PATHOLOGICAL AND PHYSIOLOGICAL STUDIES ON THE BROWN ROT FUNGUS MONILINIA FRUCTICOLA (WINTER) HONEY

by

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TABLE OF CONTENTS

INTRODUCTION

Brown rot is one of the most important diseases of peach, Prunus persica L. Batsch, and related plants. It has been reported from different countries where susceptible plants are grown. As soon as favorable conditions occur, the disease becomes serious, and partially or completely destructive to fruits and also to trees (Ezekiel, 1924; Roberts and Dunegan, 1932; and Weaver, 1950).

It is known that there are distinct organisms that cause the disease in different parts of the world. In the United States, Canada, New Zealand, Australia, Tasmania, Japan, Argentina, and in South Africa, the most important brown rot disease is caused by the fungus Monilinia fructicola (Winter) Honey (Honey, 1928; Roberts and Dunegan, 1932; Harrison, 1933 and 1935; Anderson, 1956; Walker, 1957; and Anonymous, 1963).

In the United States the disease is often called peach brown rot because it is the only rot of peaches that is of great importance to the producer, shipper, carrier, commission merchant, retailer, and consumer. All these at times suffer heavy financial losses because of brown rot. Peach brown rot is an important disease in all the peach growing sections of the eastern part and particularly the southeastern part of the United States. It is of less importance in the Middle West but is often destructive in all peach orchards except those in arid and semi-arid regions (Roberts and Dunegan, 1932).

No recent estimate for the annual losses of this disease has been reported in literature. For peaches alone, the estimated average annual

reduction in yield in the United States from 1917 to 1925, inclusive, was 3,200,000 bushels. In addition to peaches, there was an extensive loss to cherries, plums, prunes, apricots, nectarines, and pome fruits. The loss in transit was often from 5 to 25% (Anderson, 1956). In western Washington in 1936, brown rot caused a reduction of 60% in Italian prunes, 40% in sour cherries and 90% in sweet cherries (Huber and Baur, 1941).

Peck in 1881 was apparently the first one to investigate the pathogenicity of American brown rot fungus. Since that time, work has been done to study this fungus and its effect in incidence of the disease. Much difference in varietal susceptibility has been recorded, especially in peaches, plums, cherries, and pome fruits. These records are based mostly on observations and are reliable only in a very general way (Valleau, 1915; Curtis, 1928; Hesse, 1939; and Roberts and Dunegan, 1932).

We appear to have no data of the importance of pathogenic specialization in M. fructicola. There are several reports of more or less constant variation among isolates of this pathogen grown on a variety of culture media (Reade, 1908; Ezekiel, 1924; Seal, 1924; and Roberts and Dunegan, 1932). The stability of these was questioned by Roberts and Dunegan (1932). No tests of the relative pathogenicity of a series of isolates of this fungus appear to have been made by any plant pathologist (Anonymous, 1963).

In this investigation, the work was undertaken to study the variations that might exist in a series of isolates collected from different parts of the United States, their physiology, and their relative pathogenicity on different varieties of stone and pome fruits.

REVIEW OF LITERATURE

History

Brown rot fungi have been known in Europe for many years. The name Torula fructigena was given for the first time by Persoon in 1796 to the fungus which he found on the decayed fruit of Prunus domestica, Amygalus persica, and Pyrus. In 1801 Persoon changed its name to Monilia fructigena (Conel, 1914; Bartram, 1916; Ezekiel, 1924; Roberts and Dunegan, 1932; and Anderson, 1956). In 1851, another species, Monilia cinerea, was described by Bonorden (Roberts and Dunegan, 1932) as the cause of small gray, sometimes brownish, conidial tufts on fruits.

The first definite connection between the brown rot fungus and the disease in the United States was made when Peck in 1881 conducted tests in New York and demonstrated the pathogenicity of the fungus, which he called Oidium fructigenum Kunze & Schmidt.

According to Anonymous (1963), in 1883 a peach mummy bearing apothecia was collected in Pennsylvania and sent to Winter in Germany for identification. Winter named it Ciboria fructicola. In 1893 Schroter (Anonymous, 1963) placed the brown rot species in the genus Sclerotinia on the basis of their ascomycetous nature. In 1902 Norton (Anonymous, 1963) discovered the peziza cups of the ascomycetous stage of one of these fungi on the ground of an old orchard in Maryland. To the fungus which Norton found, he gave the name Sclerotinia fructigena (Anonymous, 1963), and this is the one which is known in the United States.

The difficulty in differentiating between S. fructigena and S. cinerea was pointed out by many workers (Conel, 1914; Bartram, 1916; and

others). On the basis of macroscopic and microscopic examinations, they were able to differentiate between these two species.

In 1908 Reade found only one species common in New York, but the asci and ascospores produced by it were larger than those of S. cinerea and larger than the measurements which were given by Norton. He considered it, therefore, to be S. fructigena as the dimensions agreed with the size given by Aderhold in 1904 for that species (Anonymous, 1963).

Matheny (1913) and Jehle (1913), from comparative studies of materials from Europe and the United States, concluded that there were two species, but the common one in the United States was S. cinerea. Sclerotinia cinerea was also reported by Pollock (1909) in Ann Arbor, Michigan, and by Bartram (1916) in northern Vermont. Pollock mentioned that the ascospores, asci, and conidia were smaller than that of S. fructigena in Europe, but not as small as that mentioned for S. cinerea.

In 1920 Wormald in England realized that the American form was different from these two species and he proposed to make it a variety of S. cinerea as S. cinerea (Bon) Schroet. variety Americana Wormald.

In 1924 Norton and Ezekiel raised the name S. americana (Wormald) Norton and Ezekiel for that common American species named by Wormald in 1920.

Roberts and Dunegan (1932) showed from their comprehensive studies that the American brown rot fungus should be called S. fructicola (Winter) Rehm. Harrison (1933, 1935) confirmed their work.

Honey (1928) created the genus Monilinia to include those species of Sclerotinia in which the imperfect stage consisted of monilioid conidia (i.e., in chains) and which showed a genetic relationship to the genus

Sclerotinia. He made M. fructicola the type species of that genus. He described the pseudosclerotia (entostroma) and ectostroma development as important characteristics of the new genus which includes the American brown rot fungus of stone fruits M. fructicola. He also described the development of the apothecial stage of that fungus from over-wintered, more or less specialized composite stroma (entostroma) or pseudosclerotia which are commonly formed within the mummified fruits of its respective hosts. The mycelium, macroconidia, and microconidia were described also by Honey (1928, 1936).

Although Honey (1928, 1936) made a comprehensive study on this new genus and his concepts are very understandable, some workers are still using the old name S. fructicola. Alexopoulos (1963) in his book used the name Monilinia as a new genus of family Sclerotiniaceae, order Helotiales, Epigean Inperculate Discomycetes, series Discomycetes. This name M. fructicola is used in these studies.

Variability and Strains in M. fructicola

Information was lacking as to the extent, persistence and significance of variation within this species, as was stated by Ezekiel (1924). Some variations had been reported by some workers such as Reade (1908), who reported that he observed variations within the American brown rot fungus, but he mentioned that these differences disappeared when the isolates were cultured under the same conditions.

Ezekiel (1924) mentioned that 30 strains which he collected for comparison from Maryland and other places in the United States, Holland, and England were differentiated into 6 varieties according to their

culture characteristics on rotting fruits, according to size of conidia, and to growth on potato-dextrose agar at 25°C in tube cultures and at 15°C in plate cultures. These varieties retained their differences under widely different environments and after successive passages through artificial media and fruits. Similarly, Seal (1924) distinguished at least two biological forms of American Sclerotinia, as he called it, which showed constant cultural differences when they were grown on various substrata and under different environmental conditions. Roberts and Dunegan (1932) also found cultural variations within strains on different media after a long period of repeating inoculation in cultural media.

Sharvelle and Chen (1943) observed that 16 lines obtained from 2 asci of S. fructicola fell into four distinct groups on the basis of cultural characteristics, sensitivity to sulfur fungicides, and pathogenicity to apple fruits.

Ezekiel (1924) reported the behavior of different strains when seeded in combinations of 2 per plate at different points in potato-dextrose agar plates. He did not observe any macroscopic reaction when the 2 colonies grew together. On microscopic observation he noticed anastomosing of hyphae at the meeting line with some strains but not with others.

While Ezekiel (1924) reported that M. fructicola is homothallic and one ascospore inoculation in fruits gave rise to apothecia, Roberts and Dunegan (1932) reported that this may not be true, but that the fruit inoculation might get other spores found on the fruits which gave rise to the apothecia. This suggested that we cannot say that it is homothallic

or heterothallic until we initiate the perfect stage in artificial media. Thind and Kiett (1949) remarked that the formation of sclerotial bodies of the black line preceded the development of apothecia in M. fructicola and other species of the genus. But all attempts made by them and many others failed to give positive results.

Varietal Susceptibility to Brown Rot Fungus

Differences in incidence of disease on varieties of stone and pome fruits had been reported by some workers. Valleau (1915), at the University of Minnesota, found that varieties of plums resistant to brown rot fungus had a thick and tough skin. He mentioned that the firmness of the fruit at ripening played an important role in resistance.

Willaman et al. (1925) studied the relation between susceptibility to brown rot M. cinerea in plum varieties and their physical and chemical properties. They reported that the resistant and susceptible plum varieties differed in respect to density of juice, pH, titre, oxalic acid, ash, nitrogen, and other extract contents. The crude fibers and pentosans were higher than in susceptible varieties.

In 1930 Vasudeva did not observe germination of Botrytis allii spores on apples, and M. fructigena spores did not affect onions but he stated that this did not mean immunization but that this was related to the nitrogen content of the crop and the fungi.

In 1928 Curtis investigated the morphology of fruits in resistant and susceptible varieties of plums, peaches, nectarines, and apricots. She found that the morphological characters which combined to afford some

degree of resistance varied with the variety. In none was the fungus entirely excluded by cuticle or by plugging of stomata.

