp.; agar colonies 7 days old showing deep and surface colonies by transmitted light; original x 20, reduced by engraver 3:2.

Fig. 2, Ps. medicaginis, n. sp.; agar colonies 7 days old showing deep and surface colonies by reflected light; original x 20, reduced by engraver 3:2.

Fig. 3, Petri dish showing pure culture of Ps. medicaginis colonies 2/3 natural size; original x 1, reduced by engraver 3:2.

PLATE III.—Fig. 1, Alfalfa stem, inoculated by smearing the freshly scraped stem with a 48 hour agar culture of Ps. medicaginis, x 2; 40 days after inoculation.

Fig. 2, Diseased alfalfa stem showing the yellowish, olive green color, characteristic in the early stages, x 2. Field specimen, natural inoculation.

Fig. 3, Diseased alfalfa stem showing the blackened condition in the late stages of the blight, x 2. Field specimen, natural inoculation.

Fig. 4, Alfalfa stem, inoculated with a 48 hour agar culture of Ps. medicaginis by means of needle pricks, x 2; 15 days after inoculation.

Fig. 5, Alfalfa leaf showing diseased, yellow areas, apparently of water pore or stomatal infection, x 2. Field specimen, natural inoculation.

NOTE.—Figs. 1 and 2, Plate I, were made by the writer with a Leitz Photomicrographic apparatus in connection with the Leitz microscope, 1/12 oil immersion objective, eyepiece I; Welsbach gas light; Cramer's Medium Isochromatic Plates. Figs. 3 and 4, Plate I, and Figs. 1 and 2, Plate II, were made by the writer with the Leitz Photomicrographic apparatus in connection with a Leitz Microsummar, 35 mm., f:4, 5. Plate III. was prepared by Miss M. A. Palmer and Prof. B. O. Longyear from fresh material.