FUSARIC ACID PRODUCTION IN VITRO AND IN VIVO

By *Fusarium oxysporum* f. *lycopersici*

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INTRODUCTION

Several investigators have concluded that wilt symptoms in plants with systemaic vascular diseases are caused by toxic metabolites from the invading bacteria or fungi (6, 18, 26). Many wilt pathogens in culture produce toxic compounds that can cause wilting, chlorosis, or necrosis when taken up by cuttings. An example of such a compound is fusaric acid. However, it is very difficult to relate such findings to pathogenesis in the infected plant. Plants have a very limited range of visible responses, and a variety of unrelated toxic compounds can produce similar symptoms in any one plant (3).

Fusaric acid was first isolated from culture filtrates of Fusarium heterosporum Nees (34) and has since been found as a metabolic product of several Fusarium species. There is still a controversy about the role of fusaric acid in infections by Fusarium spp. Some workers claim that it is an important factor in pathogenesis in the Fusarium wilt diseases of tomato (7, 9), cotton (13, 17), watermelon (20), and other plants. Kuo and Scheffer (16) concluded that fusaric acid does not have a direct causal role in disease development and it may not be essential for pathogenicity. Furthermore, data given by Gäumann (10) suggest that a high rate of synthesis in infected plants is necessary for a toxic concentration to be reached and maintained.

The conflicting conclusions mentioned above are examined further in this report. Kuo's work with tomato plants and Fusarium oxysporum f. lycopersici (Sacc.) Synd. and Hans. (16) was extended along several lines. The accumulation of fusaric acid in vivo and in vitro was measured by bioassay and by a spectrophotometric method. Special emphasis was given to potential concentrations of fusaric acid in vivo in relation to the stage of disease development. Use was made of several strains of the fungus that differed in ability to produce fusaric acid and in relative pathogenicity.

REVIEW OF LITERATURE

Fusaric acid has been the subject of several reviews (9, 10). Only literature pertaining to the present work is summarized below.

Fusaric acid (5-n-butyl pyridine - 2 carboxylic acid, m.w. 179) is known to be a metabolic product of several fungi in the family Hypocreaceae (9). A related compound, dehydrofusaric acid (with a terminal double bond in the butyl side chain), was detected in culture filtrates of F. oxysporum f. lycopersici and was said to be produced as the mycelium ages (30). F. oxysporum f. lycopersici produces other toxic metabolites in culture along with fusaric acid. Of these, lycomarasmin and vasinfuscarin were suggested as causal factors in disease development. However, these two compounds have never been isolated from naturally infected plants and no direct evidence for a role in disease has been presented. Since alteration in the nutrient medium can affect the presence or the yield of a specific metabolic product of a fungus, certain toxic compounds found in culture filtrates might not be present in infected plants.

The evidence for fusaric acid as a causal factor in disease development is indirect. Fusaric acid was identified in cotton plants infected with F. oxysporum f. vasinfectum as a copper complex, which was identical

chromatographically with fusaric acid-copper complex (17). Kalyanasundaram and Venkata-Ram (13) estimated by a chromatographic bioassay technique that 17 mg/kg was present in cotton plants infected with F. oxysporum f. vasinfectum. Fusaric acid was demonstrated in the rhizosphere of tomato plants infected with F. oxysporum f. lycopersici (14). Subsequently fusaric acid was detected in rhizomes of banana infected with F. oxysporum f. cubense (23), in flax infected with F. oxysporum f. lini (19), and in watermelon infected with F. oxysporum f. niveum (20). The best evidence for the presence of fusaric acid in infected tomato plants comes from the isotope dilution experiments of Kern and Kluepfel (15). Fusaric acid, if present, apparently existed in vivo in quantities too small to be isolated. Therefore, extractions were made from diseased plants previously held in the presence of C^{14} labelled CO_2. Purified fusaric acid without a label was added to the extract from diseased plants that should have contained C^{14}- labelled fusaric acid. This mixture was purified further and the radioactivity determined. The activity was consistently higher for preparations from diseased plants than for healthy controls, and this was offered as evidence for the presence of fusaric acid in vivo. The data do not rule out the possibility that the label was present in trace impurities or in closely related breakdown products rather than in fusaric acid itself.