Willison (1939) reported that fruits picked later in the season were more susceptible to brown rot than were those picked first of the season.

Angell (1950) found a great variability in incidence of M. fruticola on apricots and peaches in Australia. He suggested that the disease incidence had no relation to the weather conditions for increased inoculation or inherent susceptibility. He mentioned that susceptibility appeared to be induced by conditions peculiar to the immediate environment of the tree.

Byrde and Fielding (1951) reported that infection took place less readily on day-old injuries than on those freshly made. They also reported that susceptibility of laxons superb apples to brown rot infection in early September (mean temperature 61.6°F) was much higher than in late September (mean temperature 55.7°F).

Host Range (Plants Affected)

Brown rot fungus M. fructicola affects many species of the genus Prunus, both wild and cultivated. Although it affects chiefly peaches, plums, cherries, and apricots, it rather frequently affects ripening of apples, pears, and quinces. Roberts and Dunegan (1932), in their list of suscepts, gave 34 species and known hybrids of Prunus, one species each of Pyrus, Malus, Cydonia, Chaenomelis, Rosa, and two species of Rubus. Artificial inoculations have been reported on strawberry, loquat, tomato, persimmon, and green corn (Norton, 1914; Harrison, 1928; and Roberts and Dunegan, 1932).

Natural occurrence of the fungus on grapes has been reported in New South Wales in 1941 (Anonymous, 1963).

A recent survey of suscepts is recorded in the 1960 Index of Plant Diseases in the United States.

Physiological Studies

Importance of fungus physiological studies to clarify host-parasite relationship and its importance in the disease control program have long been accepted.

Roberts and Dunegan (1932) reported that the fungus M. fructicola grew readily on a variety of artificial media. Wormald (1920) and Ezekiel (1924) pointed out that the growth in artificial media was an aid in differentiating this species from the other brown rot species, especially M. cinerea forma pruni. In petri dishes of potato-dextrose agar the former grew rapidly, producing conidia profusely, usually in concentric zones, where the latter grew much more slowly and produced few or no conidia. The margins of M. fructicola were smooth, but those of the other were deeply scalloped.

So far as it is known, the Sclerotinia stage has not been produced in artificial cultures except that Reade (1908) and Heuberger (1934) stated that the fungus was grown on artificial media from ascospores to ascospores again.

Read (1908) appears to have been the first to give a complete description of the conidial stage. Matheny (1913), Conel (1914), and Bartram (1916) described the conidial tufts as ashy or ashy gray. Roberts and Dunegan (1932) observed the conidial tufts on a large number of

different hosts and media. They found it to be ashen gray when young, becoming darker with age. The use of conidial measurement to differentiate between M. fructicola and M. cinerea was used by Wormald (1920) and Ezekiel (1924). Ezekiel also mentioned that different strains of M. fructicola differed in respect to size of conidia.

The effect of temperature on germination of conidia and growth of the fungus was studied by some workers. Ames (1951) showed that conidia will germinate at 0°C, but the growth was very slow. The optimum temperature for growth was 25°C, and the thermal death point was 53°C. Conidia of plum strain did not germinate at 36°C and conidia of peach strain did not germinate above 30°C.

Brooks and Cooley (1917) produced measurable apple rot at 5°C in one week and at 0°C in two weeks. It also grew on cornmeal agar at 0°C, making a measurable growth at the end of the second day. Both on fruits and artificial media the optimum temperature was 25°C, with some growth at 30°C.

Ezekiel (1924) studied the effect of temperature on different strains of M. fructicola. He observed no constant differences in the cardinal temperature of these various strains. The minimum, optimum, and maximum fell to near 3°, 25°, and 33°C, respectively, for all.

Weaver (1950) found that radial growth on potato-dextrose agar was most rapid at 20-25°C, with some growth observed at 30°C but none at 35°C. Sporulation occurred in 10 days at 10°C, in 3 days at 15°C, and in 2 days at 20°C and 25°C.

The effect of the pH of the media on growth of the fungus was studied by few workers. Cooley (1914), using cherry juice with known

acidity, found that the fungus grew on the acid media faster than that on natural media. Ezekiel (1923) and Norton et al. (1923) found that production of apothecia in liquid cultures was affected by the pH of liquids, where optimum pH was 2.5.

Roberts and Dunegan (1932) found that the fungus grew best on an acid media, but the point at which increased acidity stopped growth was found to depend not only on the hydrogen ion concentration but also on the acid used. Thus, with sulphuric acid, the greatest growth was obtained at pH 2.84, with phosphoric acid at pH 3.90, and with formic acid at pH 4.37.

Tilford (1936) found that there was a correlation between the effect of temperature and hydrogen ion concentration on the growth of the fungus and that at a higher temperature the optimum pH for mycelium growth was higher than at a lower temperature. Temperature limits were also influenced, likewise, by the reaction of the media.

Isolation of Monosporic Cultures

Keitt (1915) proposed a technique for isolating monosporic cultures by using two transfer needles, one with an end like a "biscuit cutter" and the other with an end like a tiny "spatula". He used a pure agar medium in petri dishes, and then poured on a diluted spore suspension, and detected the spores by using a microscope. Later he marked it and transferred it to the new medium by using his needles.

Ezekiel (1930) reported a modification to the Keitt method. He used dilution series, selected the spores after germination, then marked it and transferred it by using two needles, one with a hollow cylindric end

to cut the agar, and the other with flattened and twisted tip to transfer the spore to another medium.

In 1924 Brown proposed his technique for isolation of single strains by cutting hyphal tips by using the microscope.

Another technique was reported by Dickinson (1933). He used dilutions of spore suspension in liquid media, then transferred one drop to different parts of a petri dish. After germination and the forming of small colonies could be detected under the microscope's low power, he transferred it to the agar medium.

MATERIALS AND METHODS

Cultures

Isolates of M. fructicola were obtained from mummies and diseased fruits collected from different parts of the United States.

Isolation of pure cultures was done without any surface disinfection of the mummies or the diseased fruits. The materials that had spores on the surface at the time of collection were isolated directly by using two methods for isolation. The materials that had no spores on the surface at the time of collection were put inside sterilized moist chambers for 16 to 24 hours. These moist chambers had been sterilized by using 0.9% sodium hypochlorite (Clorox) for three minutes. After a period of 16 to 24 hours, the fungus formed many conidia on the surface of the materials used for isolation of pure cultures.

The two methods of isolation of pure cultures can be summarized:

In the first method, conidia were transferred to different parts on potato-dextrose agar petri dishes by using a needle with straight

and flat end. The medium was acidified with hydrochloric acid 0.1 N to pH 5.0 before sterilization. The plates were incubated at room temperature (20° - 25°C) for 36 to 48 hours. The growing colonies were examined for purity, then parts from the edges of these colonies were transferred again to a new medium in plates and incubated at the same temperature. After 24 hours the growth was examined again, and then PDA slants (pH 5.0) were inoculated and incubated at 25°C for 6 days. After the incubation period the slants were kept in the refrigerator.

The second method was the streak method. Conidia were streaked directly onto potato-dextrose agar petri dishes (pH 5.0). The plates were incubated at room temperature (20° - 25°C). After 24 to 36 hours separate colonies were detected and then examined by microscope. Parts of the pure colonies were transferred to a new medium in plates, then incubated for 24 hours at the same temperature. PDA slants were inoculated from the growing colonies and incubated at 25°C for 6 days. After incubation, the slants were kept in the refrigerator.

Cleaning of Glassware

The glassware was first soaked with a detergent and kept for 24 hours, then washed by tap water thoroughly, rinsed three times by distilled water, and rinsed by de-ionized (redistilled) water three times, and left to dry for one day before sterilization.

The glassware which had just been used was sterilized before cleaning for 15 minutes at 15 pounds.

The Water Used in this Work

De-ionized water was used in this work because de-ionized water was also used for preparation of the standard media.

Media Used

1. Potato-dextrose agar (Riker & Riker, 1936)

Peeled potatoes	200 g
Dextrose	10 g
Bacto agar	17 g

De-ionized water - to complete to liter

The peeled potatoes were boiled in 600 ml water for one hour, then filtrated in a double layer of cheesecloth. To the filtrate, dextrose and agar were added, and finally completed to liter by water.

2. Pears-dextrose agar

Fresh pears	200 g
(instead of the 40 g dried prune used by Riker & Riker, 1936)	
Dextrose	10 g
Bacto agar	17 g

De-ionized water to complete to liter

3. Modified yeast extract dextrose (Hoagland and Arnon, 1950) liquid medium (standard medium or basal medium). This medium contains:

1 m mole	$(NH_4H_2PO_4)$	ammonium acid phosphate
4 m mole	$(Ca(NO_3)_2$	calcium nitrate
6 m mole	(KNO_3)	potassium nitrate

2 m mole	($MgSO_4$)	magnesium sulfate
5 ppm	Fe	as iron tartrate
0.5 ppm	Bo	as boric acid
0.05 ppm	Zn	as zinc sulfate
0.5 ppm	Mn	as manganese chloride
0.02 ppm	Cu	as copper sulfate
0.01 ppm	Mo	as molybdic acid
10 g		dextrose
5 g		yeast extract

These amounts were added to 600 ml de-ionized water, and later completed to liter. The preparation of the basal medium was done as mentioned by Hoagland and Arnon (1950) in their publication on the water culture technique. Solution 2 was used in this work. The procedure used can be summarized:

Firstly, molar solutions of $NH_4H_2PO_4$, KNO_3, $Ca(NO_3)_2$, and $MgSO_4$ were prepared. Secondly, the following amounts were added to liter:

	cc in a liter of nutrient solution
M $NH_4H_2PO_4$	1
M KNO_3	6
M $Ca(NO_3)_2$	4
M $MgSO_4$	2

Later a supplementary solution was prepared to supply the following amounts per liter of de-ionized water:

	grams dissolved in a liter of water
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$H_2MoO_4 \cdot H_2O$	0.02
$CuSO_4 \cdot 5H_2O$	0.08

One cc of this solution was added for each liter of nutrient solution.

