

ASPECTS ON INFECTION OF VERTICILLIUM
WILT OF POTATOES

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CONSTANTINE C. THANASSOULOPOULOS
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This is to certify that the

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ASPECTS ON INFECTION OF VERTICILLIUM WILT OF POTATOES

By

Constantine C. Thanassoulopoulos

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ABSTRACT

ASPECTS ON INFECTION BY VERTICILLIUM WILT OF POTATOES

by Constantine C. Thanassoulopoulos

Certain aspects of penetration and infection of potatoes by Verticillium albo-atrum Reinke and Berthold, and perpetuation of the fungus in seed tubers have been studied.

V. albo-atrum, "microsclerotial" and "dark mycelial" types, grown on potato-dextrose agar or corn meal-Perlite media, were used for inoculation. Two inoculum types were used throughout: 1) a water suspension of spores and mycelial fragments from scraped PDA cultures, and 2) a suspension obtained by crumbling a corn meal-Perlite culture and suspending in water.

Sebago, Kennebec, Cherokee, Russet Arenac, and Russet Burbank varieties were used in laboratory, greenhouse, and field experiments.

Considerable evidence was obtained histologically and by isolations that the fungus penetrated leaves of potatoes through stomata whether leaves were detached or attached to the plants, and was followed by invasion of the vascular tissue of petioles. Further evidence of systemic spread of the fungus from the leaf lamina to the stem and the

tubers was also obtained by positive isolations from stems and tubers. High inoculum concentration, low light intensity and high relative humidity were important factors in increased severity of symptom appearance and infection of leaves.

Tuber sprout infection caused the death of apical and leaf primordia meristems. Evidence was obtained suggesting direct penetration through epidermal cells of the sprouts, apparently at any location, followed by granulation and death of protoplasm. Abundant conidiophores and heavy sporulation was produced on dead sprout tissues.

Of the different methods used for field inoculation, root inoculation and seed surface contamination were the most effective, as evaluated by vascular discoloration of the tubers, and positive isolations of the fungus. The leaf inoculation method resulted in a relatively high incidence of infection, as indicated by both vascular discoloration of the tubers, and positive isolations from the discolored areas. The cut tuber method in which a tuber was cut and the opening was filled with inoculum was completely ineffective. Inoculation either by injection of inoculum with a hypodermic syringe or by inserting a tooth pick carrying inoculum in the stem of the plants were intermediate in effectiveness. The root dip method, in which plants were dug out and dipped in inoculum, was not useful for field work because of the direct influence of transplanting injury on the plant growth.

Seed of healthy appearing and vascular discolored tubers were evaluated in field trials. Isolations before planting gave no evidence of viable fungus within vascular regions of the seed. No differences between stands, vigor, and general appearance were evident during the growing season. No significant differences were obtained at harvest time in vascular discoloration of the harvested tubers. Positive isolations made from vascular discolored areas of tubers were essentially similar whether tubers were grown from healthy or from vascular discolored seed lots.

Considerable reduction of viable fungus in vascular discolored tubers was evident 4 months after harvest. A further reduction in viability of Verticillium in vascular discolored tubers was obtained 7 months after harvest. In 2 seed lots no viable Verticillium was obtained and in 2 seed lots viable fungus was obtained from approximately 10% of the tubers. This is in contrast with previous results when seed stored from the 1965 crop and used in isolation tests before planting in 1966 was completely free from viable Verticillium.

This trend suggests that survival of V. albo-atrum in seed tubers may be of relatively minor importance in transmission of the fungus in potato crops.

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INTRODUCTION

Verticillium wilt is a major disease problem in potatoes. In Michigan, the chief loss is associated with poor tuber quality because of vascular discoloration. More information is needed concerning infection and inoculum survival.

Verticillium albo-atrum Reinke and Berthold was described for the first time in 1879 as a potato parasite. Since that time it has been recognized as a soil borne pathogen invading roots or root hairs (Reinke and Berthold, 1879). This observation was confirmed later by several authors (Roberts, 1943, Ayers, 1952, van den Ende, 1958) and also it has been found recently to invade cotton through the root cap, between or within root epidermal cells, root hairs, and through hypocotyls (Garber and Houston, 1966).

Reinke and Berthold (1879) reported brown spots on potato leaves infected with Verticillium wilt, but they did not report isolation of the fungus from these brown spots. They also reported brown patches on subterranean stems due to Verticillium infection, but also they did not state whether the fungus was isolated from them. In 1918, successful isolations of the fungus were effected from yellow spots on the outer leaves of beets by Westerdijk

(from Rudolph, 1931). The first report of true leaf infection by Verticillium was made on tomato and eggplant in 1959 (Providenti and Schroeder, 1959). In their trials, the disease obtained by leaf infection was similar to the disease resulting from root infection. Sackston (1960), one year later, reported leaf infection of clover seedlings accomplished by spraying leaves with spore suspensions, but there are some doubts from his paper whether or not the fungus invaded through leaves or from lower portions of the plants. In 1963, Griffiths and Isaac confirmed the work of Providenti and Schroeder (1959) on tomato leaf infection. In spite of the fact that Verticillium has been reported in systemically invaded potato leaves even to the tips (Barrus and Chupp, 1926), no attention has been given to the possibility that leaves could also be primary sites of infection.

In 1912, Dale, in England, in a brief paper described "Blindness" as a tuber disease of potatoes due to V. albo-atrum. She claimed that the fungus attacked the tubers by means of the eyes. Pethybridge (1916) suggested also that the mycelium of the fungus grows around the outside cork of the tubers, it reaches the bases of the young sprouts and probably penetrates through the cortical portion of the young roots. Pitt et al. (1964) reported also that the abnormality of sprouts known as "coiled sprout" was caused by V. nubilum Peth. infection.

The fungus also has been reported to invade stems of young potato plants by growing directly from infected tubers (Ayers, 1952, Robinson, et al., 1957, Robinson and Ayers, 1961). The same authors (1957, 1961) have also found that this type of inoculation is not as important in inciting disease as the inoculum on the tuber surface. Friedman and Folsom (1953) found no increase of vascular discoloration during storage of Kennebec tubers.

Although it is generally recognized that V. albo-atrum may be isolated from vascular discolored areas of tubers, Edson (1920) and Muncie (1954) have reported isolations of the fungus from apparently healthy tubers.

Some method of stimulating severe incidence of V. albo-atrum in the field is highly desirable. For that reason, methods of inoculation have been attempted using among other methods those of several investigators working with different plant hosts (Brinkerhoff, 1949, Keyworth and Bennett, 1951, Robinson, et al., 1957, Robinson and Ayers, 1961, Patil et al., 1964, Erwin et al., 1965, Fronek, 1965). A critical evaluation of these methods to determine which of them is most efficient for increasing disease incidence and severity in field experiments with potatoes is necessary, in evaluating varietal resistance and in developing of methods of control.