There are evidences indicating that fusaric acid has no significant role in disease development. Wilt in Fusarium infected tomato plants is correlated with xylem dysfunction, especially in the petioles. Fusaric acid was found to have no effect on conductive ability of the xylem of treated stems and petioles (16). Fusaric acid has no specific toxicity to plants that serve as hosts of various Fusarium spp. (7, 9), in contrast to the activity of several known toxins (25). Symptoms of fusaric acid injury (9) are not similar to symptoms usually developed by infected plants. These symptoms include both external and internal symptoms and respiratory responses or biochemical symptoms. Physiological effects of infection include increased gas exchange by tissues and increased activity of isolated mitochondria (33). In contrast to the pattern in diseased plants, uptake of fusaric acid by cuttings did not cause increases in respiration, but caused decreases (2, 16). When fusaric acid was used in concentrations high enough to cause injury there was a tremendous decrease in succinicoxidase activity by mitochondria isolated from treated shoots (16).

Fusaric acid producing ability of a number of ultraviolet (UV)-induced mutants of F. oxysporum f. vasinfectum did not correlate with their pathogenicity to cotton plants (32). Kuo and Scheffer (16) also were unable to show a correlation between fusaric acid synthetic ability of UV-induced mutants of F. oxysporum f. lycopersici in vitro and

their pathogenicity to tomato plants. Some mutants did not produce fusaric acid *in vitro* but were as virulent as the high fusaric acid producers. High fusaric acid producing strains varied similarly. Two naturally occuring species, *F. lateritium* Nees and *F. scipi* Laub and Fautr., are known to cause a typical tomato wilt (24) but they do not produce fusaric acid in culture (9).

Fusaric acid seems to be rapidly metabolized in some plant tissues (1, 11, 28). Thirty per cent or less of the fusaric acid taken in by tomato cuttings was recovered (28). When smaller amounts of fusaric acid were given to tomato cuttings, smaller amounts of unchanged fusaric acid were detected. Fusaric acid was not detected when *F. oxysporum* f. *conglutinans* was grown on cabbage stem sections, nor was it detected in diseased tissues (11), although the fungus produces fusaric acid abundantly in certain culture media. Fusaric acid was rapidly metabolized after being taken up by cabbage cuttings. Fusaric acid seems to be broken down by tomato tissue, (28) and direct evidence for its presence in infected plants has not been reported. Contrasting data for cotton plants infected with *F. oxysporum* f. *vasinfectum* (17) could be attributed to higher sensitivity or lower activity in destroying fusaric acid in cotton than in tomato plants. The minimal dose for cotton is 25 mg/kg fresh weight, but for tomato it is 150 mg/kg fresh weight (8). It

is doubtful if the amount of fungus in diseased tomato plants (33) could produce this amount of fusaric acid.

The Japanese workers Tamari and Kaji (34) suggested that fusaric acid may be transformed *in vivo* by the host tissue to unknown compounds, such as water insoluble chelate complexes with metal ions (4). Therefore, identification of the free acid in the infected host tissues would be complicated.

Several assays have been used for estimation of fusaric acid content of culture filtrates. A bioassay using *Bacillus subtilis* (Cohn) Prazmowski as the fusaric acid sensitive organism was used by several workers (12, 13, 16). Selected strains of *B. subtilis* are said to be capable of detecting 5 ug fusaric acid per ml (12). This method was used in conjunction with chromatographic separation of fusaric acid (13, 14, 17), because *B. subtilis* is inhibited by several substances in fungus cultures. *Ustilago zeae* spore germination is said to be selectively inhibited by fusaric acid and this was used as the basis of a bioassay (28). Spectrophotmetric methods similar to the one described below were used by several workers (27, 28, 35).

Detection of fusaric acid in diseased plants has posed many problems. Bioassays with *B. subtilis* have been used but this method could give misleading results because of various inhibitory compounds in plant tissue. Extraction

of fusaric acid from diseased tissue and detection by the UV-spectrum was described by Matsui (19) but he gave no quantitative procedures. Kern and Kluepfel (15) were not successful in detecting fusaric acid in diseased tomato plants, but concluded that it was present on the basis of the isotope dilution technique, as described above.

MATERIALS AND METHODS

F. oxysporum f. lycopersici strain R5-6 was the parent strain used in this study. The ultraviolet (UV) induced mutants were the same as those used by Kuo (16). On the basis of Kuo's data, two highly pathogenic, two moderately pathogenic, and two mildly pathogenic strains were chosen for further work. All strains were maintained by serial subsulture on potato dextrose agar slants.