One cc of 0.5% iron tartrate was added for each liter of nutrient solution.

All these amounts were added to 600 ml water; then 10 g dextrose and 5 g yeast extract were added, and finally the solution was completed to liter.

4. Basal solid medium

To the basal medium in No. 3, 17 g of Bacto agar was added to a liter of the medium.

Distribution of the Media

The distribution of the media was done usually before sterilization. Liquid media were distributed in 125 ml Erlenmeyer flasks, usually 50 ml per flask.

Agar media were distributed in test tubes, 18 ml per tube, and plugged with cotton to be ready for sterilization. From these tubes after sterilization, the media were poured into sterilized petri dishes to prepare plate agar.

Sterilization of Media, Glassware, Soil, and Plastic Pots

The media were sterilized in the autoclave at 15 pounds for 15 minutes.

The glassware should be quite dry before sterilization. The petri dishes were wrapped in paper bags and closed with sticker tapes. The

pipettes were plugged with small pieces of cotton and kept in a steel pipette container. All the glassware was sterilized in the autoclave at 15 pounds for 15 minutes.

Usually the glassware was sterilized for at least 6 hours before using.

The soils, 50% clay and 50% sand soil, and the vermiculite were sterilized in the autoclave at 15 pounds for one hour, one week before use.

The plastic pots were sterilized with 0.9% sodium hypochlorite (Clorox) for 3 minutes.

Measurement of pH

All the pH measurements were taken by using a Beckman pH meter. The pH's of the media were adjusted before sterilization and after sterilization the pH's of the media were determined.

In the liquid media, the pH's were determined after incubation was made for the filtrates of the replicates. The means of the media were then determined.

Inoculation of Media

Solid media were inoculated by 5.0 mm disks, from 7-day-old cultures grown on the basal medium (modified Hoagland solid medium) at 25°C, unless another temperature is mentioned.

Liquid media in 125 ml Erlenmeyer flasks were inoculated by counted spore suspensions prepared from 7-day-old cultures grown on the basal medium in petri dishes or in test tube slants.

The counted spore suspensions were prepared as follows:

Twelve ml of sterilized water prepared in test tubes was poured on the cultures in petri dishes or test tubes. By a needle the spores were freed or loosened gently, then the suspensions were poured in Erlenmeyer flasks (125 ml), each containing 50 ml distilled water.

The spore suspensions were counted by using "Spencer Bright-Line Hemacytometer" slide, by using the 43X objective for counting. The technique used is described in detailed in the Hemacytometer Catalog (Catalog No. 1483 AO Spencer Bright-Line Hemacytometer). The number of spores per cubic millimeter = $\frac{\text{Number of cells counted} \times 4000}{\text{Number of small squares counted}} = \frac{\text{Number of spores counted} \times 4000}{80}$.

To inoculate any experiment by using different isolates, all flasks inoculated had the same number of spores. This was done by inoculation with different amounts of spore suspensions of the different isolates which had different concentrations of spores to get the same number of spores for all isolates.

All this work of spore suspension preparations and inoculation was done in a sterilized inoculation chamber.

Incubation of Cultures

The cultures were incubated in incubators adjusted at the temperature of the experiment.

Growth Measurements

In agar media, the linear growth of the fungus was measured. The two diameters in right angles of each petri dish were determined in

millimeters, and the means were taken. Four replicates for each isolate in each treatment were measured and the means of the replicates determined.

In liquid media, the drying weights of the mycelium mats were determined in mg. The filter papers were dried at 70 to 72°C for 24 hours, then cooled in a desiccator which had calcium chloride. Later its dry weights were determined in mg. The mycelium mats were harvested by filtration, by using vacuum and screen disks to support the filter papers. The mats were washed two times by water, then dried in an oven at 70 to 72°C for 24 hours. The dried mats were cooled in a desiccator, then weighed in mg by using an analytical balance. The net dry weights of the mycelium mats were determined by subtracting the dry weights of filter papers from the total dry weights. The means of the replicates were determined.

Reducing Sugars Determination

The procedure used in this work was the same procedure mentioned in "Association of Official Agricultural Chemists Official Method of Analysis (9th ed. 1960), Secs. 13.028, 13.029, pp. 162-164" (Horwitz, 1960). The analysis of reducing sugars was done first for starch and cellulose. Secondly, the amounts of reducing sugars were determined in the filtrates after the growth of isolates for 7 days and the mycelium mats were harvested. The filtrates were completed to 50 ml by water, then 10 ml were used for determination of reducing sugars. Two replicates were done and the means were determined. The net reducing sugars were determined by

subtracting the reducing sugars in the starch and cellulose from the total per flask.

The reducing sugars were expressed as mg of maltose per 50 ml filtrate.

Total Nitrogen Determination in Fruits

The procedure used in this work was the macro Kjeldahl method mentioned in "Association of Official Agricultural Chemists Official Method of Analysis (9th ed. 1960), Secs. 2.035, p. 12 and 20.002(c), p. 262" (Horwitz, 1960).

The samples were prepared as mentioned in page 262, by mixing 80 g of fresh fruits in 200 ml water with a high speed blender. Fifty ml of the juice was used for determination of total nitrogen by the Kjeldahl method, p. 12. Two replicates were made for each determination, and the means were determined. The amounts of total nitrogen were determined in mg per 100 g of fresh weight of fruit.

Isolation of Monosporic Cultures

Many methods have been used for the isolation of bacteria and fungi in monocellular cultures. Fungi with hyaline spores were difficult to isolate. Many methods have been used by many workers, as mentioned in the literature review. Some of these methods were tried for isolation of monosporic cultures of M. fructicola but none were suitable for use in this work under the laboratory conditions where this work was done and because of its complications.

The following method was devised and used by the author in this work. The method was:

The basal medium was prepared in liquid form in test tubes, 9 ml per each, and in solid form in petri dishes, 10 ml per plate (prepared in test tubes, sterilized, and later poured in sterilized petri dishes and then kept in the refrigerator for at least two hours for complete solidification before use). The liquid and solid media were prepared and filtrated in double layers of cheesecloth before its distribution and sterilization.

Four series of dilutions were made from the spore suspensions of the isolates, which were prepared from one-week-old cultures in test tubes containing 9 ml liquid medium each to maintain about two to four spores per ml in the last dilutions.

One drop from the last dilution was introduced to the surface of agar medium in each plate and three petri dishes were distributed for each isolate. The plates were shaken on the table to spread the drops on the surface. Sometimes the loop needle was used to help prevent cracks from being produced in the medium. The plates were left on the table from 40 to 60 minutes to let the solution be absorbed into the medium.

From the experience of the author in that work, it was found that the hyaline spores were hard to detect on the surface of the medium. It was found that by adjusting the focal length of the low power of the microscope, germinating spores could be seen on the surface of the medium. (This was done by introducing a drop of spore suspension to the surface of agar medium in the petri dish and left for 24 hours.) The spores

were detected in the same focal length by using the fine adjustment. The detection of the spores was done by putting the plates on the stage where the bottoms were facing the objective. The spores were marked with a fine pen by making a circlet around the space of the spore where no other spores were lying. These marked parts were cut by a sharp needle, transferred to agar slant medium, and incubated at 25°C for 7 days.

Inoculation of Fruits

Two methods were used for inoculation of fruits after the surface disinfection of the fruits. Disinfection was done by dipping fruits for three minutes in 95% ethyl alcohol, which acts as a wetting agent, then dipping for another three minutes in 0.9% sodium hypochlorite (Clorox). The fruits were put on a sterilized towel paper, which had been sterilized in the autoclave at 15 pounds for 15 minutes.

The first method of inoculation used was the common method used by many workers (Peck, 1881; Ezekiel, 1924; Roberts and Dunegan, 1932; Smith, 1936; Kung-Hsiang, 1942; Byrde, 1952; Corbin, 1963; and others). The sterilized fruits were inoculated by conidia from 7-day-old cultures grown on potato-dextrose agar in petri dishes or in test tubes, incubated at 25°C. By using a needle with a flat sharp end, the conidia were introduced into the fruits. The needle was sterilized before and after each inoculation by direct Bunsen flame. The fruits were covered by a double layer of moistened cheesecloth and placed on a table under laboratory conditions (20 to 27°C).

The second method of inoculation used in this work was a modified method of inoculating fruits published by a Japanese pathologist, Toshikazu Tani (1963). The sterilized fruits were pricked open by a sharp sterilized needle to about one centimeter deep, then inoculated with 0.5 cm disks from 7-day-old cultures grown on the basal media at 25°C. The inoculated parts were covered with sterilized cotton fibers. Small amounts of sterilized water (1 ml water) were dropped over the inoculums to keep them moist. The fruits were put on a sterilized paper towel in an incubator at 25°C and covered with a double layer of sterilized cheesecloth.

EXPERIMENTAL RESULTS

Isolation of Cultures

Thirteen isolates of M. fructicola were obtained from mummies and diseased fruits collected from different parts of the United States by using two methods of isolation, one by direct transfer of conidia to PDA medium and the other by streaking conidia on PDA medium. The medium used was acidified to pH 5.0 before sterilization by using hydrochloric acid. The best isolations were obtained by using the streak method.

The isolates obtained were compared and identified according to the work published by Ezekiel (1924), Roberts and Dunegan (1932), Honey (1928, 1936), Harrison (1928, 1933), and Hewitt and Leach (1939).

The rate of growth on PDA medium, germination of conidia, branching of germ tubes, color of conidia, and inoculation of fruits were used for this identification.

The 13 isolates and their dates of collection, geographical origin, hosts from which they were first isolated, and their morphological characteristics on PDA medium incubated at 25°C for two weeks, are listed in Table 1.

From the data in Table 1 it was observed that some isolates showed different morphological characteristics on PDA medium with regard to the color of mycelium, color and amount of macroconidia and microconidia, and time required for spore formation. These isolates were Nos. 1, 2, 11, 12, 14, 16, and 23. Some isolates were nearly similar to each other, and to one or more of the isolates mentioned before. Isolates Nos. 3, 4, 5, 6, and 10 were similar to isolate No. 2. Isolate No. 13 was similar to isolate No. 12.