This study deals with certain aspects of infection of potatoes by V. albo-atrum: 1) leaf and tuber sprout infection; 2) methods of inoculation in the field; and

3) perpetuation in seed tubers during the storage and incidence of disease in the subsequent crop.

PART I

LEAF AND SPROUT INFECTION

Materials and Methods

Isolates of V. albo-atrum from potato both microsclerotial (MS) and dark mycelial (DM) types were grown on potato agar (PDA), or on corn meal-Perlite which contained respectively 400 g and 250 g of each in 1000 ml water.

Two types of inoculum were used throughout the experiments: 1) "spores"--from PDA culture, a suspension of mycelial fragments and conidia, approximately $1-1.2 \times 10^5$ per ml, prepared by scraping spores from the surface of colonies; and 2) "resting bodies"--from corn meal-Perlite culture, a suspension of mycelial fragments and spores adjusted to the same spore concentration as 1), but having in addition abundant resting bodies (microsclerotia or dark mycelial structures), and also nutrients from the corn meal. The second type was prepared by crumbling and suspending in water a culture grown on corn meal-Perlite.

Leaves or usually the upper 2/3 of the plants were dipped in an inoculum suspension. Sprouts of potato tubers were inoculated with "resting bodies" type inoculum

from an MS isolate of the fungus. Inoculations were made either on detached sprouts growing in a moist chamber, or surface infected tubers were planted between 2 layers of damp sphagnum moss infested with inoculum

Isolations from potato petioles and stems were made on PDA adjusted to approximately pH 5 with 25% lactic acid. All plant material used for isolations, leaf petioles, sprouts, stems etc., had been previously sterilized with 1% sodium hypochlorite for 1 min.

Histological sections were cut with microtome for cutting fresh plant material (Hooker, in press) and mounted in distilled water without fixation. Photographs of fresh, unfixed, infected plant material are shown.

Results

Symptoms of leaf infection.--Similar symptoms developed in laboratory, greenhouse, and field inoculations (Fig. 1). In the field, typical symptoms appeared when the inoculation was made beneath the leaf canopy. Certain aspects of field trials are treated in greater detail in another section of this paper.

Early symptoms became evident 48 hours after inoculation as small bronze spots on leaf lamina. These areas enlarged during the next 2-3 days and the center of the lesion became darker brown with a watersoaked appearance. The leaf became bronze to yellow in color within a few days after inoculation. In a few cases



Fig. 1. Bronzing and necroses of potato leaves 5 days after field inoculation with "resting bodies" inoculum of V. albo-atrum.

chlorosis was most prominent in the interveinal areas. The larger veins maintained the green color somewhat longer. By the fifth day after inoculation, some dead leaflets were present, and infection may have progressed sufficiently to kill the entire leaf. In some cases, the whole leaf was dead within 5-6 days. Usually most of the leaves were dead within 15-20 days after inoculation.

Vascular discoloration following leaf infection was most common in the leaf petiole, but was also present in stems and in tuber stolon attachments. The fungus was readily isolated from vascular discolored areas, especially from those of leaf petioles.

Heavy sporulation of V. albo-atrum, evident macroscopically, formed on wounded detached leaves under conditions of low light and high relative humidity. It also developed on wounded attached leaves in greenhouse plants under similar conditions. Macroscopic sporulation did not develop under the other environmental conditions and apparently wounding was a prerequisite, under the conditions of the trials.

Dead leaves in the field were very soon invaded by different saprophytic fungi, especially Botrytis and Alternaria, making diagnosis of verticillium in infected leaves difficult. Invasion by other fungi may possibly have accounted for the fact that sporulation was not observed in the field. Abundant microsclerotia were formed on dead leaves in laboratory experiments if the

leaves were kept for 1 month or more under moist chamber conditions.

Infection of detached leaves.--In a preliminary laboratory trial in petri plates, infection of detached potato leaflets of the Russet Arenac variety was demonstrated by positive isolations from the petioles in 20 of 30 inoculated leaflets. A drop of "spores" inoculum was placed near the tip of leaflet, and 48-72 hours later a bronze colored spot around the inoculation point suggested some infection. Positive isolations made from the leaflet petioles, 3-10 days after inoculation, indicated that there had been infection as well as some fungus movement through the vascular system of the leaflets.

In later inoculation tests with the Russet Arenac variety, green leaves with 5-7 leaflets were detached and the petioles were placed in small glass vials (1.5X5.5 cm) containing tap water. Water was added once or twice a day to replace transpiration loss. Care was taken during and following inoculation to avoid contamination of the water. Water was added carefully and the inoculated portions of the leaves were arranged so that the leaflets hung over the side of the vials.

Factors studied in relation to infection (Table 1) were: 1) inoculum type, "spores" or "resting bodies"; 2) light intensity, 100 ft-c approximately vs. 20-25% ft-c each at 12 hours per day; 3) humidity; and 4)

Table 1.--Influence of inoculum type, light intensity, humidity, and wounding on infection of detached inoculated potato leaves by V. albo-atrum

Factors compared	Leaves tested	Symptoms on leaves ^{a/}			Dead Leaves		V. albo-atrum in petioles after 10 days ^{b/} 30 days	
		no.	no.	% C/ %	%	% C/ %	%	% C/ %
Innoculum control	117	0	0	0		13 b	0	0
"resting bodies"	90	51	57a	35a	16	78a	41	83a
"spores"	84	14	17 b	5 b	3	6 b	3	3 b
Light low, control	81	0	0	0	7	16 b	0	0
low, inoculated	96	54	56a	36a	15	46a	40	43a
high, control	36	0	0	0	0	3 b	0	0
high, inoculated	78	11	14 b	5 b	6	21 b	7	43a
Relative humidity in moist chamber, control	81	0	0	0	4	10 b	0	0
inoculated	96	51	53a	31a	13	36a	35	45a
no moist chamber, control	36	0	0	0	8	16 b	0	0
inoculated	78	14	18 b	10 b	11	32a	12	42a
Wounding infection wounded, control	60	0	0	0	5	12 b	0	0
inoculated	90	40	44a	24a	14	52a	24	45a
not wounded, control	57	0	0	0	5	12b	0	0
inoculated	84	25	30a	17a	13	49a	24	45a

^{a/} Symptoms on leaves 10 days after inoculation.

^{b/} Presence of fungus in petioles established by isolations on PDA.

^{c/} Similar letters indicate results not differing significantly from each other at the 1% level of probability. Interaction of four factors was significant at the 1% level of probability.

influence of wounding the leaf surface. Treatments were planned in all possible combinations of these 4 factors. For each treatment, 3-7 leaves were used with 3 replications in a factorial design.

