SOME FUNGI ISOLATED FROM ARIZONA COTTON SEED: THEIR RELATIONSHIP TO SEEDLING BLIGHT AND FREE FATTY ACIDS

by

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INTRODUCTION

The presence in Arizona cotton seed of numerous fungi has been known for some time. The high frequency of internal infection of cotton seed is due to a pore at the chalazal end of the seed. This pore, protected at the most by a plug of thin-walled cells, furnishes an easy route of infection (5).[1] In as much as these fungi could kill the germ or be a source of seedling diseases and of inoculum for later infections, it was thought desirable to determine the kinds of fungi present and to obtain some idea of their pathogenic potentialities to cotton seedlings.

Additional experiments were undertaken because of a problem that arose. Oil pressed from Arizona cotton seed grown during the 1954 season showed high free fatty acid content. The seed contained a higher percentage of "slick" seed (not covered with lint) than normal. These had a free fatty acid content of 25 per cent or more. The average content of free fatty acids in seeds was from 2-3 per cent as compared with a normal average 0.5-1 per cent (19). A high content of free fatty acids, a type of

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1. Numbers in parenthesis refer to Literature cited.

rancidity, decreases the commercial value of the oil. It was therefore thought desirable to determine whether the cotton seed fungi were capable of utilizing cotton seed oil as a substrate.

LITERATURE REVIEW

In 1923 Crawford (6) noted numerous fungi borne internally in cotton seed. In his list of fungi isolated were Fusarium vasinfectum, Collectotrichum sp., Diplodia gossypii (= D. gossypina), three unidentified species of Fusarium, Cephalothecium sp. and Alternaria sp. Ray and McLaughlin (13) made isolations from seeds, seedlings and bolls, and studied the pathogenicity to seedlings in greenhouse tests. Their list of fungi in seeds included Glomerella gossypii and seven species of Fusarium. It is interesting to note that although Aspergillus spp. were listed as occurring on seedlings and bolls, none was listed as occurring in seeds. Arndt (2) isolated Collectotrichum gossypii, Fusarium moniliforme, Fusarium spp., Rhizopus spp., Penicillium spp., Diplodia theobromae, Alternaria spp. and Aspergillus spp. from cotton seed. Christensen et al. (5) isolated Fusarium vasinfectum, Aspergillus glaucus, A. versicolor, A. candidus, Penicillium sp. and Glomerella gossypii from internally infected seeds.

The relationship between moisture content and high free fatty acid content has been known for some time. Simpson (16), in connection with studies of longevity of cotton seed in storage, noted that seeds stored under

moist conditions had a higher free fatty acid content than seeds with a low water content. Karon and Altschul (10), studying factors affecting the development of high free fatty acid content, noted that the seeds which had a high moisture content developed a high fatty acid content. These seeds also became moldy. No attempt apparently was made to identify the fungi involved. They did, however, consider that these organisms might be responsible for the development of the fatty acids. Other explanations were also suggested. Christensen et al. (5) in their excellent paper showed that internal infection of cotton seed by various fungi could account for the increase in free fatty acids under moist conditions.

METHODS OF ISOLATION

Isolations were made from various lots of Arizona cotton seed. The seeds used were of four types: (a) undelinted, (b) machine delinted, (c) burner delinted (lint removed from the seeds by passage through a gas flame), and (d) acid delinted. Since almost all of the seed used carried as a surface contaminant Rhizopus sp., which by its rapid growth obscured evidence of the presence of other fungi and rendered their isolation difficult, surface sterilization was necessary. In surface sterilization seeds were immersed in a beaker filled with Rada's Solution, prepared according to the formula given by Barducci and Rada (3), for several minutes, and then rinsed with two changes of sterile distilled water. Lint-covered seeds were delinted with concentrated sulfuric acid, rinsed in running tap water and used without further sterilization. The seeds were then placed on agar, usually Potato Dextrose Agar ("Difco"), either in petri plates, ten seeds to a plate; or in agar slants, one seed to each. The use of agar slants facilitated the obtaining of pure cultures of the organisma. In one series Czapek's Solution Agar with 20 per cent sucrose, prepared according to the formula given by Thom and Raper (22), was used in an attempt to isolate

members of the Aspergillus glaucus Group, which do not grow well on media of low osmotic pressures.