The work was undertaken to investigate the stability of these differences between the isolates mentioned above and the significance of these variations on the infection of different varieties of fruits.

Variations in Pathogenicity of Seven Isolates on Peaches and Plums

After 7 days from inoculation time, the morphological characteristics of the diseased fruits were recorded. The part infected and its color, the amount of spores produced on the surface of fruits and their color, were all taken into consideration. The data obtained are summarized in Table 2.

From these data it was observed that isolates Nos. 1, 2, 3, 11, 14, and 16 showed morphological variabilities between each other in infecting peaches, where isolate No. 4 showed close morphological characteristics

Table 1. Thirteen isolates of M. fructicola, their dates of collection, geographical origin, morphological characteristics on PDA incubated at 25°C for two weeks.

No. of isolate	Date of collection	Geographical origin	Host	Morphological characteristics
1	7-14-63	Arkansas	Diseased Elberta peach fruit	Mycelium is light gray color, macroconidia and microconidia formed, zoned +[a]
2	"	"	"	Mycelium is dark in color, zoned, macroconidia and microconidia formed +++[b]
3	"	"	"	"
4	"	"	"	"
5	"	"	"	"
6	"	"	"	"
10	7-18-63	"	"	"
11	7-26-63	Yakima, Wash.	Diseased nectarine fruit	Mycelium is dark brown, macroconidia and microconidia formed in spots ++++[c]
12	7-27-63	Arkansas	Diseased Elberta peach fruit	Mycelium gray in color, macroconidia and microconidia formed ++[d]
13	"	"	"	"
14	8-4-63	California	Diseased peach fruit	Mycelium light in color, macroconidia gray, and microconidia formed ++[d]

Table 1. (Concl.)

No. of isolate	Date of collection	Geographical origin	Host	Morphological characteristics
16	8-4-63	Lawrence, Kansas	Peach mummy	Mycelium gray in color, macroconidia and microconidia formed in spots +[a]
23	10-19-63	Manhattan, Kansas	"	Mycelium dark gray, macroconidia and microconidia formed, zoned +++++[e]

[a] Slight spores formed in two weeks.

[b] Medium amount of spores formed in two weeks.

[c] Heavy spores formed in two weeks.

[d] Little amount of spores formed in two weeks.

[e] Heavy spores formed in one week only.

Table 2. The pathogenicity of 7 isolates of M. fructicola on Elberta peaches and Italian prunes incubated for 7 days.

No. of isolate	Pathogenicity symptoms	
	On Elberta peaches	On Italian prunes
1	Infected 1/2 of the fruit, brown in color, produced heavy gray spores	Infected 1/2 of the fruit, brown in color, produced few light gray spores
2	2/3 of fruit infected, dark brown, heavy dark gray spores	Whole fruit infected, dark to black, produced heavy dark gray spores
3	Infected 1/2 of fruit, dark brown, produced moderate gray spores	Infected 2/3 of fruit, dark to black, produced moderate gray spores
4	As No. 3	As No. 1
11	Infected 2/3 of fruit, dark brown with black area around inoculation, heavy dark gray spores produced	Infected the whole fruit, black in color, produced moderate gray spores
14	Infected 2/3 of fruit, light brown in color, produced few light gray spores	Infected the whole fruit, brown in color, produced moderate light gray spores
16	Infected 1/2 of fruit, brown in color, produced few gray spores	As No. 1

to isolate No. 1. On plums isolates Nos. 1, 2, 3, 11, and 14 showed morphological variabilities between each other, where isolates Nos. 4 and 16 showed close morphological characteristics to isolate No. 1. These morphological variabilities among the isolates might have been due to the chemical nature of fruits. This conclusion agreed with that of Angell (1950), Byrde and Fielding (1951), Vasudeva (1930), and Willaman et al. (1925).

Isolation of Monosporic Cultures and Measurements of the Size of Macroconidia

Six isolates which had different morphological characteristics on peaches, plums, and on PDA medium, were isolated in monosporic cultures. The type of growth and sporulation of these isolates are shown in Fig. 1. Isolate No. 1 showed whitish mycelium and slight amount of spores mainly in the center. Isolate No. 2 showed dark brown mycelium and heavy amount of spores formed in zones. Isolate No. 11 showed dark gray mycelium and a medium amount of spores mainly in the center, with some spores in a concentric area in the rest of the culture. Isolate No. 14 showed light gray mycelium and a light amount of spores in the center. Isolate No. 16 showed whitish mycelium and a very slight amount of spores in the center. Isolate No. 23 showed gray mycelium and a medium amount of spores not zoned.

Thirty spores which were selected at random from each isolate were measured and the means determined. These measurements are listed in Table 3. The size of macroconidia of these isolates is shown in Fig. 2.

From these results it was found that the size of macroconidia was variable for the different isolates and ranged from 12.0 to 17.8 by

EXPLANATION OF FIG. 1

The type of growth and sporulation of monosporic cultures of 6 isolates of M. fructicola (Winter) Honey after two weeks at 25°C on the water culture agar plates and test tubes. 1) From Arkansas. 2) From Arkansas. 11) From Yakima, Washington. 14) From California. 16) From Lawrence, Kansas. 23) From Manhattan, Kansas.

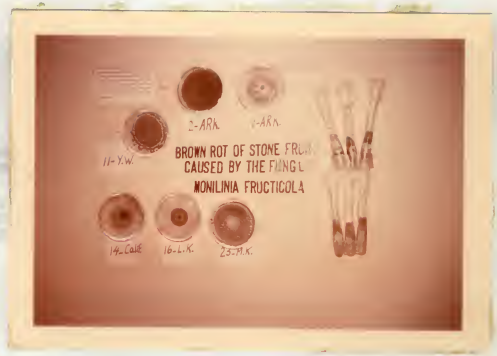
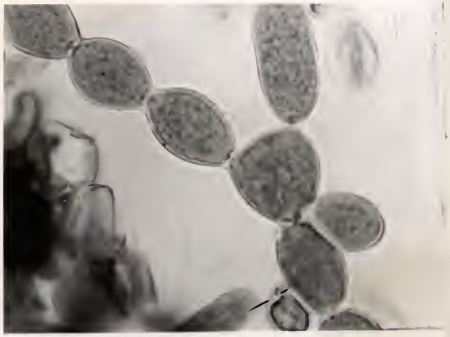

2-ARK.
4-ARK.
11-Y.W.
BROWN ROT OF STONE FRUI
CAUSED BY THE FUNGU
MONILINIA FRUCTICOLA
14-Coke
16-L.K.
23-M.K.

Fig. 1

EXPLANATION OF FIG. 2

The size of macroconidia of different isolates of M. fructicola after 7 days growth on water culture agar medium (X - 1,900). a) Macroconidia of isolate No. 1 from Arkansas. b) Macroconidia of isolate No. 2 from Arkansas. c) Macroconidia of isolate No. 11 from Yakima, Washington. d) Macroconidia of isolate No. 14 from California. e) Macroconidia of isolate No. 16 from Lawrence, Kansas. f) Macroconidia of isolate No. 23 from Manhattan, Kansas.

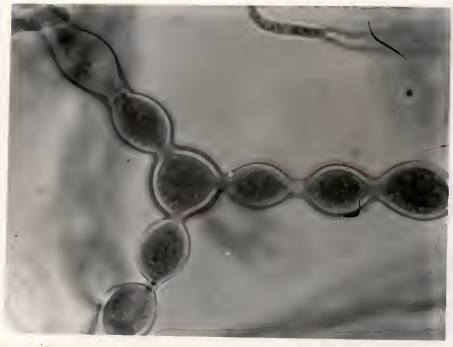
a



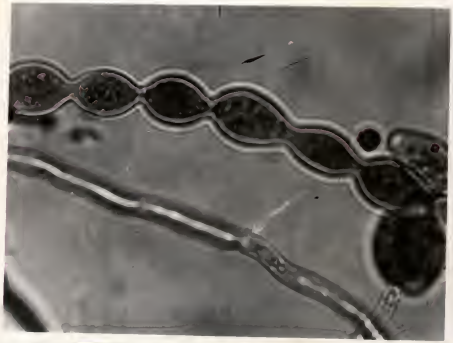
b

Fig. 2

c



d



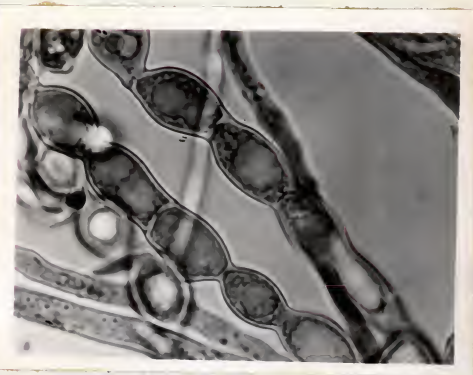


Table 3. The size of the macroconidia of 6 isolates of M. fructicola grown on the basal medium at 21°C for 7 days.

No. of isolate	Size of macroconidia in microns*
1	16.2 x 9.5
2	17.8 x 8.1
11	12.0 x 7.6
14	13.7 x 7.2
16	14.5 x 12.0
23	14.0 x 10.2

* These are the means of 30 spore measurements.

7.2 to 12.0 microns. These measurements agreed fairly well with those obtained by Ezekiel (1924) and differed from those obtained by Reade (1908), Roberts and Dunegan (1932), Matheny (1913), and Harrison (1928).

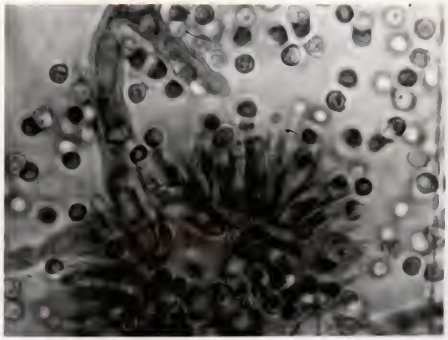
These differences in measurements obtained by different workers might have been due to different isolates they worked with and technique used.