Leaf infection was determined by isolations from the petioles. Isolations were made when the inoculated leaflets were dead or almost dead. At this time, the noninoculated basal leaflets and the lower part of the leaf petiole still appeared to be in good health. Significant differences were determined by the Tuckey test (Guenther, 1964). A disease index was developed by classifying diseased leaves into 0-healthy, to 5-dead, and expressing this on a percentage basis. Intermediate values, 1, 2, 3, and 4, were assigned according to increasing severity of symptoms on the leaflets. The leaf was considered systemically invaded when the fungus could be recovered from the petiole below the inoculated leaflets.

More severe symptoms and higher infectivity was obtained with "resting bodies" type inoculum than with "spores" inoculum. Leaf survival with "spores" after 30 days was good and was very similar to that of the controls. In contrast, leaf survival with "resting bodies" inoculum was low. Systemic infection after 10 or 30 days was high with "resting bodies" inoculum and was very low when "spores" were used for inoculation.

More severe symptoms and more frequent systemic infections (10 day) were obtained with low than with high

light intensity. After 30 days the amount of systemic infection was similar. Leaf survival in high light intensity after 10 and 30 days was significantly higher than in low light intensity and essentially similar to that of the noninoculated controls.

Leaf symptoms were less severe in low than in high humidity. The number of dead leaves, however, was essentially similar after 10 or 30 days, and in either case the number of dead leaves following inoculation was higher than in the noninoculated controls. Systemic infection was lower outside the moist chamber at 10 days but after 30 days differences were not significant.

Wounding of leaves had slight or no influence on symptom appearance, survival of leaves, or systemic invasion either 10 or 30 days after inoculation.

The interaction of the above 4 factors was significant. Most severe infection was obtained with heavy inoculum in low light, high relative humidity, and following wounding.

Younger leaves seemed to be more easily infected, while older leaves were more quickly killed.

Infection of attached leaves.--In preliminary greenhouse experiments, infection of attached potato leaflets of Russet Arenac variety by V. albo-atrum was readily obtained. This was evident by symptoms developing on 52 of 60 inoculated leaflets, each leaflet being on a separate plant. A drop of "spores" inoculum was placed on

30 apical leaflets and a small piece of "resting bodies" inoculum was placed on the other 30. Half of the plants inoculated with "spores" inoculum and half of those inoculated with "resting bodies" were placed in the dark and the other in 50-80 ft-c (14 hours light). All plants were covered by plastic bags. Similar treatment was given to control plants. The plants remained under these low light intensity and high humidity conditions for 5 days, and then they were placed under regular greenhouse conditions. Three to 4 days after inoculation a bronze area was evident around the point of inoculation on leaflets infected with both inoculum types. Leaflets inoculated with "resting bodies" inoculum type and held in the dark had more severe symptoms than leaflets kept in 50-80 ft-c light intensity. One week to 10 days after inoculation 10 of 30 leaves with inoculated leaflets held in the dark abscised and 2 of 30 leaflets from the plants in 50-80 ft-c were also abscised. No symptoms were evident in controls except that of a slight chlorosis, from which the leaves soon recovered after exposure in full light. No leaves from controls abscised within 10 days after inoculation. No isolations were attempted.

Later experiments were designed to determine if systemic invasion of stems following leaf infection was common. Sebago, Kennebec, and Cherokee varieties were inoculated with "resting bodies" type inoculum of either

MS or DM culture types. Since the DM and MS culture types caused similar types and frequency of infection the data were combined (Table 2). Plants were kept in moist chamber conditions by placing them in plastic bags for 3 days after inoculation. Half of the plants were placed under the greenhouse bench with 10-50 ft-c light intensity during the day (14 hours light), while the other plants were kept in day light but not in direct sun light at 1000-5000 ft-c. Except for the 2 basal leaflets for each leaf, every other leaf of each plant was inoculated by dipping into inoculum suspension. The alternate noninoculated leaves served as controls.

Random isolations from leaf petioles before inoculation established that V. albo-atrum was not originally present in the plant. Early symptoms on leaves were visible 2 days after inoculation and they became clearly evident by the third day (Fig. 2). Isolations from leaf petioles 3 days after inoculation were positive for V. albo-atrum in 6 of 9 leaves. Isolations from leaf petioles of inoculated leaves were also made 7 and 14 days after inoculation. These data are combined (Table 2). The fungus was isolated from 24 of 40 leaf petioles at low light intensity. At high light intensity petioles of only 2 of 36 leaves were systemically infected. Mycelium and some granulation of vascular elements were present within xylem in sections of leaf petioles, while such structures and textures were

Table 2.--Infection of attached leaves and petioles following inoculation of leaf lamina by V. albo-atrum

Variety and light intensity	Inoculated leaves	Leaves with symptoms after 3 days	Leaves with <u>V. albo-atrum</u> in petioles ^{a/}
	no.	no.	no.
Sebago			
low light	12	10	9
high light	11	0	0
Kennebec			
low light	17	17	10
high light	16	0	2
Cherokee			
low light	11	9	5
high light	9	0	0

^{a/} Presence of V. albo-atrum established by isolations to PDA after 7 and 14 days, and the data were combined.



Fig. 2. Bronzing symptoms on potato leaves 3 days after greenhouse inoculation with V. albo-atrum.

absent in the controls. The influence of high light intensity on systemic invasion of petioles of attached leaves was quite similar to that obtained in detached leaves. This is evident by comparing the 10 day observation (Table 1) of systemic infection with data obtained within 14 days after infection (Table 2). Resistance of the leaves to systemic infection evident on detached and attached leaves is apparently increased by light.

Systemic invasion of potato stems by V. albo-atrum following infection of aerial part of plants was established (Table 3). Plants of the varieties of Sebago and Russet Arenac were inoculated by dipping the upper 2/3 of the plant into inoculum consisting of "resting bodies" of the MS type. To avoid soil contamination, pots were covered with an aluminum foil sheet tied around the plant stem. The plants were watered by subirrigation. Non inoculated controls were similarly dipped into tap water. Plants were kept under conditions favorable for infection, i.e., high relative humidity and low light intensity, for 4 days.

Systemic infection of 11 of 32 plants was demonstrated by isolations made from different places on the main stem of the plant 2 months after inoculation. In 9 of 11 infected plants the fungus could only be isolated from the part of the plant above the inoculated leaves; in 1

Table 3.--Systemic invasion of potato stems by V. albo-atrum following leaf inoculation

Variety and treatment	Plants	Plants with <u>V. albo-atrum</u> In the stem ^{a/}
	no.	no.
Sebago		
control	8	0
inoculated	16	6
Russet Arenac		
control	8	0
inoculated	16	5

^{a/}Significant differences at the 1% level of probability with t test.

case it was isolated from both above and below, and in 1 plant it was recovered only from the lower portion of the stem but not from the roots. In 5 of these 11 infected plants, distinguishable vascular discoloration was evident. Similar isolations from control plants were negative and tissue was believed to be sterile.