IDENTITY OF ORGANISMS ISOLATED

Some twelve fungi, representing four genera, were obtained in pure culture. The number of infected seeds per lot was variable, from almost none to almost all. In the descriptions, color terminology is in general according to common usage. When the use of the terminology of Ridgway (14) was felt to be meaningful, the color name is followed by that author's name in parentheses.

A species of Alternaria developed regularly from the seeds. The conidia, which are 20-50 x 13-18 μ., vary greatly in shape and septation (Fig. 1). Faulwetter (8) identified an Alternaria occurring on cotton leaves as Alternaria tenuis Nees. Although the measurements of the conidia of the Alternaria isolated do not agree closely with those given by Faulwetter, the conidia are similar in shape and septation to his illustrations. It is the writer's opinion that the two fungi may be identical. In addition to causing leaf spots an Alternaria has been reported as causing boll rot of cotton by Owens (12).

The Aspergilli were well represented in respect to number of species, although some of the species did not occur frequently. Descriptions of members of this genus, unless otherwise noted, are from colonies grown on Czapek's

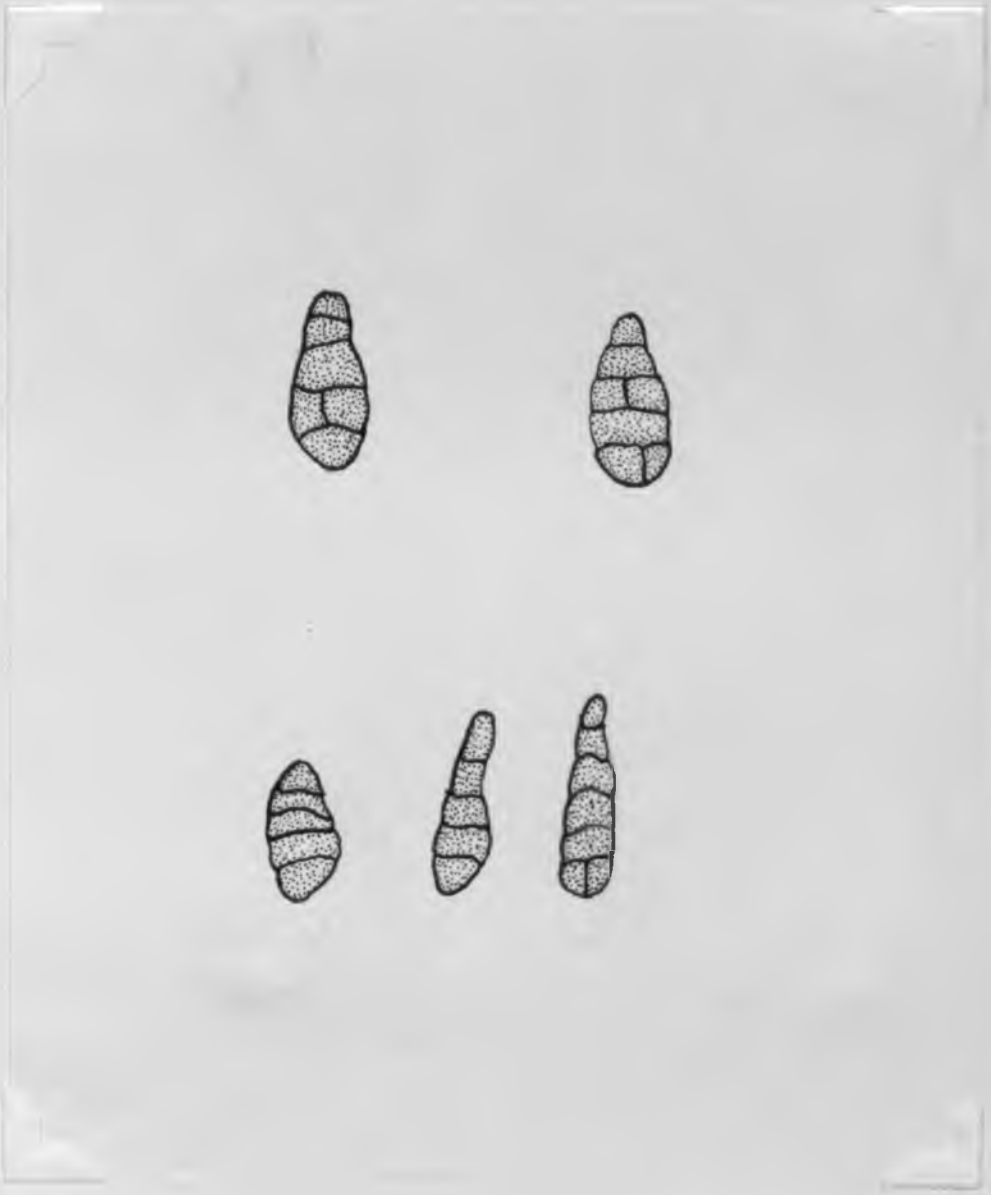

Fig. 1. Characteristic spores of the Alternaria sp. isolated from cotton seed. 800 X.

Solution Agar with 3 per cent sucrose (22).

The Aspergillus glaucus Group was represented by two species. Members of this group do not develop well on ordinary media, but require concentrated media such as Czapek's Solution Agar with 20 per cent sucrose (22). In attempts to isolate members of this group from cotton seed on the above medium, difficulty was experienced with more rapidly growing fungi.

The first member of this group isolated was grown on Potato Dextrose Agar through several transfers. During this time the chief characteristic was the restricted growth of colonies. After several transfers colony growth on Potato Dextrose Agar was freer and perithecia were observed for the first time, enabling the proper placement as to group. This