It was observed in this work that there were some differences in the size of the microconidia of some isolates as in Fig. 3 but no exact measurements were done.

EXPLANATION OF FIG. 3

The size of microconidia of two different isolates of M. fructicola after 7 days growth on water culture agar medium (X - 1,900).
a) Microconidia of isolate No. 2 from Arkansas.
b) Microconidia of isolate No. 14 from California.

a



b

Fig. 3

Effect of Different Media on the Rate of Growth

In these studies different media were used: a) potato-dextrose agar, b) pears-dextrose agar, c) complete Hoagland and Arnon medium (basal medium), and d) basal medium minus yeast extract. The plates were inoculated, then incubated at 21°C for 6 days.

The morphological characteristics of the isolates on PDA and the basal medium are shown in Fig. 4. It was observed that these isolates differed in their type of growth on these media and that they produced different colors in the media after their growth, as shown in Fig. 5.

The data obtained are summarized in Tables 4a and 4b. From these data it was found that the growth was inhibited on the basal medium minus yeast extract, but normal growth was obtained on natural media and also on the basal medium which had yeast extract. This indicated that these isolates need a growth factor or factors for their growth and PDA and pear media do have this or these growth factor or factors. Also, yeast extract supplied these growth factors in the basal medium.

These isolates showed significant differences between each other on the same medium and between the same isolate on these different media. It was found that no one medium was the best for the growth of all isolates. Potato-dextrose agar medium was the best for the growth of the isolates Nos. 11, 14, and 23 and was the same as the basal medium for isolate No. 16. The pear medium was the best for the growth of isolate No. 2.

EXPLANATION OF FIG. 4

The rate of growth of 6 isolates of M. fructicola on two different media, incubated at 21°C. a) On PDA medium after 6 days. b) On water culture medium after 3 days. c) On water culture medium after 6 days. The isolates were: 1) From Arkansas. 2) From Arkansas. 11) From Yakima, Washington. 14) From California. 16) From Lawrence, Kansas. 23) From Manhattan, Kansas

Fig. 4

b


16-L.K.
14-Calif.
23-M.K.
Growth after 3 days
on water culture
media

c


16-L.K.
14-Calif.
23-M.K.
Growth after 6 days
on w.c. media

EXPLANATION OF FIG. 5

The effect of the growth of 6 isolates of *M. fructicola* on the staining of two different media after 6 days at 21°C. a) On Water culture medium. b) On PDA medium. These are the bottom of plates.

a


16-L.K.
14-Calif.
23-M.K.
Growth after 6 days
on w.c. media

b


1-ARK.
2-ARK.
11-Y.W.
14-Calif.
16-L.K.
23-M.K.
Growth after 6 days on

Fig. 5

Table 4a. The effect of different media on the rate of growth of 6 isolates of M. fructicola incubated at 21°C for 6 days.*

Media	Isolates					
	1 Ark.	2 Ark.	11 Yakima, Wash.	14 Calif.	16 Lawrence, Kans.	23 Manhattan, Kans.
PDA	71	77	74	84	79	61
Pear medium	82	60	72	58	52	56
Complete Hoagland and Arnon (basal) medium	77	84	73	72	79	39
Complete Hoagland and Arnon medium minus yeast extract	46	36	43	39	35	21

* These are the means of four replicates without subtracting the inoculum diameter (5.00 mm).

Table 4b. Analysis of variance.

Source of variation	DF	Ss	Ms	F	Significant at 5%	Significant at 1%
1-Media	3	20804	6934	889	Yes	Yes
2-Isolates	5	6029	1205	154	Yes	Yes
3-M x I	15	4483	298	38	Yes	Yes
4-Error	72	561	7			

Media $LSD_{5\%}$ = 1.6

Isolates $LSD_{5\%}$ = 2.0

M x I $LSD_{5\%}$ = 3.9

These results agreed with those of Roberts and Dunegan (1932), and with that of Willaman (1920) on pure salt medium.

Effect of Temperature on the Rate of Growth

The effect of temperature on the rate of growth of the isolates was studied by using the basal medium in petri dishes. The plates were incubated at 5°, 21°, 25°, 30°, and 35°C for 6 days. The plates at 5° and 35°C did not show any growth after the incubation period. These plates were transferred and incubated at 30°C for another 6 days to test the effect of temperature changes on the growth of these different isolates. The results obtained are presented in Tables 5a and 5b.

From these results it was found that the temperature had a great effect on the growth of the isolates. There was no growth at 5° and 35°C. The growth generally slowed down for all isolates at 30°C. The optimum temperature for growth differed with respect to the different isolates, and ranged from 20° to 25°C; for isolates Nos. 1, 14, 16, and 23 it was 20°C, and for isolates Nos. 2 and 11 it was 25°C.

Differences within the isolates at the same temperature and within the same isolate at different temperatures proved that they were highly significant.

The growth of the isolates was affected by the change in temperature from 35° to 30°C and from 5° to 30°C. The change of temperature from 35° to 30°C permitted the growth of all isolates to some limited extent. This might have been due to the harmful effect of this high temperature (35°C) on the growth of the isolates. Most isolates grew well when the temperature raised from 5° to 30°C, except isolate No. 14 which did not

Table 5a. The effect of temperature on the growth of 6 isolates of M. fructicola on water culture medium for 6 days.*

°C	Isolates					
	1 Ark.	2 Ark.	11 Yakima, Wash.	14 Calif.	16 Lawrence, Kans.	23 Manhattan, Kans.
5	0.0	0.0	0.0	0.0	0.0	0.0
21	75.0	56.0	46.0	76.0	79.0	74.0
25	55.0	63.0	67.0	52.0	41.0	46.0
30	17.0	38.0	13.0	31.0	18.0	20.0
35	0.0	0.0	0.0	0.0	0.0	0.0
5 to 30	27.0	36.0	18.0	0.0	32.0	22.0
35 to 30	8.0	22.0	5.0	28.0	6.0	10.0

*These are the means of four replications measured in mm and by subtracting the diameter of inoculum (5.0 mm) from the measurements.

Table 5b. Analysis of variance.*

Source of variation	DF	Ss	Ms	F	Significant at 5%	Significant at 1%
1-Temperature	4	44749	11187	1538	yes	yes
2-Isolates	4	1843	460.95	63	yes	yes
3-T x I	16	6513	407	56	yes	yes
4-Error	75	545	7			

* Statistical analysis was done with omitting isolate No. 14 and temperatures 5° and 35°C from the analysis.

Temperature $LSD_{5\%} = 1.7$
Isolates $LSD_{5\%} = 1.7$
T x I $LSD_{5\%} = 3.8$

show any growth by this change. This growth was due to changes in the physiological activities of the fungus, which explained that the low temperature had no harmful effect on the fungus except on isolate No. 14; it only slowed down the growth to limits that could not be detected by the unaided eye.

Effect of pH on the Amount of Growth

Effect of pH on the amount of growth was studied by using the basal medium in liquid state. Adjustments were made to inoculate the flasks with the same amount of spores (4,000 spores). The data obtained are presented in Tables 6a, 6b, and 6c.

From these results it was found that the effect of pH on the amount of growth differed with respect to the different isolates. The differences between the isolates at the same pH, and between the same isolate at different pH's were highly significant. It was found that pH 7.5 inhibited the growth of all isolates to some extent but they still were able to grow at this level of pH. The optimum pH for isolate No. 1 was 6.4, for Nos. 2, 11, and 16 was pH 4.8, and for Nos. 14 and 23 it was 3.8.

The data on final pH in Table 6c indicated that pH always shifted to the acid side for all initial hydrogen-ion concentrations. This showed that the growth of all isolates made the medium more acidic.

Effect of the Carbon Source on the Amount of Growth

Effect of carbon source on the amount of growth was studied by using the basal medium minus dextrose as the control medium. The

Table 6a. Effect of pH on the amount of growth of 6 isolates of *M. fructicola* grown on W. C. medium for 7 days.*

No. of isolate	pH			
	3.8	4.8	6.4	7.5
1-Ark.	253.0	255.0	317.0	123.0
2-Ark.	257.0	261.0	122.0	150.0
11-Yakima, Wash.	219.0	235.0	200.0	166.0
12-Calif.	240.0	202.0	211.0	144.0
16-Lawrence, Kans.	192.0	200.0	118.0	101.0
23-Manhattan, Kans.	274.0	260.0	200.0	175.0

* These are the means of 5 replicates measured in mg dry weight.

Table 6b. Analysis of variance.

Source of variation	DF	Ss	Ms	F	Significant at 5%	Significant at 1%
1-pH	3	180277	60092	210	Yes	Yes
2-Isolates	5	86972	17394	61	Yes	Yes
3-pH x I	15	106827	7121	24	Yes	Yes
4-Error	95	27091	285			

pH $LSD_{5\%}$ = 8.7
Isolates $LSD_{5\%}$ = 10.6
pH x I $LSD_{5\%}$ = 21.2

Table 6c. The final pH after 7 days incubation of 6 isolates of M. fructicola.*

Initial pH	Isolate					
	1	2	11	14	16	23
	Final pH of the isolates					
3.8	3.5	3.2	3.4	3.5	3.9	3.6
4.8	4.3	4.2	4.6	4.4	4.6	4.3
6.4	4.5	4.9	5.2	5.5	5.5	5.7
7.5	5.3	6.2	5.3	5.1	5.3	6.4

* These are the means of 5 replicates.

different carbon sources used were d(-) fructose, d(-) mannitol, soluble starch, and cellulose of filter paper (Alce- V 42870). All these compounds were used at 10 g per liter, except cellulose at 1 g per liter. The media prepared in flasks were inoculated with 4,000 spores per flask, and then incubated at 25°C for 6 days. The dry mycelial mats were determined. The results obtained are presented in Tables 7a and 7b.