Infected leaves had a tendency to absciss early. This resulted in defoliation of plants following severe leaf infection. Isolations attempted from petioles of dead abscissed leaves were almost always negative, with respect to recovering of Verticillium.

Penetration and subsequent progress of the fungus.--

Detached or attached leaves inoculated with "resting bodies" inoculum of both types of the fungus and kept under low light intensity and high moisture were sectioned. Penetration of V. albo-atrum through leaf stomata (Fig. 3) of either the upper or lower surface was demonstrated. Infection of stomata was apparently accomplished by a number of hyphae growing between the guard cells. A heavy mass of hyphae was present in the substomatal chambers at least by the third to fourth day after inoculation. From this mass, hyphae spread intercellularly to the neighboring cells. Necroses of cells around infection points were microscopically evident about the same time that penetration had been completed by the fungus. When "spores" was used as inoculum the same pattern of penetration was observed histologically by mycelium within the stomata was not as abundant.

Fungus hyphae were present in xylem and xylem parenchyma of vascular bundles of leaf lamina 4 days after inoculation (Fig. 4). In control plants, the presence of fungus hyphae could not be demonstrated in vascular tissue of leaf lamina by histological examination, nor in that of petioles by histological examination or by isolation.

Infection of sprouted tubers.--A stunting of young sprouts and roots was obtained in preliminary inoculation experiments.

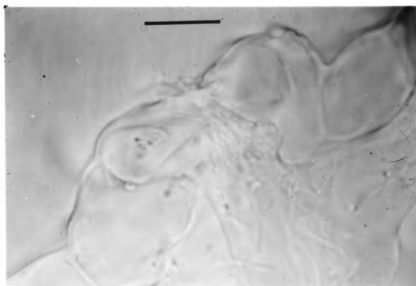
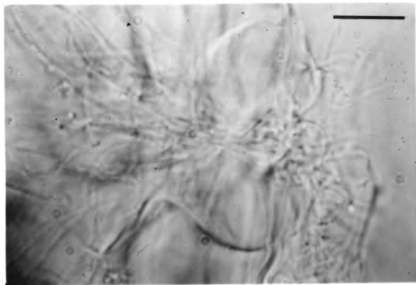


Fig. 3. Penetration of *V. albo-atrum* through leaf stomata, above-upper surface, below-lower surface, 4 days after inoculation. (Reference line indicates 10 μ).

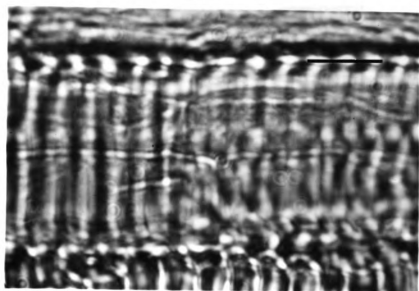
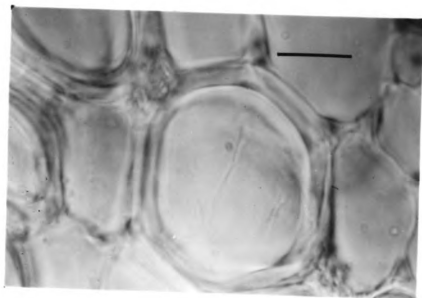


Fig. 4. Mycelium in vascular tissue of potato leaf
upper-cross section of vascular elements
lower-lingitudinal section of xylem vessels,
4 days after inoculation. (Reference line
indicates 10 μ).

In more extensive trials, tubers from Sebago, Kennebec, and Russet Burbank varieties with sprouts approximately 0.5 cm long were placed on a 1 cm layer of damp sphagnum, had been treated with abundant corn meal-Perlite inoculum. When the tips of sprouts of controls had grown above the layer of sphagnum, 15-16 days later, tubers were removed carefully, washed thoroughly in running water, and observed. In all cases sprouts from inoculated tubers were less vigorous than those from control tubers. In some cases, the number of sprouts per tuber was decidedly reduced in infected tubers as compared to controls. Tips of sprouts which were present at the time of inoculation were usually dead. Sprouts grown from inoculated tubers grew almost exclusively as lateral or secondary branches from the original sprout, and their length was markedly reduced in comparison with the noninoculated tubers (Fig. 5).

In several tests, approximately a 20% reduction of tuber germination was obtained. These results are in good agreement with observations in field experiments with the Sebago variety and are described elsewhere.

When tubers with 0.5 cm sprouts were inoculated, the length and fresh weight of roots were noticeably reduced. On the other hand, when tubers were completely dormant at the time of inoculation no root reduction was observed. This may suggest that within a short time,

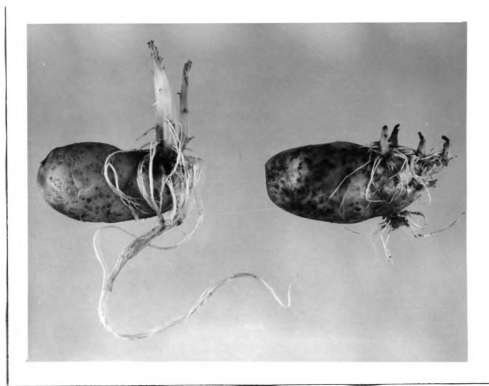


Fig. 5. Infection of tuber sprouts by V. albo-atrum and reduction of sprout growth.

viable inoculum was considerably reduced and that root primordia escaped contamination because they grew later than primordia of leaves and tips of growing sprouts.

Infection of detached sprouts.--Visual symptoms of infection on Sebago and Kennebec sprouts consisted of browning of the surface of the tips.

Sprouts 2-3 cm long were detached from the tuber, disinfected in 1% sodium hypochlorite for 1 min and thoroughly washed twice in sterile water. The upper half of the sprouts was then dipped into inoculum and the portion bearing root primordia was inserted in water agar. Without exception apical meristems were necrotic when exposed to infection in this way. Lateral buds were usually killed but when they were not, they germinated producing secondary sprouts. There was no inhibition of root formation, as evidenced by comparison with the controls. Controls grew normally and sprouts had vigorous green tips.