species was then maintained on Czapek's Solution Agar with 20 per cent sucrose. This fungus can be characterized as producing colonies, lemon-yellow at first due to the abundant perithecia, later becoming orange changing to a brownish-red from the encrustment of the mycelium with pigment particles (Fig. 2); perithecia, globose, 83-133 μ. in diameter; asci, 12-15 μ. x 8-11 μ; ascospores, lenticular, smooth, with shallow equatorial groove, and low equatorial ridges, 5.5-7.2 μ. x 3.8-5.0 μ; conidial heads, scattered in colony, pale-bluish; vesicle, 15-25 μ. in diameter; sterigmata, in a single series, flaskshaped,

Fig. 2. Colony characteristics of *Aspergillus ruber*.

7.8-13.3 μ. x 4.4-7.2 μ; conidia, ovate, spinulose, 5.0-6.6 μ. in length. The ascopores are intermediate in size between those of Aspergillus ruber (Bremer) Thom et Raper, 5.2-6.0 μ. and those of A. mangini (Mangin) Thom et Raper, 6.6-7.4 μ. With exception of ascospore size these two species are very similar. The isolate under discussion undoubtedly represents a transitional strain between the two species. For the sake of convenience it will be referred to as A. ruber.

The second member of the Aspergillus glaucus Group was represented by three isolates, one of which had to be discarded because of contamination. One isolate may be characterized as having colonies on Czapek's Solution Agar with 20 per cent sucrose producing olive-green conidial heads freely, obscuring the yellow perithecia; perithecia more or less globose, 100-165 μ. in diameter; asci, 10.0-12.8 μ. in long axis; ascospores, lenticular, convex surfaces rough; equatorial furrow, deep, V-shaped, and equatorial ridges, prominent, irregular; vesicles, subglobose, 15-24 μ. in diameter; sterigmata in a single series, 6.2-8.7 μ. in length; conidia, globose to subglobose, spinulose, 3.7-7.5 μ. The other isolate differs from the preceding only in having colonies producing conidial heads less freely, and in having vesicles and sterigmata of somewhat smaller size. These isolates are considered to be representatives of Aspergillus amstelodami (Mangin) Thom et

Church.