From these results it was found that these different isolates had different capacities in utilizing different carbon sources. All isolates did not show any visible growth on the control medium which was deficient in carbon source. Germination of spores and some branching of term tubes were detected by microscopic observations, but no further growth. The differences among the different isolates in utilizing any carbon source, also within the same isolate in utilizing different carbon sources, were shown to be highly significant.

Table 7a. Effect of carbon source on the growth of 6 isolates of M. fructicola grown on W. C. medium at 25°C for 6 days.*

Source of carbon	Isolates					
	1 Ark.	2 Ark.	11 Yakima, Wash.	14 Calif.	16 Lawrence, Kans.	23 Manhattan, Kans.
Fructose	118	257	306	143	345	326
Mannitol	70	143	211	114	143	204
Starch	117	129	139	226	158	145
Cellulose	119	56	70	86	64	120

*These are the means of four replicates measured in mg dry weight.

In the control medium, no visible growth was detected after the time of incubation.

Table 7b. Analysis of variance.

Source of variation	DF	Ss	Ms	F	Significant at 5%	Significant at 1%
1-Carbon source	3	326869	108956	766	Yes	Yes
2-Isolates	5	90937	18187	128	Yes	Yes
3-C x I	15	19976	13317	93	Yes	Yes
4-Error	72	10229	142			

Carbon $LSD_{5\%} = 6.9$
Isolates $LSD_{5\%} = 8.4$
C x I $LSD_{5\%} = 16.8$

Table 7c. Effect of amylase and cellulase enzymes produced by 6 isolates of M. fructicola on starch and cellulose.*

Isolate	Amount of reducing sugars per 50 ml filtrate	
	Starch medium	Cellulose medium
1-Ark.	40.0	48.7
2-Ark.	34.7	63.5
11-Yakima, Wash.	35.5	53.0
14-Calif.	46.5	59.7
16-Lawrence, Kansas	55.6	49.5
23-Manhattan, Kansas	43.7	56.8

* These are the means of two replicates expressed as mg of maltose per 50 ml filtrate.

For most isolates, fructose was the best carbon source. Starch was the best carbon source for isolate No. 16.

The utilization of starch and cellulose by these different isolates as shown in Fig. 6, and the determination of the amount of reducing sugars in the filtrates as shown in Table 7c, indicated that these isolates produced amylase and cellulase enzymes but in different amounts, when starch or cellulose were present as a sole source of carbon in the media.

These results which indicated the production of amylase and cellulase enzymes by different isolates of this fungus seems to be the first work to be done. This was similar to the findings of Cooley (1914) who indicated the production of cellulase by Sclerotinia cineres (Bon.) Schroter (syn. to Monilinia laxa (Aderh. & Ruhl.)) Honey.

EXPLANATION OF FIG. 6

The growth of 6 isolates of M. fructicola on water culture media using two carbon sources: (a) using starch as a sole source of carbon, (b) using cellulose as a sole source of carbon. The isolates are from left to right: 1) Arkansas, 2) Arkansas, 11) Yakima, Washington, 14) California, 16) Lawrence, Kansas, and 23) Manhattan, Kansas.

These results reflected some ideas about the wide specialization in brown rot fungus M. fructicola (Wint.) Honey as it infects different varieties of fruits at different stages.

Effect of Nitrogen Concentration on the Amount of Growth

Effect of nitrogen concentration on the amount of growth was studied by using the basal medium in liquid form minus nitrogen source as a control. Different amounts of nitrogen were added to the control medium. The results obtained are presented in Tables 8a and 8b.

From these results it was found that all isolates were able to grow, even in the absence of nitrogen in the medium. Most isolates grew well on the complete basal medium more than in any other concentration. Isolates Nos. 2, 11, 14, and 23 grew best on the complete basal medium (A); isolates Nos. 1 and 16 grew best on half concentration of nitrogen in the basal medium (D). The growth generally slowed down at low nitrogen concentration.

There were differences between the different isolates at a certain level of nitrogen, and within the same isolate at different levels of nitrogen concentrations. These differences were highly significant. This indicated that these different isolates grew best at different levels of nitrogen, mostly at high concentrations of nitrogen.

a

b

Fig. 6

Table 8a. Effect of nitrogen concentration on the growth of 6 isolates of M. fructicola grown on W. C. medium at 28°C for 7 days.*

Nitrogen concentration	Isolates 1 Ark.	2 Ark.	11 Yakima, Wash.	14 Calif.	16 Lawrence, Kans.	23 Manhattan, Kans.
A	241	254	350	265	265	286
B	205	216	328	241	259	256
C	161	189	264	157	217	291
D	253	217	243	202	317	243
E	184	125	169	187	198	220
F	77	55	91	73	100	121

* These are the means of three replicates measured in mg.

A = complete basal medium; B = double A; C = three times A; D = 1/2 A; E = 1/4 A; F = minus nitrogen (control).

Table 8b. Analysis of variance.

Source of variation	DF	Ss	Ms	F	Significant at 5%	Significant at 1%
1-Nitrogen concentration	5	425861	85172	789	Yes	Yes
2-Isolates	5	73110	14622	135	Yes	Yes
3-Nitrogen concentration x I	25	70072	2802	25	Yes	Yes
4-Error	72	7769	107			

Nitrogen concentration $LSD_{5\%}$ = 6.9
Isolates $LSD_{5\%}$ = 6.9
Nitrogen concentration x I $LSD_{5\%}$ = 16.9

The Relative Pathogenicity of 6 Isolates of *M. fructicola* on Different Varieties of Fruits with Respect to their pH and Nitrogen Content

After twelve months of transferring the isolates on artificial media, their relative pathogenicities were studied on different varieties of fruits. The pH's and the nitrogen content of the fruits were determined and the results obtained are presented in Table 9.

The fruits were inoculated by using the method used by Toshikazu Tani in Japan (1963), then incubated at 25°C. The results obtained are presented in Tables 10a through 10f.

From these results it was found that all varieties of fruits were infected; none of the fruits was excluded by the different isolates. But it was found that these isolates infected the different varieties of fruits with different capacities in developing the disease on these fruits. The incubation period, which was determined as the time required for the development of brown spot, 5 mm in diameter around the inoculation on at least 50% of the fruits, varied within the same isolate on the different varieties of fruits and among the different isolates on the same variety. Elberta peaches, Early Elberta peaches, Moorpark apricots, and Anjou pears were the most susceptible varieties for all isolates. The incubation period for most isolates on these varieties was about 12 hours. The different isolates differed in the time required for complete rot of fruits, with a range of 4-9 days on both varieties of peaches, and on apricots, where that on pears was 8-15 days. Also, the different isolates produced different amounts of

Table 9. Analysis of total nitrogen and pH of 7 varieties of stone and pome fruits.*

Variety of fruits	pH	Total nitrogen in mg per 100 g fresh weight
1-Early Elberta peach	3.9	165
2-Moorpark apricot	3.5	170
3-Beauty plum	2.8	102
4-Anjou pear	4.4	73
5-Santa Rosa plum	3.1	86
6-Winesap apple	3.2	79
7-Elberta peach	3.8	105

* These are the means of 2 replicates of pH measurements and 2 replicates of nitrogen determination by the Kjeldahl method.

EXPLANATION OF SYMBOLS USED
IN TABLES 10a THROUGH 10f

The symbols used in Tables 10a through 10f are as follows:

A. The numbers given to the fruit varieties are:

1. Early Elberta peach
2. Moorpark apricot
3. Beauty plum
4. Anjou pear
5. Santa Rosa plum
6. Winesap apple
7. Elberta peach

B. The symbols given to the relative production of conidia are:

++++ indicates the production of abundant conidia.
+++ indicates the production of heavy spores.
++ indicates the production of medium amounts of conidia.
+ indicates the production of few conidia.
+ indicates the production of slight amount of conidia.

The conidia were produced at the point of inoculation and away from the point of inoculation, or might have been at the point of inoculation only, or not produced at any part of the fruits. The conidia produced might have been in concentric circles or not in concentric circles, but as a mass of spores.

C. The symbols given to the relative amounts of nigrescence of the skin of the fruits are:

++++ indicates black nigrescence of at least 2/3 of the fruit.
+++ indicates light black nigrescence of 1/2 of the fruit.
++ indicates light black nigrescence of 1/3 of the fruit.
+ indicates light black nigrescence of 1/4 or less the fruit.
+ indicates light black nigrescence of very small spot on the fruit.

The nigrescence might have been produced at the point of inoculation and away from the point of inoculation, or at the point of inoculation only, or not produced at all.

Table 10a. The relative pathogenicity of the isolate No. 1 of M. fructicola on 7 varieties of stone and pome fruits.

Fruit variety	Incubation period in hrs.	Hrs. required for spore formation	Days required for complete rot	Production of conidia at the end of 3 weeks		Nigrescence at the end of 3 weeks	
				at point of inoculation	away from point of inoculation	at point of inoculation	away from point of inoculation
1	18	36	9	+++ not concentric	+	++	---
2	18	72	9	++ not concentric	++	++	++
3	24	72	12	+ concentric	+	++++	++++
4	12	36	15	++++ not concentric	++	+++	+++
5	24	48	10	+++ concentric	+++	++++	++++
6	24	--	17	---	---	+	---
7	12	24	5	+++++ not concentric	+++++	+	---

Table 10b. The relative pathogenicity of the isolate No. 2 of M. fructicola on 7 varieties of stone and pome fruits.

Fruit variety	Incubation period in hrs.	Hrs. required for spore formation	Days required for complete rot	Production of conidia at the end of 3 weeks		Nigrescence at the end of 3 weeks	
				at point of inoculation	away from point of inoculation	at point of inoculation	away from point of inoculation
1	12	18	6	++++ not concentric	+++	+++	---
2	12	24	4	++++ not concentric	++	++	++
3	36	72	9	+ concentric	+	++++	++++
4	12	49	12	++ concentric	---	+	---
5	36	72	10	+ concentric	---	+++++	+++++
6	48	--	18	---	---	++++	++++
7	12	12	5	+++++ not concentric	+++++	---	---

Table 10c. The relative pathogenicity of the isolate No. 11 of *M. fructicola* on 7 varieties of stone and pome fruits.