The tomato variety Clark (Fusarium wilt susceptible) was used in all experiments. Plants were grown in the greenhouse in peat-sand mixture and were fertilized with a complete nutrient solution. When cuttings were required, they were taken from plants at the four leaf stage. Stems were cut with a razor blade under water and were placed immediately in the test solution. Inoculation of tomato plants was done by the root-dip method (29). The plants were uprooted, the roots were washed and dipped into a bud-cell suspension (16, 29), and the plants were re-set in pots containing the sand-peat mixture.

Inoculum was produced by growing each isolate in 250 ml Erlenmeyer flasks in 50 ml modified Richard's solution. Fungus was grown in shake culture for 4-5 days, which resulted in enough bud-cell suspension for inoculation. The liquid medium used was a modified Richard's solution with

the following composition in g/l: glucose, 50; NH_4NO_3, 10; KH_2PO_4, 5; $MgSO_4 .7H_2O$, 2.5; and Fe $CI_3 .6H_2O$, 0.02. Cultures were seeded with small pieces of mycelial mats.

Growth of the fungus was measured by dry weight determination. The mycelium was filtered through a dried, weighed filter paper, washed thoroughly with cold water, dried along with the filter paper at 80°C for 24 hours, and re-weighed.

Kuo's (16) fusaric acid preparation was used. This preparation was reasonably pure, as shown by chromatography, biological activity, UV-spectrum, and by comparison with synthetic fusaric acid.

Two strains of Bacillus subtilis (Cohn) Prazmowski were used as fusaric acid bioassay test organisms. The sensitivity of the first strain was such that it could be used in the range 100 - 800 ug/ml. The second strain was more sensitive, and could be used in the range 40-500 ug/ml. For bioassay estimation of fusaric acid in tomato cuttings and in infected plants, the plant tissue was ground in minimal amounts of distilled water in a Waring blender for 2-3 minutes. The clear extract obtained after centrifugation for ten minutes at 1,000 x g was brought to pH 3.0-4.0 with 5 NHCl. The plant extract was then concentrated under vacuum at 37°C. The acidified concentrated solution was extracted three times with anhydrous purified ether. The ether extract was then evaporated and the residue was

dissolved in 0.5 ml sterilized distilled water. The latter solution was assayed for fusaric acid using a procedure similar to Kuo's (16). A standard antibiotic assay paper disc, 11 mm in diameter, was allowed to take up 0.1 ml of the test solution and then dried. These discs were then placed on agar plates freshly seeded with B. subtilis cells from a 20 hour old culture on potato sucrose agar. The plates were kept at 4°C overnight, then removed to room temperature for 1 hour, and finally incubated at 30°C. The diameters of the inhibition zones were measured after 24-30 hours. Solutions containing a range of known concentrations of fusaric acid were assayed as controls each time (22). A standard bioassay curve was determined (Fig. 1) and the amount of fusaric acid in solutions was calculated by comparison with the standard bioassay curve (12).

For semi-quantitative spectrophotometric estimation of fusaric acid the following procedure was used after much experimentation with different known amounts of fusaric acid. The method is a slight modification of that used by Matsui (19), Sanwal (27), Sandhu (28), and Zähner (35). Culture filtrate was concentrated under vacuum at 37° C, then brought to pH 3.0-4.0 with 5N H Cl. The acidified filtrate was extracted three times by repeated shaking with anhydrous purified ether (Mallinckrodt A.R.)using fresh solvent each time. Some commercially available was not satisfactory because of impurities that gave a UV-spectrum similar to

that of fusaric acid. The total volume used was always three times that of the sample. The ether extract was then evaporated to dryness, and the solid residue was taken up in 0.5 ml ethanol. This solution was applied (0.1 ml) to Whatman No. 1 chromatographic paper. Chromatograms were developed with butyl alcohol: acetic acid: water (4 : 1 : 1) for 16-20 hours. Fusaric acid alone was chromatographed at the same time as a control. The position of fusaric acid was located on the dried chromatograms under ultraviolet light. Spots with R_f 0.84-0.88 (comparable to the R_f of pure fusaric acid) were cut into small pieces, and placed in 5 ml 80% ethanol in a test tube for 24 hours. The UV-spectrum in the 220-320 mu range for the eluted solution was determined with a Beckman DB spectrophotometer. The amount of fusaric acid was estimated by comparing the height of the 268 mu peak in absorbance with the standard curve made with known concentrations of fusaric acid. (Fig 2.)