A member of the Aspergillus terreus Group, isolated by Mrs. M. H. Simmonsen,[2] was obtained. It produces somewhat wrinkled colonies with cream-colored mycelium predominating in the center, but with margins becoming covered with columnar, conidial heads (Fig. 3). The conidial area of the colony is at first light vinaceous fawn (Ridgway) becoming in age avellaneous (Ridgway). It is characterized as having vesicles, hemispherical, 12-20 μ. in diameter; sterigmata in two series, developing on upper half of vesicle; primary, 5.5-8.8 μ. x 1.6-2.7 μ.; secondary, 4.4-8.3 μ. x 1.1-2.7 μ.; conidia, smooth, subglobose, 1.6-3.3 μ. Although on the basis of colony color, this fungus could be considered as being Aspergillus carneus (v. Tiegh.) Blochwitz, on the basis of the close correspondence in size of various structures it is perhaps best to consider it a strain of Aspergillus terreus Thom.

Aspergillus niger van Tieghem occurred frequently in the cotton seed. The isolate used in the various tests (Fig. 4) is characterized as having vesicles, globose, 40-67 μ. in diameter; sterigmata in a single series only, 11-26 μ. in length; conidia, globose, rough, 2.7-4.5 μ. in diameter. Isolates having the normal double series of

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Fig. 3. Colony of *Aspergillus terreus*.

Fig. 4. Colony characteristics of *Aspergillus niger*.

sterigmata were also observed. This fungus is a common cause of boll rots (15).

Aspergillus flavus Link was frequently observed. The isolate used in tests is characterized as producing dark yellowish-green colonies (Fig. 5), having vesicles, more or less globose, 22-62 μ. in diameter; sterigmata, in one or two series; single sterigmata, 8.7-18.5 μ; primary sterigmata 8.7-16.3 μ; secondary, 5.0-10.0 μ; conidia, globose, finely roughened, 3.3-4.4 μ. Although there are some divergences from the description of Thom and Raper (22), it is probably best to consider the isolate under discussion as belonging to this rather variable species. It has recently been reported as causing a yellow stain of cotton fibers by Bollenbacher and Marsh (4). This problem was also investigated by Allen (1).

Two members of the Aspergillus ochraceus Group were isolated. Of these one produced colonies that were predominantly sclerotial (Fig. 6). The sclerotia are globose, yellow becoming a rufous shade in old cultures. The sulfur-yellow conidial heads are sparsely produced. Vesicles are globose, 33-40 μ. in diameter; sterigmata in two series; primary, 10-22 μ. in length; secondary, 8-13 μ; conidia, smooth-walled, globose, 2.2-3.8 μ. It probably represents a strain of Aspergillus quercinus (Bainier) Thom et Church.

Fig. 5. Colony characteristics of *Aspergillus flavus*.