Fruit variety	Incubation period in hrs.	Hrs. required for spore formation	Days required for complete rot	Production of conidia at the end of 3 weeks		Nigrescence at the end of 3 weeks	
				at point of inoculation	away from point of inoculation	at point of inoculation	away from point of inoculation
1	12	24	7	+++ not concentric	+++	++	++
2	12	12	4	+++++ not concentric	+++++	++	++
3	36	48	9	+ concentric	---	++++	++++
4	12	12	9	++++ not concentric	+	---	---
5	24	36	9	+ concentric	---	++++	++++
6	36	--	14	---	---	+++	+++
7	12	12	4	+++++ not concentric	+++++	++	++

Table 10d. The relative pathogenicity of the isolate No. 14 of *M. fructicola* on 7 varieties of stone and pome fruits.

Fruit variety	Incubation period in hrs.	Hrs. required for spore formation	Days required for complete rot	Production of conidia at the end of 3 weeks		Nigrescence at the end of 3 weeks	
				at point of inoculation	away from point of inoculation	at point of inoculation	away from point of inoculation
1	36	48	9	++ concentric	---	+	---
2	12	24	4	++++ not concentric	++++	++	---
3	48	72	10	+ concentric	---	++++	++++
4	24	48	12	++ concentric	---	++	---
5	48	48	9	+++ not concentric	---	++++	++++
6	60	--	18	---	---	+++	+++
7	12	24	6	+++++ not concentric	+++++	---	---

Table 10e. The relative pathogenicity of the isolate No. 16 of M. fructicola on 7 varieties of stone and pome fruits.

Fruit variety	Incubation period in hrs.	Hrs. required for spore formation	Days required for complete rot	Production of conidia at the end of 3 weeks		Nigrescence at the end of 3 weeks	
				at point of inoculation	away from point of inoculation	at point of inoculation	away from point of inoculation
1	36	36	9	++ concentric	---	+++	---
2	24	24	6	+++ concentric	+++	+++	+++
3	36	72	12	+ concentric	+	+	+
4	12	24	8	+++++ not concentric	+++++	++	---
5	18	24	7	++++ concentric	+++	+++++	+++++
6	36	--	18	---	---	++	---
7	12	12	4	+++++ not concentric	+++++	+	---

Table 10f. The relative pathogenicity of the isolate No. 23 of M. fructicola on 7 varieties of stone and pome fruits.

Fruit variety	Incubation period in hrs.	Hrs. required for spore formation	Days required for complete rot	Production of conidia at the end of 3 weeks		Nigrescence at the end of 3 weeks	
				at point of inoculation	away from point of inoculation	at point of inoculation	away from point of inoculation
1	12	24	6	+++++ not concentric	+++++	+++	---
2	12	24	6	+++++ not concentric	+++++	++++	++++
3	48	72	10	+ concentric	---	+++	+++
4	36	72	14	++ concentric	++	++	---
5	36	84	11	+ concentric	---	---	---
6	24	--	20	---	---	+++++	+++++
7	12	12	4	+++++ not concentric	+++++	+++	---

spores on these different fruits. The different isolates infected Beauty plum, Santa Rosa plum, and Winesap apple with different capacities, but in general they were slower on these varieties. The incubation period ranged from 24-48 hours on Beauty plum, 18-48 hours on Santa Rosa plum, and 24-60 hours on Winesap apple; the time required for complete rot ranged from 9-12 days on Beauty plum, 7-11 days on Santa Rosa plum, and 14-20 days on Winesap apple. The amount of spores was small on plums whereas no spores were produced by any isolate on apples (Fig. 7).

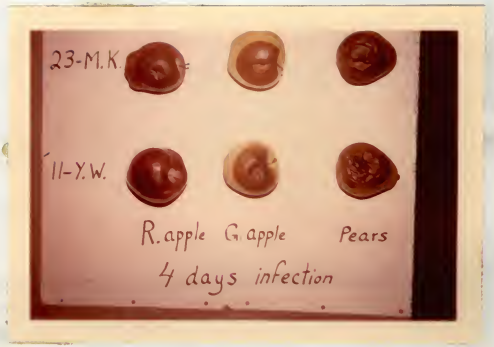
The incidence of the disease was affected by the pH and by the nitrogen content of the fruits. It was found that the pH of fruits at or near the optimum pH and with high nitrogen content of fruits, favors rapid development of the disease, as that on Early Elberta peach; also the pH at or near the optimum favors the disease development, even with low nitrogen, as that on pear and the Elberta peach. The pH far from optimum inhibits the disease development to limited extent and delays its progress and inhibits the spore production as that on plums and apples.

Reactions between Different Isolates of *M. fructicola* in Soils

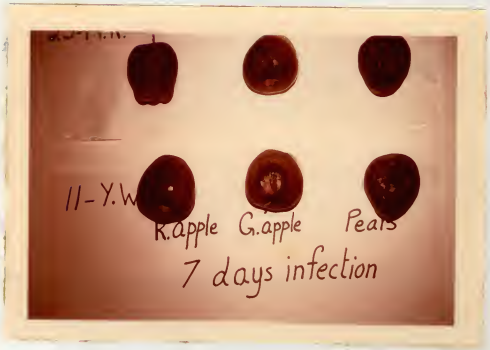
Some workers observed reactions between different strains of this fungus in petri dishes. Ezekiel (1924) made combinations of 2 strains per plate inoculated at different points in potato-dextrose agar plates but he did not observe any microscopic or macroscopic reactions. He mentioned that anastomosing occurred between the 2 colonies of some

EXPLANATION OF FIG. 7

The pathogenicity of two isolates of M. fructicola, No. 11, from Yakima, Washington and No. 23 from Manhattan, Kansas, on the pome fruits, red apples, Golden Delicious apples, and Anjou pears incubated at 25°C.
a) The symptoms after 4 days incubation.
b) The symptoms after 7 days incubation at the face of the point of inoculation.
c) The differences in the advancement of the disease on the back face of the point of inoculation after 7 days incubation.


23-M.K
11-Y.W.
R.apple G.apple Pears
4 days infection

a


11-Y.W
R.apple G.apple Pears
7 days infection

b

Fig. 7


23-M.K.
11-Y.W
R.apple
G.apple
Pears
7 days infection

strains but not with the others and that no further reactions accompanied these anastomoses.

Thind and Keitt (1949) found that a dark line formed between 2 colonies from the four different spore pairs in each asous of M. fructicola but not between the two spores of one pair. The mycelium of the dark lines seemed to be sclerotial in character and produced abundant microconidia. The formation of dark lines might be a result of physiological, chemical, or sexual response when 2 different strains came in contact with each other.

The formation of the dark mycelium was observed by the author in different media at different periods for only one isolate in plate. These were identified as sclerotial bodies.

The reactions between the different isolates were studied in soil. Two soils were used, 50% clay and 50% sand soil and vermiculite. These soils were distributed in plastic pots 4 inches in diameter to be 2/3 full.

Different combinations were made by using 2 months inoculated mummies which had viable spores and by seeding the isolates alone. The 6 isolates used were No. 1 and No. 2 from Arkansas, No. 11 from Yakima, Washington, No. 14 from California, No. 16 from Lawrence, Kansas, and No. 23 from Manhattan, Kansas. Four replicates were made for each combination.

The inoculated pots were divided into halves; one stayed inside the greenhouse and the other was put outside the door. This was done twice, once by using clay soil and the other time by using vermiculite.

The pots were irrigated once every week with 1/2 concentration Hoagland and Arnon solution No. 2 (1950).

From the time of inoculation in January 1963 to December 1963, observations were made on the inside and outside pots for any reactions that might occur, as shown in Fig. 8.

No reactions occurred between any isolates but just the growth of the mycelium of the isolates in the pots was observed. Negative results were obtained from this experiment.

DISCUSSION

Variations among isolates of the fungus M. fructicola (Winter) Honey were observed under different conditions. It was found that these isolates showed morphological variabilities between each other on PDA medium and on peaches and plums. These morphological differences among the isolates might have been due to the chemical nature of the fruits. This confirmed work of Angell (1950), Byrde and Fielding (1951), Vasudeva (1930), and Willaman et al. (1925).

It was found that the size of macroconidia was variable for the different isolates and ranged from 12.0 to 17.8 by 7.2 to 12.0 microns. These measurements agreed fairly well with those obtained by Ezekiel (1924) who reported a range of 12.5 to 16.5 by 8.5 to 11.8 microns for the different strains he worked with. Different measurements have been reported by different workers (Reade, 1908; Roberts and Dunegan, 1932; Matheny, 1913; and Harrison, 1928). These differences in measurements obtained by the different workers might have been due to different isolates they worked with and technique used.

EXPLANATION OF FIG. 8

Combinations between 6 isolates of *M. fructicola* by using two-month mummified peaches and plums in plastic pots 4" in diameter 2/3 full with clay soil or vermiculite placed in the greenhouse for one year. No reactions were observed.

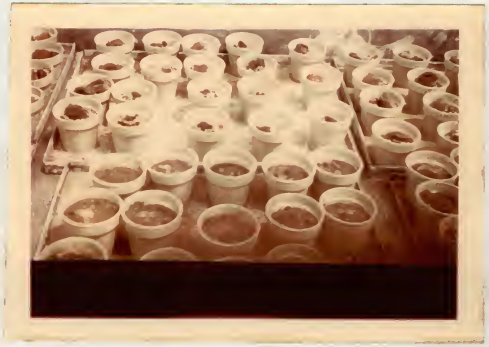


Fig. 8

The growth of these isolates was inhibited on mineral salt medium deficient in a growth factor or factors. This was corrected by adding yeast extract to the salt medium and also by using PDA and pear media. The growth of these isolates depends on a growth factor or factors which must be added to achieve their growth on mineral salt medium. These results agree with that obtained by Roberts and Dunegan (1932) and by Willaman (1920).