The experimental error of the method was 5% or less, as shown by adding known amounts of fusaric acid to culture filtrates before extraction.

A similar procedure was used to estimate fusaric acid taken in by tomato cuttings, and in tomato plants infected with F. oxysporum f. lycopersici. The plant tissue was ground in a Waring blender and extracted by the same procedure used for filtrates. Plant tissue extract was concentrated under vacuum at 37°C and extracted with ether.

EXPERIMENTAL RESULTS

Pathogenicity of the UV induced mutants: Tomato plants were inoculated at the four leaf stage 21-32 days after seeding (33). In each of three experiments 16 tomato plants were inoculated with each of the six individual mutants as described in materials and methods. Plants treated in a similar way without inoculation were used as controls. All the plants were kept in the greenhouse under conditions favorable for disease development. A disease index for each mutant was calculated. (Table 1.) The parent strain R5-6 and mutants 28 and 30 were found to be highly pathogenic. Mutants one and ten were moderately pathogenic while mutants two and 12 were almost non-pathogenic. These results agreed essentially with the data of Kuo, who tested the same isolates for pathogenicity. These particular isolates therefore appear to be genetically stable.

Fusaric acid production in vitro by UV-induced mutants: Small pieces of mycelial mats of each isolate were transfered to 50 ml modified Richard's medium in 250 ml Erlenmeyer flasks. Still cultures were grown in the dark at 28°C. After 10, 15, 19, 24 and 30 days growth, replicate flasks of each isolate were harvested and filtered. Dry weight

of mycelium and fusaric acid content for each isolate was determined by the spectrophotometric method described previously. The UV-spectrum in every case was similar to that of fusaric acid. (Fig. 3.) Results (Table 2) showed that the parent strain, which was highly pathogenic, produced much less fusaric acid than did the weakly pathogenic isolate 5. There was no correlation between pathogenicity of the six isolates and their fusaric acid producing ability. (Table 1,2) Results also showed that fusaric acid production reached a maximum in 19-24 days for the isolates tested. (Fig. 4.) Fusaric acid level did not drop while the mycelium was still growing. This suggests that fusaric acid is a product of active growth and not of autolysis (28). Isolates 30 and 12 showed a second rise in fusaric acid production. The second peak might have resulted from dehydrofusaric acid, which usually follows production of fusaric acid in culture (30).

Recovery of fusaric acid from treated tomato cuttings: A preliminary experiment was done to detect the percentage of recovery of fusaric acid from healthy tomato homogenates. This would indicate the extent of fusaric acid destruction by homogenates and by the extraction procedure. A known concentration of fusaric acid (10^{-3}M) was added to homogenates of healthy tomato stems and roots. The mixture was extracted as described in materials and methods, and the fusaric acid content was estimated spectrophotometrically.

Fifty to 70% of the fusaric acid added to homogenates was recovered.

A second preliminary experiment was done to determine the amount of fusaric acid that could be recovered from cuttings exposed to different known concentrations of fusaric acid. A knowledge of the level of recovery should tell whether or not fusaric acid is metabolized by tomato plants. If toxic levels of fusaric acid are detectable by the method, then the compound should be detectable in diseased plants, if it is a direct cause of symptoms.

Tomato cuttings were taken from greenhouse-grown plants at the four leaf stage. Cuttings were allowed to take up with the transpirational stream measured amounts of 10^{-3} and 10^{-4} M solutions of fusaric acid. Cuttings were exposed to each concentration for 4, 9, 19 and 48 hours in the first experiment and for 6, 18, 24 and 33 hours in the second experiment. In this way the amount of fusaric acid taken in by each cutting was calculated. Fresh weights of the cuttings were taken before they were allowed to take up fusaric acid so that the dilution factors could be calculated. All cuttings were maintained under similar conditions of temperature, humidity and light. Cuttings exposed to 10^{-4} M fusaric acid showed no signs of injury even after 48 hours. Those exposed to 10^{-3} M fusaric acid had a collapse of the lower parts of the stems followed by leaf injury. After the cuttings were removed from fusaric

acid solutions, they were carefully blotted with paper towel, and the leaves and stems were separated and frozen. Extraction for leaves and stems for each treatment was done separately by the procedure described above. Estimation of fusaric acid content was done spectrophotometrically and by bioassay in order to compare the methods and to determine to what extent each would be useful.