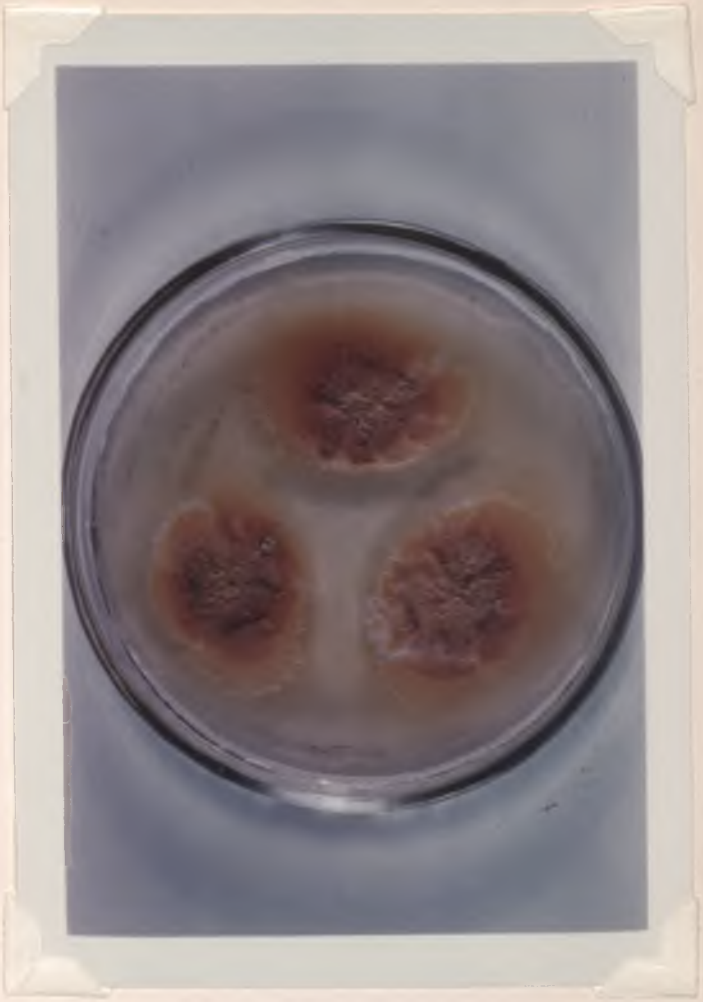

Fig. 6. Colony characteristics of *Aspergillus quercinus*.

The other member of this group produced ochraceous conidial heads freely (Fig. 7). It may be characterized as having globose vesicles, 37-62 μ. in diameter; sterigmata in two series; primary 12-37 μ; secondary, 6-12 μ. in length; conidia, globose, with finely roughened walls, 2.7-3.8 μ. It is considered to represent a strain of Aspergillus ochraceus Wilhelm.

A Diplodia occurred frequently. On Potato Dextrose Agar it produced a gray floccose mycelium, in which pycnidia were produced in two to three weeks. The spores are dark, two-celled, with delicate striations in the wall, and measure 11-16 μ. x 20-29 μ. These characteristics agree well with the description of Diplodia gossypina Cooke as quoted by Stevens (20), who, after further study, considered it to be the imperfect stage of Physalospora rhodina (Berk. et Curt.). Cooke and Stevens thought the fungus to be identical with the Diplodia occurring on citrus, known as D. natalensis Pole-Evans, and possibly other species (21). It is also probably identical with D. tubericola (Ell. et Ev.) Taub., the cause of Java Black Rot of sweet potatoes (6). The Diplodia isolated from cotton seed produced typical rots on both oranges (Fig. 8) and sweet potatoes (Fig. 9). It is thus very similar, if not identical, to the one which has as its perfect stage P. rhodina. Since the perfect stage was not observed positive identification is not possible.

Fig. 7. Colony characteristics of *Aspergillus ochraceus*.