The growth of the isolates was inhibited at 5° and 35°C. But when they transferred to suitable temperature, different results were obtained. Those cultures transferred from 5° to 30°C grew well on the new temperature except isolate No. 14 which was affected by the low temperature (5°C); those cultures transferred from 35° to 30°C grew very slowly to some limited extent. This indicated that the high temperatures have harmful effects on these isolates with different degrees. The optimum temperature for growth differed with respect to the different isolates and ranged from 20° to 25°C. These results agreed with that obtained by Weaver (1950) who reported that the optimum temperature was about 20° to 25°C and did not observe any growth at 35°C, and with that reported by Roberts and Dunegan (1932) who obtained a growth at 3°C on PDA. This might have been due to the effect of medium and the isolates they worked with. Also, these results agree with that reported by Brooks and Cooley (1921) on agar medium.

The different isolates grew best on the acid medium, where the natural or basic media inhibit their growth. The optimum pH for growth differed with respect to the different isolates, in a range of pH 3.8 - 6.7. The growth of all isolates made the medium more acidic.

These results agreed with that reported by Thind and Keitt (1949), Mader and Teller (1947), and Cooley (1914).

The different isolates have different capacities in utilizing different carbon sources. No growth was observed on carbon free medium. The utilization of starch and cellulose by these different isolates and the determination of the amount of reducing sugars in the filtrates indicated that these isolates produced different amounts of amylase and cellulase enzymes when starch or cellulose was present as a sole source of carbon. These results seem to be the first indication on the production of these enzymes by this fungus. A similar report on the production of cellulase in culture medium by Sclerotinia cinerea (Bon.) Schroter (syn. to Monilinia laxa (Aderh. & Ruhl.) Honey was published by Cooley (1914).

This new concept on the production of these enzymes by this fungus reflects some ideas about the wide specialization in brown rot fungus M. fructicola (Winter) Honey as it infects different varieties of fruits at different stages of maturity.

The isolates differ in their amount of growth with respect to the nitrogen concentration of the medium. Some growth was detected in nitrogen-free medium for all isolates but in different amounts. These results agree with that of Willaman et al. (1925), Vasudeva (1930), and Curtis (1928).

It was found that these different isolates were able to infect 7 varieties of stone and pome fruits to different degrees with respect to the incubation period, the time required for complete rot, time required for spore formation, amount of spores produced at the point of

inoculation and away from the point of inoculation, and with respect to the amount of nigrescence produced. Peaches, apricots, and pears were the most susceptible to infection, where plums and apples were rotted slowly with the production of slight amount of spores, or no spores produced, as on apples. The incidence of the disease was affected by the pH and the nitrogen content of the fruits. The pH at or near the optimum for growth favors the disease development and its progress and the production of spores, even with low concentration of nitrogen, as that on peaches and on pears. The pH far from optimum inhibits or slows the disease development and spore production. These results agree with that of Ezekiel (1924), Valleau (1915), Willaman et al. (1925), Vasudeva (1930), Curtis (1928), Angell (1950), Smith (1936), and Corbin (1963).

SUMMARY

Thirteen isolates of the fungus M. fructicola (Winter) Honey were obtained from mummies and diseased fruits collected from different parts of the United States.

Isolation of pure cultures was done without any surface disinfection of the mummies or the diseased fruits. The materials that had no conidia on their surface were kept inside sterilized moist chambers for a period of 16 to 24 hours.

Two methods of isolating pure cultures were used, both by using an acidified PDA medium by using HCl (0.1 N) to pH 5.0 before sterilization of the medium. One method was done by direct transfer of conidia to

different parts of PDA petri dishes. The other method was done by streaking the conidia on PDA petri dishes.

The isolates obtained were identified and compared according to their habits and rates of growth on PDA medium, germination of conidia and branching of germ tubes and their characteristics on peaches and plums.

Some isolates showed different characteristics under the same conditions, but others had similar or closely related characteristics.

From the isolates that showed different characteristics under uniform conditions, 6 isolates were selected for further work.

Six monosporic cultures were obtained from the 6 pure cultures selected by using a modified method devised by the author for this work and similar work with fungi which formed hyaline spores.

The growth of the different isolates was inhibited on media deficient in a growth factor or factors needed for their growth. The growth on natural media proved that these media contained this or these growth factor or factors. By addition of yeast extract to a synthetic medium, normal growth was obtained. The yeast extract supplied the growth factor or factors needed for the growth of the isolates. The isolates showed significant differences in their growth on the different media used and also the differences were observed within each isolate on the different media.

The high temperatures inhibited the growth of all isolates even when they were returned to a suitable temperature for their growth. The low temperatures did not permit any growth of the isolates, but when they were returned to a suitable temperature for their growth, they grew well.

The optimum temperature for the isolates ranged from 21° to 25°C. The different isolates showed significant differences between each other under a certain temperature and these differences were observed within each isolate at the different temperatures.

The isolates grew best on the acid medium but the neutral or basic medium inhibited the growth of the isolates. The isolates showed differences in their growth under a certain pH, also differences were detected within each isolate under different pH's. The optimum pH for growth differed with respect to the different isolates.

The different isolates had different capacity in utilization of different carbon sources. These isolates were able to produce different amounts of amylase and cellulase enzymes when they were grown on media that had starch or cellulose as a sole source of carbon. Although the isolates differed in their amount of growth on these sources, they differed also in the amount of reducing sugars detected in the filtrates of their growth.

The growth of the isolates was detected under wide concentrations of the nitrogen content of the media and even in the absence of the nitrogen source. The isolates differed in the amount of nitrogen required for their best growth.

The different isolates had shown wide variations in affecting different varieties of stone and pome fruits under controlled conditions. In spite of the fruits being different in their pH and nitrogen content, the incidence of the disease was more related to the pH of the fruits than to their nitrogen content.

Combinations between these different isolates on peach and plum fruits were made in pots with clay soil while others had vermiculite. Two replicates were put outside the greenhouse, and two replicates were kept inside the greenhouse. All the pots were irrigated weekly with Hoagland and Arnon solution for nearly one year. No reactions between these different isolates occurred.

It is concluded from this work that the strains within the brown rot fungus M. fructicola do exist. These different strains retained their differences under widely different environments and after successive passages through artificial media and fruits. This confirmed the work done by Ezekiel (1924), Seal (1924), and Roberts and Dunegan (1932). These different strains have shown different effects on the incidence of the disease not only on stone fruits as reported by many workers but also on pome fruits. This new concept on the incidence of the disease by the different strains on pome fruits as on stone fruits is of importance in the development of resistant varieties.

CONCLUSION

Variations among isolates of the brown rot fungus M. fructicola (Winter) Honey do exist and affect the incidence of the disease on different varieties of fruits. These different isolates studied retained their differences under widely different environmental conditions and after successive passages through artificial media and fruits. The variations between the different isolates were highly significant under the different conditions tested. Some workers observed some of the

variations between different isolates such as the rate of growth on PDA medium in plates and test tubes, color of mycelium, branching of germ tubes, color of conidia, size of conidia, oxidase production, and morphological characteristics on fruits. On these bases Ezekiel (1924) grouped the different isolates which showed some of these characteristics, even when there was no significant differences between these isolates, into 6 varieties and he used the term strains for these groups. On the same basis, Seal (1924) grouped the different isolates into 2 physiological forms. Also, Roberts and Dunegan (1932) used the term strain to that group of isolates which differed from another group on the basis of morphological characteristics.

From these studies, on the basis of morphological differences, which were highly significant between the different isolates such as the size of macroconidia, rate of growth on PDA, pear medium, mineral salt medium, amount of growth at different pH levels, production of amylase and cellulase enzymes, effect of nitrogen concentration on the amount of growth, and incubation period on fruits, time of spore formation, time required for complete rot, amount of spores, and amount of nigrescence of fruits, and following Ezekiel (1924), these isolates are some of the strains present in the United States. These different strains have different effect on the disease development on the different varieties of fruits. Some isolates have shown that they infect some pome fruits such as pears, very easily and produce heavy amounts of spores which act as inoculum for spreading the disease. These new hosts should be taken into consideration in further work to be able to control the disease

on them. Also, it was found that these different strains produce different amounts of amylase and cellulase enzymes which reflect the idea that they are able to infect different varieties of stone and pome fruits at different stages of maturity. It is clear that the host range must be studied separately for the strains present in each area in the United States to be able to control this disease.

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PATHOLOGICAL AND PHYSIOLOGICAL STUDIES ON THE BROWN ROT FUNGUS MONILINIA FRUCTICOLA (WINTER) HONEY

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The brown rot caused by the fungus Monilinia fructicola (Winter) Honey is one of the most destructive diseases of peaches and related plants. It is reported from different countries where susceptible plants are grown. In the United States the total losses have been estimated to be as much as $5,000,000 annually.

There are several reports of more or less constant variations among isolates of this pathogen grown on a variety of cultural media. The stability of these variations between the different isolates and its significance on the incidence of the disease on different varieties of fruits were questioned by many workers.

In this investigation, work was undertaken to study the stability of the variations that may exist in a series of isolates of this fungus collected from different parts of the United States under different conditions, and the effect of these isolates on the incidence of the disease on fruits.

Six isolates which showed different morphological characteristics under uniform conditions were selected from the 13 isolates obtained in pure cultures from mummies and diseased fruits collected from different parts of the United States, and isolated in monosporic cultures by using a modified method devised by the author.

These six isolates were tested under different cultural conditions and inoculation of different varieties of fruits. It was found that these isolates retained their differences under these different conditions, and statistical analyses showed that these differences were highly significant.

It was concluded that these isolates should be considered as some of the different strains that exist in the United States and should be considered in further work. Some of these strains infected pome fruits in the same manner that they infected stone fruits; however, many workers considered stone fruits to be the most important hosts for this pathogen. This new idea is important in the revision of the disease control program in the United States.