In the first experiment no attempt was made to concentrate the plant tissue extract. No trace of fusaric acid was detected in leaf extracts by bioassay or spectrophotometrically except in cuttings left for 48 hours in 10^{-3} M fusaric acid. In this case a large amount of fusaric acid (313.2 gm/kg fresh wt) was taken up by the cutting and hence it is not surprising to detect some in the leaves. Fusaric acid was detected readily in the stems. Results (Table 3) showed not more than 33% of fusaric acid was recovered from the whole plant, as determined spectrophotometrically. A representative UV-spectrum of one of the cutting extracts is shown. (Fig. 3.) This spectrum is identical with the UV-spectrum of pure fusaric acid.

In the second experiment, the cutting extracts were concentrated under vacuum at 37°C before ether extraction. In this experiment fusaric acid was detected in leaves as well as in stem extracts. Not more than 27% of the fusaric acid taken in was detected, with one exception. After only six hours exposure, 25 mg fusaric acid per kg fresh

weight was taken in, and 87% of this was recovered from the plant. (Table 3.) This high percentage of recovery may be because the compound taken in did not have time to be broken down. It is also possible, but unlikely, that some of the fusaric acid on the plant surface was not thoroughly removed. The low percentage of recovery in both experiments as detected spectrophotometrically indicates a rapid breakdown or transformation of fusaric acid by tomato plants as in some other plant tissues (11).

Portions of the same extracts used in the spectrophotometric assay were dissolved in sterilized distilled water and assayed against B. subtilis as described in materials and methods. The estimated amounts of fusaric acid were calculated from the standard curves (Fig 1), using two different strains of B. subtilis. In the first experiment the test organism used was capable of assay in the range 100-800 ug/ml. In the second experiment the sensitivity of the strain of B. subtilis used covered the range 40-500 ug/ml. The bioassay showed a much lower percentage of fusaric acid recovery than did the spectrophotometric method. (Table 3.) Other workers used a strain of B. subtilis which could be used in the range 5-200 ug/ml (12). A possible explanation of the difference in the recovery between the bioassay and the spectrophotometric methods could be a transformation of part of the fusaric acid to a non-antibiotic compound which gave the same spectrum as fusaric acid.

Fusaric acid was not detected either spectrophotometrically or by biossay when cuttings were exposed to 10^{-4} M fusaric acid. At 6, 18, 24 and 33 hours cuttings took in 4.2, 14.9, 17.9, and 26.6 mg/kg fresh weight, respectively. It seems likely that these levels of fusaric acid were broken down so rapidly that none could be recovered unchanged.

Fusaric acid content of infected tomato plants: Sixteen tomato plants at the four leaf stage were inoculated with each of the seven isolates that varied in relative pathogenicity. Plants were kept in the greenhouse under conditions favorable for disease development. Symptoms were first evident 12-14 days after inoculation. Three to six plant samples were harvested at different times after inoculation, when the plants showed slight, moderate, and severe symptoms. Care was taken to select plants with uniform symptoms for each harvest. Non-inoculated plants were harvested at the same times and used as controls. Fresh weight of each group of harvested plants was determined and the plants were frozen and held for processing, not over ten days after harvest. The frozen plants were ground in distilled water with a Waring blender and centrifuged to remove debris. The supernatant was concentrated under vacuum at 37°C and extracted with ether as described previously. Fusaric acid determination for roots and stems was done separately using both the spectrophotometric method and the bioassay method. Fusaric acid was

not detected spectrophotometrically in stem or root extracts in three separate experiments. (Table 4.)

The dried ether extracts of the infected and control plant tissues were taken up in 0.4 ml sterilized distilled water and bioassayed for antibiotic activity against the fusaric acid sensitive strain of B. subtilis. Only root extracts gave clear inhibition zones. These zones were compared with the inhibition caused by known concentrations of fusaric acid used to prepare a standard curve (Fig. 1), which was made at the same time the tissue extracts were tested. On the unlikely assumption that the inhibition zones from tissue extracts were caused by fusaric acid, the possible concentrations of fusaric acid were calculated. (Table 4.) In all cases the possible fusaric acid level was very low, with a maximum concentration of 10^{-5} moles/kg fresh wt of roots (0.0018 gm/kg). Plants inoculated with the parent strain R5-6 and the highly pathogenic isolate 28 showed slight increase in inhibition zone sizes as symptoms developed from slight to severe. Plants inoculated with the five other isolates had either a slight decrease or no significant change in size of inhibition zones with symptom development. Generally there did not appear to be a correlation between inhibition zones and symptoms, especially since the non-inoculated controls sometimes gave inhibition zones. (Table 4.)