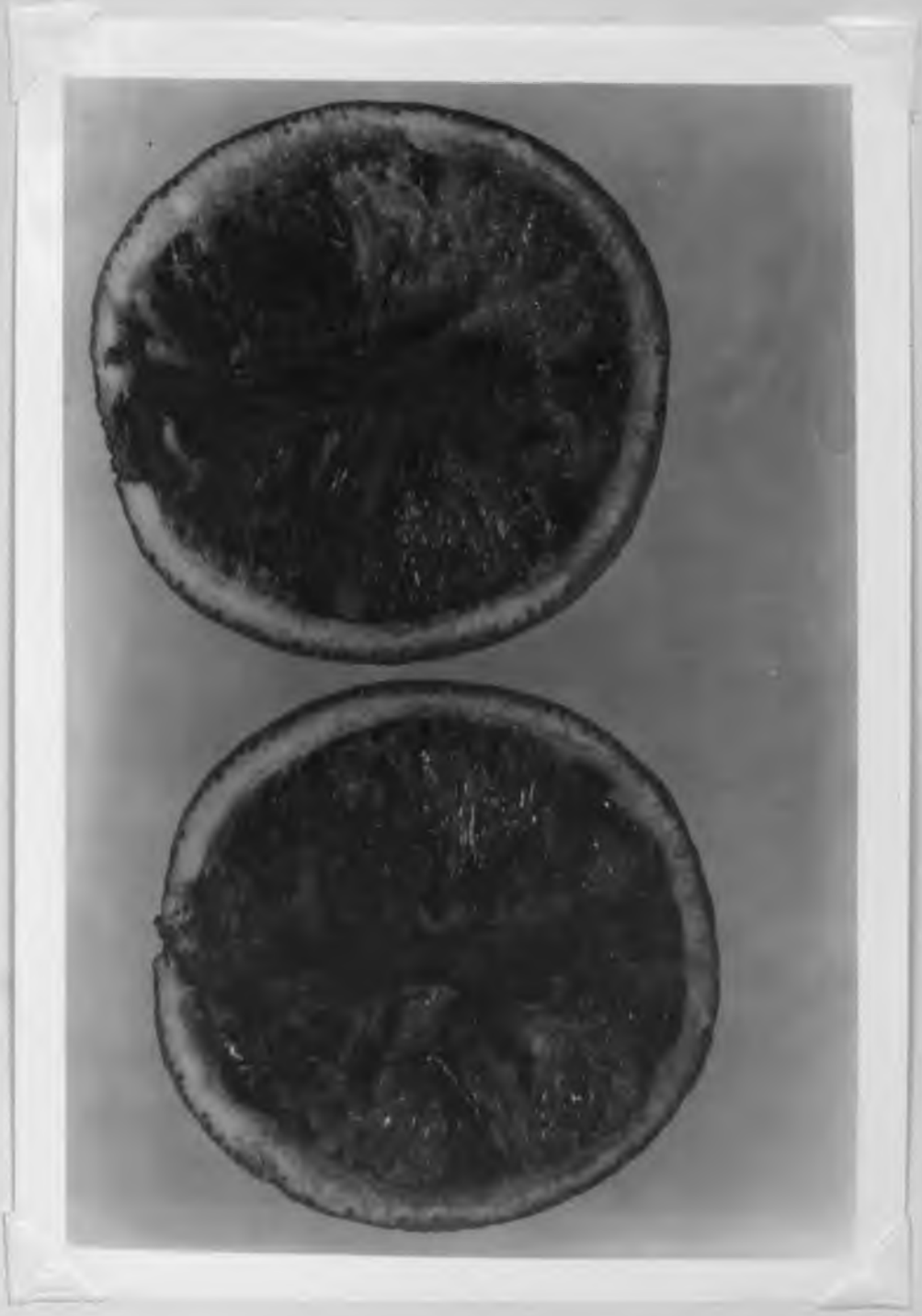

Fig. 8. Orange infected with the cotton Diplodia.

Fig. 9. Sweet potato infected with the cotton Diplodia.

Several Fusaria were isolated. In view of the unsettled state of the classification of the genus Fusarium it was not felt desirable to attempt identification, except in the case of one isolate which showed signs of pathogenicity in preliminary tests. The original isolate was obtained from lesions on the cotyledons of a seedling, which developed from a seed which had been surface sterilized, inoculated with Aspergillus ruber, and planted in sterile soil. Further isolations of this Fusarium were made in the usual manner. It is characterized by producing thin, floccose colonies, white to pink, on Potato Dextrose Agar, which it discolors a deep wine at times; more or less pyriform microconidia are freely produced; macroconidia, rarely; conidiophores, verticillate; intercalary and terminal chlamydospores occur. These characteristics place it in the Gruppe Sporotrichiella of Wollenweber and Reinking (23). This group in the revision of Snyder and Hansen (17) forms the species Fusarium tricinctum. A similar Fusarium has been reported on cotton seed under the name of F. chlamydosporum Wr. et Rg. (- F. tricintum sensu Snyder and Hansen) (13).