The following sequence of events obtained in sprouts grown on water agar: Verticillium hyphae, either those growing from a mycelial mass or from the germ tube of germinating spores, were capable of penetrating epidermal cells of the sprouts apparently at any location (Fig. 6). Some evidence also was obtained that infection may have taken place through hair cells on the surface of the sprout developed a brown color and shortly after that

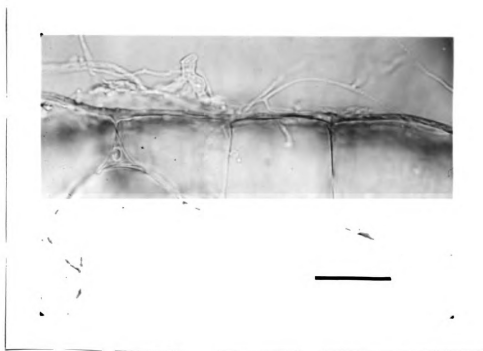


Fig. 6. Penetration of *V. albo-atrum* through tuber sprout surface. (Reference line indicates 25 μ).

protoplasm of the cells which the hypha had penetrated became granulated (Fig. 7), turned brown and died. This was followed by death of neighboring cells. The fungus progressed intracellularly. In many cases necrotic tissues were limited to the superficial layer of the sprout. Further growth of the sprout was stopped since the growing cells of apical meristems and leaf primordia had been killed (Fig. 8). Soon after cells had been killed the fungus developed conidiophores on which abundant spores were produced (Fig. 9). Necrotic tissue extended into the sprout and was observed in close proximity to the vascular bundles. This suggested that systemic invasion by V. albo-atrum could be anticipated (Fig. 10). Of 20 isolations attempted, 2 positive isolations were made from the central vessels of the sprouts as soon as 7 days after inoculation.

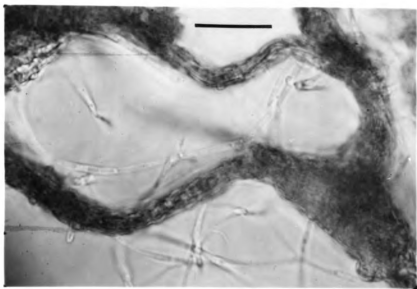
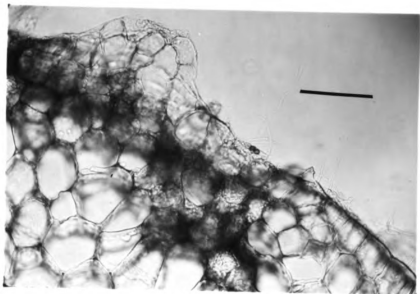


Fig. 7. Hyphae of *V. albo-atrum* within cells of tuber sprout. Note granulation of protoplasm. (Reference lines indicate, upper 25 μ , lower 100 μ).

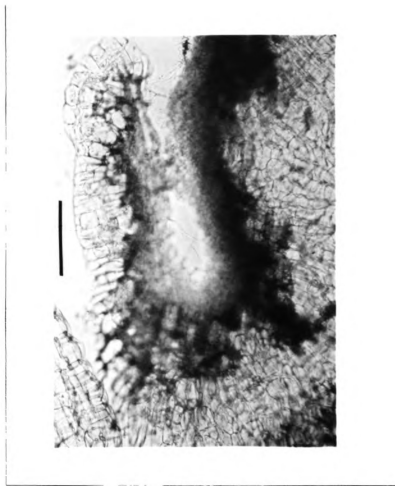


Fig. 8. Apical meristem and leaf primordium of a tuber sprout following V. albo-atrum infection. (Reference line indicates 100 μ).

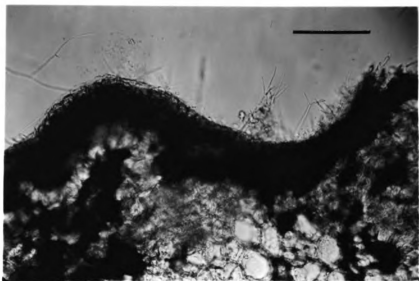


Fig. 9. Conidiophores of *V. albo-atrum* developing on necrotic potato sprout after infection. (Reference line indicates 100 μ).

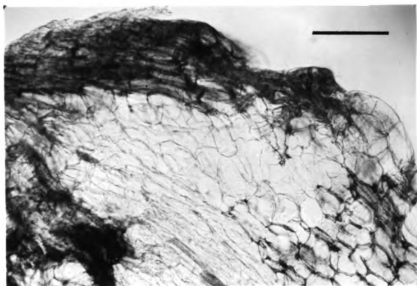


Fig. 10. Necrotic peripheral cells of apical meristem of a tuber sprout. Note proximity of necrotic tissue to vascular elements. (Reference line indicates 100 μ).

PART II

COMPARISON OF METHODS FOR FIELD INOCULATION

Materials and Methods

Healthy appearing tubers of the Sebago (moderately susceptible) and Kennebec (susceptible) varieties were planted on muck soil at the Michigan State University Muck Experimental Station at Bath. Before planting, all tubers were cut at the stolon attachment, and if a suggestion of vascular discoloration was present, tubers were discarded. Sebago tubers were planted in a randomized block design with 25 tubers for each treatment in 6 replications, and with Kennebec 3 replications of 10 tubers for each treatment.

For inoculum, V. albo-atrum, MS type, originally isolated from diseased potatoes from Michigan, was grown on corn meal-Perlite medium for about 2 months. Unless otherwise indicated, this inoculum, consisting of spores, mycelial fragments, microsclerotia, corn meal, and Perlite, was crumbled and shaken with seed tubers or suspended in water for spraying and root dipping.

Inoculation procedures were:

- 1) Control. No inoculation.
- 2) Root inoculation. Soil containing roots near the plants was cut with a shovel, inoculum poured into the

freshly cut soil and presumably over the cut ends of roots, and the soil pressed back into place.

3) Root dip. Plants were dug and roots dipped into inoculum suspension and replanted.

4) Seed surface contamination (5 day). Whole seed tubers were shaken in inoculum contained in a plastic bag, air dried, and planted 5 days later.

5) Seed surface contamination (0 day). Same as 3 but seed were planted immediately after inoculation.

6) Tuber cut. Seed tubers were cut once between apical eyes with a sharp knife contaminated with inoculum, tuber opening filled with inoculum, and the tubers planted 5 days later.

7) Leaf inoculation. Inoculum mixed with 400 mesh carborundum sprayed on leaves at close range with an atomizer at 70 lbs pressure.

8) Hypodermic syringe. 0.5 ml spore and mycelial suspension (12×10^4 spores /ml) injected with a hypodermic syringe into the first or the second above ground node of each plant.

9) Tooth pick inoculation. Inoculum grown in potato dextrose broth culture on tooth picks which were inserted into the stem of each plant.

Inoculation 2), 3), 7), 8), and 9 were made 40 days after planting, when plants were 15-20 cm tall. Treatments 4), 5), and 6), were made before planting.