The dried ether extracts of the infected tomato plants as well as the control plants were dissolved in 0.5 ml

80% ethanol. The usual procedure of loading, developing, and elution of chromatograms was the same as that used for the cuttings described above. The characteristic spot of fusaric acid could not be detected on the chromatograms of the infected tomato plant extracts with ultraviolet light. The portion of the dried chromatograms having an R_f between 0.80-0.90 was cut out and eluted and the UV spectrum was determined as described previously. The UV-spectrum of the infected plant root extracts were similar to those of the non-infected control plants. No absorption peaks were obtained in the range 250-320 mμ, indicating that no fusaric acid was present. The eluted alcohol solution was dried under vacuum at 37°C, dissolved in sterilized distilled water and bioassayed for antibiotic activity. No inhibition zones were obtained. Since fusaric acid is soluble in both water and ethanol, this result is a further indication that no fusaric acid was present.

DISCUSSION AND CONCLUSIONS

Bioassays and spectrophotometric methods have been used successfully in the past to estimate levels of fusaric acid in culture fluids (13, 19, 27, 28). However, the satisfactory use of such methods for estimating the level of fusaric acid in plant tissues has not been reported. An important part of my research was to adapt and evaluate a spectrophotometric assay for detection of fusaric acid in plant tissues. Comparative experiments with a bioassay and the spectrophotometric method showed the latter to be far more sensitive, although there is some reason to believe the spectrophotometric method gives a high estimate of the level of fusaric acid in tissues. Related compounds will give a similar UV-spectrum.

To test the methods for detection of the compound in plants, known amounts of fusaric acid were given to tomato cuttings. When a concentration of fusaric acid high enough to cause visible injury was used, the compound was recovered and detected by the spectrophotometric method. Approximately 1/3 of the fusaric acid taken up was detected in most cases. When concentrations below the toxic level were used (10^{-4} M), no fusaric acid was recovered. Apparently in these cases the plant tissue was able to destroy the compound faster than it was accumulated by transpirational pull. Fusaric

acid is known to be metabolized rapidly by some plants (1, 11) and by the fungus itself (1).

The data with cuttings can be used to interpret results with diseased plants. Fusaric acid could not be detected in diseased plants by the spectrophotometric method, although this method appeared to be more reliable than bioassay. The data with cuttings indicated that the compound should be detectable in diseased plants, if it was present in concentrations high enough to cause direct injury. Therefore, if the compound is produced in diseased plants, it appears to be broken down long before it reaches toxic levels. These data support the conclusions of Kuo and Scheffer (16).

There are several reasons to believe that bioassay is not a reliable method for detecting fusaric acid in diseased fusaric acid in diseased plants. Compounds that inhibit bacteria and other microorganisms are known to be present in many plant tissues (21). The inhibition zones obtained by the bioassay method could be caused by ether soluble antibacterial compounds found in normal tomato plants, as indicated by the fact that extracts from control plants in two of three experiments gave inhibition zones comparable to those from infected plant tissue extracts. A logical candidate for the antibacterial compound is tomatin, but this substance is ether insoluble (5) and must be eliminated as a possibility. Antibiotic compounds produced by

host tissue as a result of disturbances could also give misleading results as far as fusaric acid is concerned.

According to Kalyanasundarum (13) fusaric acid at an estimated concentration of 17 mg/kg was found in cotton plants infected with F. oxysporum f. vasinfectum. This small quantity was detected by the chromatographic bio-assay method, a procedure of doubtful validity. However, it is possible that fusaric acid can reach much higher levels in cotton than in tomato because cotton does not break down the compound. Still, fusaric acid as a general factor in the Fusarium wilt diseases can hardly be supported.

These results with infected tomato plants together with much data in the literature support the conclusion that fusaric acid has no direct causal role in symptom development in tomato wilt.