PATHOGENICITY TESTS

Methods

In order to determine the pathogenicity of the fungi isolated to cotton seedlings, greenhouse tests were made. Cotton seed used in the tests was from lots known to have a low percentage of infection. The seed was surface sterilized with Rada's Solution, rinsed several times with sterile distilled water, and inoculated. The inoculum used was from colonies of the different fungi grown on Potato Dextrose Agar in petri plates. With the exception of the Diplodia, seeds were simply placed in the plates on the surface of the colonies. Brief agitation was sufficient to coat the seeds thoroughly with spores. The Diplodia pycnidia were first broken open with sterile disecting needles to release the spores before placing the seeds on the colony. The seeds were then planted, ten seeds to a pot, in sterile soil. Ten pots were planted for each treatment, making 100 seeds in all, except in certain cases noted in Table I. The pots were watered regularly with tap water. The pots were kept in a greenhouse where some temperature control was possible. Three series were run. In the first two tests temperature were below optimum for cotton seed germination. In the third

test the temperatures were maintained close to the optimum temperatures for germination of cotton seed.

Results

From the results as given in Table I, it is apparent that Fusarium tricinctum was the only organism which consistently showed pathogenicity. In addition to decreasing the percentage germination, it produced pinkish masses of conidia in lesions on the more or less deformed cotyledons (Fig. 10 and 11). In severe cases more or less normal development occurred. In the case of Alternaria sp. the isolates used were different in the two tests. The isolate used in test A had been in culture for several months, while the one used in test B was a recent isolate. Spores of this fungus could be obtained from black marginal lesions in a few cases. Seeds, inoculated with Diplodia sp., showed slight reduction in germination. No symptoms ascribable to this fungus were observed. Aspergillus terreus was the only Aspergillus species which proved to be pathogenic.

Fig. 10. Cotyledons of cotton seedlings with lesions produced by Fusarium tricinctum.

Fig. 11. A reisolation of Fusarium tricinctum from infected cotton cotyledons.

TABLE I

RESULTS OF GREENHOUSE PATHOGENICITY TESTS

Data represent in each case percentage germination of 100 seeds, except where otherwise noted. A. Av. soil temperatures 13-21 deg. C.; readings taken after 20 days. B. Av. soil temperatures 10-15 deg. C. at start of test and then adjusted upwards to 21-27 deg. C. the last week of test; readings taken after 30 days. C. Av. soil temperature 15-27 deg. C.; readings taken after 22 days.

Fungi	A	B	C
Alternaria sp.	72		34
Aspergillus ruber		54	50
A. terreus			39
A. niger		53	50
A. flavus		52	63
A. quercinus		48	53
A. ochraceus		48	55
Diplodia sp.	25		49[a]
Fusarium tricinctum	0	21	35[b]
Control	40	44[a]	54[b]

a. Av. of 200 seeds.

b. Av. of 4 isolates, 100 seeds per isolate.

UTILIZATION OF COTTONSEED OIL

Methods

To test for lipolysis and fat utilization three media were used: Spirit Blue Agar, Czapek's Solution Agar with cottonseed oil ("Wesson's") as sole carbon source, and Czapek's Solution with cottonseed oil. Spirit Blue Agar was prepared in the manner described by Starr (18). A positive test is indicated by the change of the medium from a lavender shade to a deep blue. The Czapek's Solution Agar was prepared according to the formula of Thom and Raper (22), except for the substitution of cottonseed oil for the sucrose. In one series 5 ml. cottonseed oil to 50 ml. agar in a 125 ml. flask was used; in a second series, 1 ml. of the oil to 50 ml. of agar, were the proportions used. After the flasks containing the medium had been autoclaved, they were agitated while cooling. Good dispersal of the oil in the agar was obtained by this method. The medium containing the 5 ml. of oil did not solidify well, but was otherwise satisfactory. For use as a control Czapek's Solution Agar was prepared without any carbon source except for the agar or impurities in the agar. As a confirming test of fat utilization Czapek's Solution was prepared without agar or sucrose. Twenty

milliliters of this solution was placed in petri plates. To this was added 2 ml. of cottonseed oil. The plates were then autoclaved.