Stand counts were made 1 month after planting. Average height of plants from each inoculation treatment was expressed as a per cent ratio of the average height of control plants. The extent of tuber vascular discoloration after harvest was determined from a random sample of approximately 200 tubers by cutting the stolon end 0.5 cm below the point of attachment. Analysis of variance and the Duncan test were used for statistical analysis.

V. albo-atrum infection of tubers was determined by isolations made by planting 3 pieces from each tuber into PDA adjusted to approximately pH 5 with 25% lactic acid.

Results

Stands of Sebago (Table 4) were significantly reduced when seed were covered with surface borne inoculum 5 days before planting. Stands from tubers similarly inoculated just before planting were slightly reduced. No other treatments reduced stands. Plants which grew from seed which had been surface contaminated (0 day) were significantly smaller (1% level) in size 1 month after planting as compared to controls. Plants growing from surface contaminated seed (5 day) were slightly stunted. Root dip inoculation which entailed a transplanting type of treatment at an unfavorable time in plant development caused the greatest reduction in plant vigor and growth of any of the inoculation treatments.

Table 4.--Effectiveness of different inoculation methods for field infection of Sebago potato by V. albo-atrum

Treatment	Plants		Yield per plant lbs.	Vascular Discoloration			
	Total no.	Mean stand plot no. ^{a/}		Tubers examined no.	Tubers affected % ^{a/}	Tubers for isolation no.	Verticillium infected %
1) Control	276	23.0 ^{a/}	2.8a	424	5.4 e	36	0
2) Root Inoculation	137	(22.6) ^{c/}	2.7a	407	32.7a	18	11.1
3) Root dip	135	(22.8)	1.6 b	216	33.8a	18	11.1
4) Seed surface contamination (5 day)	113	18.9 b	3.3a	186	23.5 bc	18	16.6
5) Seed surface contamination (0 day)	131	21.8ab	3.1a	176	25.5ab	18	33.2
6) Tuber cut	144	24.0a	3.0a	198	12.6 de	18	0
7) Leaf inoculation	138	(23.0)	2.9a	203	22.9 bc	18	16.6
8) Hypodermic syringe	138	(23.0)	2.6a	201	16.0 cd	18	16.6
9) Tooth pick inoculation	142	(23.7)	2.9a	213	19.8bcd	18	27.7

^{a/} Similar letters indicate no significant differences at the 0.1% level of probability.

^{b/} Average stands from 6 plots. 25 tubers per plot.

^{c/} Figures in parenthesis indicate that plants were inoculated after stands had been determined.

Yields from plants following root dip inoculation were significantly reduced as compared to controls. Yields from other treatments were not significantly different from yields of control plots. Except for the cut tuber method, vascular discoloration of tubers was significantly higher (0.1% level of probability) than that of control tubers with each of the inoculation procedures tested. The highest percentage of vascular discoloration was obtained by the methods of root dip, root inoculation and seed surface contamination (0 day).

Isolations from vascular discolored areas of the tubers showed that Verticillium was present in tubers of all treatments except those of the control and cut tuber inoculation. In all cases of positive isolations, the same type of fungus used for inoculation was subsequently isolated.

The least variation as determined by incidence of vascular discoloration was obtained following inoculations by the hypodermic syringe, leaf inoculation, and both seed surface contamination methods. Greatest variability was encountered with the tooth pick method of inoculation.

The leaf inoculation method provided relatively high levels of infection as measured by both vascular discoloration of the tubers and positive isolations of the fungus from vascular discolored areas. Furthermore, results were quite uniform between replications. This method could be useful for further work. The hypodermic

syringe method was not so effective in stimulating vascular discoloration and the isolations of the fungus was relatively limited, but the method had the advantage of considerable uniformity. The tooth pick method, quite similar in some respects with the hypodermic syringe, gave better results than the hypodermic syringe in stimulating vascular discoloration and incidence of infection, but had the disadvantage of great variability. This may have been due to variation in quantities of inoculum inserted into each stem. The tuber cut method was completely ineffective for inoculation.

Stands of Kennebec (Table 5), were not influenced significantly by any of the inoculation methods tested. In other respects, results were essentially similar to those obtained with Sebago. Leaf inoculation, hypodermic syringe, and tooth pick inoculation methods were negative in isolation tests. Possibly differences between responses of Sebago and Kennebec have been due to the small number of tubers available for isolation.

The root dip method for inoculating both varieties was the most effective as measured by incidence of vascular discoloration. Infection of tubers as measured by frequency of positive isolations was no more effective than that with certain other methods. Plants did not recover from the shock of inoculation and transplanting. Two to 3 days after inoculation plants were wilting.

Table 5.--Effectiveness of different inoculation methods for field infection of Kennebec potato by V. albo-atrum

Treatment	Plants		Yield per plant lbs.	Vascular discoloration			
	Total no.	Mean stand no./plot ^a		Tubers examined no.	Tubers affected %	Tubers for isolation no.	Verticillium infected %
1) Control	60	10.0 ^{b/}	3.0a	135	12.7 c	18	0
2) Root inoculation	30	(10.0)	3.1a	75	36.5 b	9	11.1
3) Root dip	29	(9.6)	2.0b	90	47.6a	9	22.2
4) Seed surface contamination (5 day)	30	10.0a	3.1a	86	13.8 bc	9	11.1
5) Seed surface contamination (0 day)	28	9.3a	3.3a	81	27.2 b	9	11.1
6) Tuber cut	30	10.0a	3.0a	81	11.4 c	9	22.2
7) Leaf inoculation	29	(9.6)	2.9a	86	26.1 b	9	0
8) Hypodermic syringe	30	(10.0)	2.9a	86	18.8 bc	9	0
9) Tooth pick inoculation	30	(10.0)	3.1a	84	19.3 bc	9	0

^{a/} Similar letters indicate no significant differences at the 0.1% level of probability.

^{b/} Average stands from 3 plots, 10 tubers per plot.

^{c/} Figures in parenthesis indicate that plants were inoculated after stands had been determined.

This method is relatively undesirable for field work, because of the inherent injury to plants, large amount of inoculum needed, and relative high labor requirements.

PART III

THE IMPORTANCE OF VASCULAR DISCOLORED SEED TUBERS IN TRANSMISSION OF VERTICILLIUM WILT OF POTATOES

Materials and Methods

Tubers from 2 lots of Sebago, and 1 each from Kennebec, Cherokee, and Russet Burbank, were separated into 2 groups, those apparently healthy and those with vascular discoloration. For this, tubers were sectioned at the stolon attachment, 0.5 cm below the surface, and were separated by macroscopic observation. The extent of seed transmission of *Verticillium* wilt was determined by planting these 2 groups of seed in paired rows of 10 hills each with the Sebago seed lots and of 5 hills each with Kennebec, Cherokee, and Russet Burbank. Standard potato culture practices were followed throughout the growth period. At harvest, the yield of each plot was weighed and a random sample of 30-50 tubers from each plot were used for evaluating vascular discoloration.