Results

The organisms tested on Spirit Blue Agar were Aspergillus ruber, A. amstelodami, A. terreus, A. niger, A. quercinus, A. ochraceus, A. flavus, Alternaria sp., Diplodia sp., and Fusarium tricinctum (two isolates). All of the Aspergilli listed above have been reported as utilizing fats except A. ruber, and A. quercinus (7,9,11). Three plates per organism were inoculated. All of these fungi gave positive results for lipolysis with the exception of A. ruber, A. amstelodami and Alternaria sp. The A. ruber and A. amstelodami on some plates gave possible positive results; on others, negative. The Spirit Blue Agar is not suitable for the growth of these two Aspergilli because of the low concentration of solutes. In the case of the Alternaria sp. the negative results might have been due to the dark color of the colonies.

In the tests using Czapek's Solution Agar with cottonseed oil the same organisms were used with the exception of A. amstelodami. None of the fungi grew on Czapek's Solution Agar without carbon source. Good growth was shown by all on both concentrations of cottonseed oil with the exception of A. ruber.

In the test using Czapek's Solution with cottonseed oil, in which the fungi tested were the same as in the preceding test, all the fungi developed with the exception of A. ruber. In connection with A. ruber it should be remembered that the above media are not suitable for the growth of this organism, and that the negative results in the above tests do not eliminate the possibility of utilization of fats under more favorable conditions. The manner of growth of the colonies on Czapek's Solution with cottonseed oil was of interest. At the beginning of the experiment the oil was spread in a thin droplet on the surface of the water. When growth of the organisms had begun, the droplet was gathered into a round drop enclosed by the mycelium, which grew at the water-oil interface. Inasmuch as growth of the mycelium continued to be predominantly at the interface, the resulting colonies formed shallow trays containing the oil droplet.

Conclusions

In the tests on fat utilization all the fungi, with the exception of A. ruber and A. amstelodami were shown to be capable of metabolizing cottonseed oil. In the light of this it is reasonable to believe that one or more of these may be responsible for the development of free fatty acids in Arizona cotton seed.

DISCUSSION

The significance of the organisms occurring in cotton seed is somewhat difficult to assess. Although the relation of these fungi to the deterioration of cotton seed in storage was not investigated, it is quite possible that they are responsible for deterioration of cotton seed stored under moist conditions, such as described by Simpson (16).

Their ability to utilize cottonseed oil indicates their probable involvement in the increase of free fatty acids under suitable conditions. The problem of free fatty acids in Arizona cotton seed, however, is far from being solved at the present time. It may be a result of growth conditions without any organisms being involved. If organisms are involved, it is not known at this time whether the damage occurs in the field or in storage or both. Further, it is not known whether the organisms which were isolated from stored cotton seed are representative of the fungi occurring in seeds in field. Studies need to be made to determine the relation of weather to the formation of free fatty acids, and whether damage occurs in the field or in storage. In addition, it would be desirable to culture seeds and bolls from various areas throughout the season. Whether an organism or organisms are responsible for "slick" seeds should be determined, and the organisms

identified.

From the pathogenicity tests it seems apparent that the majority of these fungi are probably not an important cause of seedling blight, with the exception of the Fusarium tricinctum. However it should be remembered that reactions of organisms in pure culture under greenhouse conditions are not necessarily identical with the reactions of the same organisms under field conditions.

Finally, these organisms must be considered in relation to boll rots. Several of these are known incitants of boll rots; the rest are potential causes of boll rots. In view of this planting lots of seed heavily contaminated with fungi would seem undesirable. At least they would have a low per cent of germination. It is quite possible, however, that these fungi may be so widely distributed that the infected seeds are not important as a source of inoculum.

SUMMARY

Fungi isolated from cotton seed grown in Arizona were found to include Aspergillus ruber, A. amstelodami, A. terreus, A. niger, A. flavus, A. quercinus, A. ochraceus, Alternaria sp., Diplodia sp., and Fusarium spp. Although Rhizopus sp. can be isolated from unsterilized seed it is extremely rare internally.

From greenhouse pathogenicity tests it was found that Fusarium tricinctum was the only likely cause of seedling blight. Possible involvement in the problem of free fatty acid in Arizona cotton seed was shown by tests, which indicated that, with the exception of A. ruber and A. amstelodami, the other fungi were capable of utilizing cottonseed oil. Their possible importance was discussed, and suggestions for future work on the problem of free fatty acids were made.

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