The presence of viable *Verticillium* in the tubers was established by isolating from the vascular region near the stolon at 3 random locations from each tuber. For this, a random sample of tubers from each group

was selected. Isolations were made from vascular discolored tubers to a petri plate of potato dextrose agar adjusted to approximately pH 5 by adding a few drops of 25% lactic acid.

Isolations were made after harvest (Sept. 15) from a random sample and the balance of the tubers was held in a potato storage maintained under usual conditions of 4 C temperature. From stored tubers random samples were taken for isolations after 4 months (Jan. 5) and 7 months (April 5).

Results

Both seed lots were of similar seed quality. There were no missing hills with either seed lot, healthy or vascular discolored. Throughout the growing season, no differences were apparent between plants grown from healthy or grown from vascular discolored seed. Particular attention was given to differences in appearance, vigor, foliage color, etc. The frequency of vascular discoloration of tubers and tuber yields were similar from both vascular discolored seed and from seed healthy in appearance (Table 6).

No positive evidence was obtained from isolations made before planting (April, 1966) that the seed tubers contained viable V. albo-atrum in the vascular region (Table 7). Over 600 isolations were attempted from over 200 tubers with vascular discoloration and an equal number

Table 6.--Yield and vascular discoloration of potato tubers after harvest grown from healthy and vascular discolored seed

Variety and seed tuber appearance	Tuber seed planted ^{a/}	Average yield per plant	Tuber symptoms	
			Tubers examined	Vascular discolored
	no.	lbs ^{b/}	no.	%
Sebago, lot 1				
healthy	180	2.7	710	27
vascular discolored	180	2.5	834	29
Sebago, lot 2				
healthy	130	2.8	626	29
vascular discolored	130	2.9	646	34
Cherokee				
healthy	45	2.3	415	64
vascular discolored	45	2.4	452	69
Kennebec				
healthy	70	2.7	374	12
vascular discolored	70	3.0	388	15
Russet Burbank				
healthy	25	3.5	241	6
vascular discolored	25	3.5	212	11

^{a/}Hills per replication-10 for Sebago, 5 for other varieties.

^{b/}No significant differences were obtained.

TABLE 7.--Frequency and type of V. albo-atrum isolated from potato tubers at different times

Variety and seed tuber appearance	Tubers tested	From seed before planting				At harvest				After 4 months in storage				After 7 months in storage				Type of fungus isolated								
		no.	%	Frequency of isolations from vascular discolor			Infected tubers	Type of fungus isolated		Infected tubers	Frequency of isolations from vascular discolor			Infected tubers	Frequency of isolations from vascular discolor			Infected tubers	MS	DMZ						
				1	2	3		no.	%		no.	%	no.		1	2	3				no.	%	no.	1	2	3
Sebago, lot 1	55	0	53	0	0	1	1.8	1	0	30	0	0	0	0	0	0	100	0	0	0	0	0	42			
healthy																										
vascular																										
discolored	55	0	53	0	0	1	1.8	1	0	30	0	0	0	0	0	0	100	0	0	0	0	0	0			
Sebago, lot 2	54	0	30	7	2	0	23.0	0	9	21	2	1	1	12.5	0	3	90	5	3	2	10	0	10			
healthy																										
vascular																										
discolored	54	0	30	4	2	3	23.0	3	6	18	3	2	1	25.0	0	6	87	5	6	2	13	0	13			
Cherokee	45	0	12	6	11	7	68.0	5	19	21	1	1	1	12.5	0	3	94	4	2	0	6	0	6			
healthy																										
vascular																										
discolored	54	0	11	5	11	9	64.5	11	14	19	2	1	2	20.8	0	5	90	2	5	3	10	0	10			
Kennetec	25	0	40	0	2	0	4.8	2	0	15	0	0	0	0	0	0	36	0	0	0	0	0	0			
healthy																										
vascular																										
discolored	25	0	40	1	1	0	4.8	2	0	15	0	0	0	0	0	0	36	0	0	0	0	0	0			
Russet Burbank	25	0	15	0	6	0	0	0	0	10	0	0	0	0	0	0							(no tubers available)			
healthy																										
vascular																										
discolored	25	0	15	0	6	0	0	0	0	10	0	0	0	0	0	0							(no tubers available)			

a' Isolations from tubers were made from 3 random places of the vascular region near stolon attachment.

b/MS indicates "microsclerectal" type, and DM indicates "dark mycellur" type.

from tubers free from vascular discoloration. All isolations attempted were negative concerning Verticillium.

Immediately after harvest, there were no significant differences in frequency of fungus isolation from vascular discolored tubers grown from vascular discolored of from healthy appearing seed. Also the frequency of isolations of the fungus from the vascular regions of the tubers suggests incomplete invasion of the tuber, with roughly the same number of tubers yielding the fungus from 1 location or from all the 3 locations and a slightly higher number of tubers yielding the fungus from only 2 of the 3 locations.

It is interesting that the DM type of the fungus was isolated at harvest somewhat more frequently than the MS type.

Some 150 isolations were attempted from approximately 50 tubers which were free from macroscopically evident vascular discoloration. All but 3 isolations representing 3 different tubers were negative in respect to Verticillium. The 3 isolates were 2 of the MS and 1 of the DM type.

The percentage of Verticillium isolations was considerably lower at the time of the second isolation from stored tubers, as compared to the first isolations 4 months earlier. Slightly more tubers were infected with viable fungus in the vascular discolored seed lot of Sebago and Cherokee than tubers grown from healthy

appearing seed. Viable Verticillium was not obtained from 1 lot of Sebago, from Kennebec, nor from Russet Burbank.

In isolations 4 months after harvest, the MS type was not recovered and apparently this culture type did not survive in the tuber as well as did the DM type.

Isolations made 7 months after harvest (April, 1967), i.e., a short time before the usual planting dates in Michigan, were fewer than those made 4 months after harvest in the seed lots from Sebago and Cherokee. Also, slightly more tubers were infected with viable fungus in the vascular discolored seed lot than in the tubers grown from apparently healthy seed.

In April isolations only the DM type was isolated.

The trend of reduced survival of Verticillium in stored tubers supports the observations made before planting in the previous year (1966) in that Verticillium apparently did not survive throughout the storage period. However, the data are not in complete agreement because there was some viable tuber borne inoculum even at this late date in storage with plot Sebago and Cherokee. The supply of tubers in 1967 were exhausted and further isolations were not possible.

DISCUSSION

Foliage symptoms of Verticillium wilt of potato have been described as those of typical wilt disease (Pethybridge, 1916, McKay, 1926, Rudolph, 1931, Ayers and Hurst, 1939, Keyworth, 1952, Robinson, et al., 1957). These symptoms consist of: 1) epinasty and wilting of the lower leaves, gradually progressing to the whole stalk; 2) loss of bright green color of the leaves with development of an irregular yellowing followed by browning, withering and flagging; 3) sometimes stalks may ultimately have only a small bunch of leaves at their tops; 4) severely affected plants are stunted and in many cases plants succumb after a period of time; 5) a severe vascular discoloration of stems with complete or partial wilting; 6) yield reduction and vascular discoloration of tubers.

Severe symptoms as described in certain of the above reports were not seen during field observations made in different potato areas in Michigan, during the summer of 1966. Even though severe symptoms were lacking, considerable vascular discoloration in harvested tubers was common. Similar observations have been made over a number of previous years (Hooker, personal communication). In the field inoculation trials, only the root dip method produced severe symptoms similar as described by other workers.

It is difficult in the light of this work and that published elsewhere (Robinson et al., 1957, Robinson and Ayers, 1961), to account for the relatively high

incidence of tuber symptoms at harvest on the basis of internally borne inoculum in vascular discolored seed tubers.

Increased disease from seed surface contamination, at least as measured by incidence of vascular symptoms in tubers are in agreement with results of others (Robinson et al., 1957, Robinson and Ayers, 1961). These investigators believe that the tuber surface inoculum is one of the most important in wilt epidemiology.

Tuber surface inoculation may well be the most efficient method of evaluating varietal resistance. The ease with which tubers may be infected is a distinct advantage and severity of disease was as great as any of the method attempted.

Reduction of tuber germination in the field followed suitable seed infestation at the Muck Farm and followed sprout infection in the laboratory. This suggested that seed surface contamination could be more important than formerly believed.

Leaf infection may be of greater importance in potatoes than previously suspected. In these laboratory experiments formation of resting structures of V. albo-atrum on potato leaves has been observed. This is in agreement with other observations, that resting structures are formed on dead potato plant debris (Reinke and Berthold, 1879, Pethybridge, 1916, Rudolph, 1931,

Robinson et al., 1957, Guthrie, 1960) or on dead debris of other plants as tomato, lucerne, hop, cotton, etc. (Bewley, 1922, Rudolph, 1931, Keyworth, 1942, Isaac, 1957, Wilhelm and Taylor, 1965). Evans et al., (1966) found microsclerotia were formed on cotton plants even in the leaf mesophyl, and they report production of conidia on moribund tissues. They concluded that these resting structures doubtless serve to disperse the fungus and contribute to the inoculum reservoir in the soil. They also suggested that, although experimental evidence are lacking, conidia produced on tissues probably serve for dissemination of the fungus. Wilhelm (1954) found that the fungus produces aerial microsclerotia from conidial germ tube anastomosis in agar media and that these also developed on plant tissues, suggesting air dispersion of them. Isaac (1957) and Davis and Isaac (1958) trapped a sufficiently high number of spores in the air over lucerne crops to suggest that air dissemination could be very significant. In the present work abundant sporulation in both laboratory and greenhouse inoculation experiments was observed on leaves and sprouts of potato. The high number of spores produced also on dead meristematic tissue of young sprouts suggests a possible source of spores and possibly also a source of mycelial inocula.

A leaf infection with spores has been obtained in greenhouse experiments, suggesting that spores could be a source of infection.

It seems very probable that early infection of young leaves could result in an increase in the amount of disease during growing season. Infection of older senescent or near senescence leaves probably does not lead to a serious outbreak of the disease under normal conditions but may simply be involved in fungus dissemination and overwintering on dead leaf tissues. In cases of serious outbreaks of disease it could be hypothesized that both root infection and leaf infection, under favorable environmental conditions early in the season, could contribute to the overall disease situation.

A characteristic late season symptom of Verticillium wilt on potatoes common in Michigan, is the flagging of the leaves. The same symptom was apparent in some cases in greenhouse inoculation experiments. This suggests that leaf infection in the field may be more frequent than previously suspected.

The isolation of viable fungus from leaf petioles 3 days after inoculation, which is a distance twice as great as the fungus grows on agar media in the same time, suggests that the fungus is carried as spores or other structures through vascular elements. This is further supported by recent evidence on cotton, where spores of

the fungus were identified in sections near the tip of the plants a few hours after root inoculation (Sewel and Wilson, 1964, Garber and Houston, 1966, Presley, et al., 1966).

It has been shown by several workers (Lacy and Horner, 1965, Wilhelm and Taylor, 1965, Garber and Houston, 1966, Varney, 1967), that severity of the Verticillium disease resulting largely from root infection, increases as inoculum potential increases. In this work with leaf and sprout infection essentially similar results were obtained. Spore inoculum was held constant in the "spores" inoculum and the "resting bodies" inoculum contained the same number of spores as well as an unidentified number of resting bodies and mycelial fragments in the corn meal inoculum. The latter was considerably more infectious.

It was reported by Fronek (1965) that only the DM type had been isolated previously from potatoes in Michigan. From the present experiments with naturally infected potato tubers it was clear that both types MS and DM, in a ratio approximately 1:2 were present in at least 2 varieties. The DM type in this study was more resistant as measured by survival in the vascular region of the tubers than the MS type. This may well account for the wider distribution of the DM than MS type. In isolations of this work from naturally infected tubers the white type of isolate (Horner, unpublished data) was not obtained.

In some instance following artificial inoculations with the MS type certain isolates appeared to be of the "white type." The culture was not held sufficiently long to establish this with certainty.

SUMMARY

Certain aspects of penetration and infection of potatoes by Verticillium albo-atrum, and perpetuation of the fungus in seed tubers have been studied.

Considerable evidence was obtained with 4 potato varieties, histologically and by isolations that the fungus penetrated leaves of potatoes through stomata whether leaves were detached or attached to the plant. This was followed by invasion of the vascular tissue of petioles. Further evidence of systemic spread of the fungus from the leaf lamina to the stem and the tuber was also obtained.

Evidence was obtained suggesting direct penetration through epidermal cells of the sprouts, followed by granulation and death of protoplasm and subsequent death of apical and leaf primordia meristems.

Of the different methods used for inoculation, root inoculation and seed surface contamination were the most effective. The leaf inoculation method resulted in a relatively high infection. The other methods used, (hypodermic syringe, tooth pick inoculation, root dip, etc.), were intermediate or ineffective in establishing infection.

No significant differences between stands, vigor, general appearance, yield, vascular discoloration of tubers,

and the presence of viable fungus in the tuber, were obtained from healthy or from vascular discolored seed lots.

Considerable reduction of viable fungus in stored vascular discolored tubers was evident 4 and 7 months after harvest.

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