SUMMARY

Bioassays and assays based on spectrophotometry were evaluated as methods for detecting and estimating the concentrations of fusaric acid in cultures and in Fusarium-infected tomato plants. The spectrophotometric method appeared to be more reliable and more sensitive than the bioassay method. The compound reached maximum concentrations in cultures in 19-24 days. Isolates of Fusarium oxysporum f. lycopersici varied in ability to produce fusaric acid, but this was not correlated with relative pathogenicity. Approximately 30% of the fusaric acid taken up by tomato cuttings was detected by the spectrophotometric method. The remainder of the compound taken in by cuttings apparently was changed or destroyed by the plant tissue. No fusaric acid was detected spectrophotometrically in infected plants, and only a trace was detected by bioassay. The bioassay results in this case were shown to be questionable. Presumably the compound is either not produced in infected plants or is inactivated as fast as it is formed. Results are considered as further evidence against fusaric acid as a causal factor in disease development.

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APPENDIX

TABLE 1.--Comparative pathogenicity of Fusarium oxysporum f. lycopersici strain R5-6 and six UV-induced mutants to tomato plants (var. Clark).

Isolates	Disease index[a]) at day 12	15	20	30
R5-6	29	64	72	81
28	32	65	79	95
30	7	31	64	78
10	19	37	65	69
1	19	42	50	50
12	5	7	7	11
5	2	2	2	2
control	0	0	0	0

a) Each plant showing symptoms was put in one of the following disease classes: 0=healthy; 25=slightly wilted; 50=moderately wilted; 75=severely wilted; 100=dead. The disease index was the sum of the number of plants in each class times the class value, divided by the total number of plants in the group.

TABLE 2.--Production of fusaric acid in culture by selected isolates of *Fusarium oxysporum* f. *lycopersici*.

Isolates	Growth, mg[a)]	Fusaric acid production mg/l at day					mg fusaric acid /g dry wt at 15 days
		10	15	19	24	30	
R5-6	104	13.8	8.5	15.3	13.3	11	4.08
28	90	11.3	7.8	17	40.5	6.3	10.8
30	74	7.8	21	80	65	89	14.1
10	83	10	12.3	7.3	6.8	9.3	7.4
1	88	13.3	23.8	32	33	26.8	14.1
12	70	12	32	26	44	46	22.5
5	81	35.3	15.3	105	60	96.5	9.4

a) Cultures were grown in 50 ml modified Richard's solution in 250 ml flasks at 28°C. The values given are dry wts of mycelium per flask after 15 days growth.

TABLE 3.--Recovery of fusaric acid (F. A.) from treated cuttings as determined spectrophotometrically and by bioassay.

Time[a] (hr)	F. A. uptake mg/kg	F. A. recovery mg/kg		% F. A. recovery	
		spectro.	bioassay	spectro.	bioassay
Exp. I					
4	49	12.9[b]	--	26.4	--
9	55	14.1[b]	6.3	25	11.4
19	114	25.2[b]	5.9	22	5.2
48	313	105.2	29.6	33	9.4
Exp. II[c]					
6	29	25.2	6.9	87	23.6
18	164	40.9	13.7	25	8.5
24	319	88	26.5	27	8.3
33	347	59.4	30.5	17	8.7

a) Hours cuttings were allowed to take up 10^{-3} M fusaric acid solution by transpirational pull.

b) No fusaric acid was detected in leaves. In all other cases it was detected in both leaves and stems.

c) In this experiment the water extracts from cuttings were concentrated under vacuum at 37°C before extraction with ether.

TABLE 4.--Apparent concentration of fusaric acid in roots of infected tomato plants as determined by bioassay. Every sample was assayed spectrophotometrically as well, but no fusaric acid was detected in any case.

Isolate	Days after inoculation	Symptom	Fusaric acid concentration[a)]
R5-6	13	slight	0.0
	17	moderate	0.13
	22	severe	0.50
28	13	slight	--
	17	moderate	0.13
	22	severe	0.11
30	13	slight	0.0
	17	moderate	0.10
	22	severe	0.11
10	13	slight	0.17
	17	moderate	0.18
	32	moderate	0.14
1	13	slight	0.15
	17	moderate	0.16
	32	moderate	0.09
12	13	slight	0.10
	17	slight	0.0
	32	moderate	0.09
5	13	slight	0.0
	17	slight	0.17
	32	slight	0.08
Control	13	0	
	17	0	
	32	0	

a) Calculated as mg fusaric acid per 100 gm fresh wt of roots, based on the size of inhibition zones as compared to zones caused by known concentrations of fusaric acid.

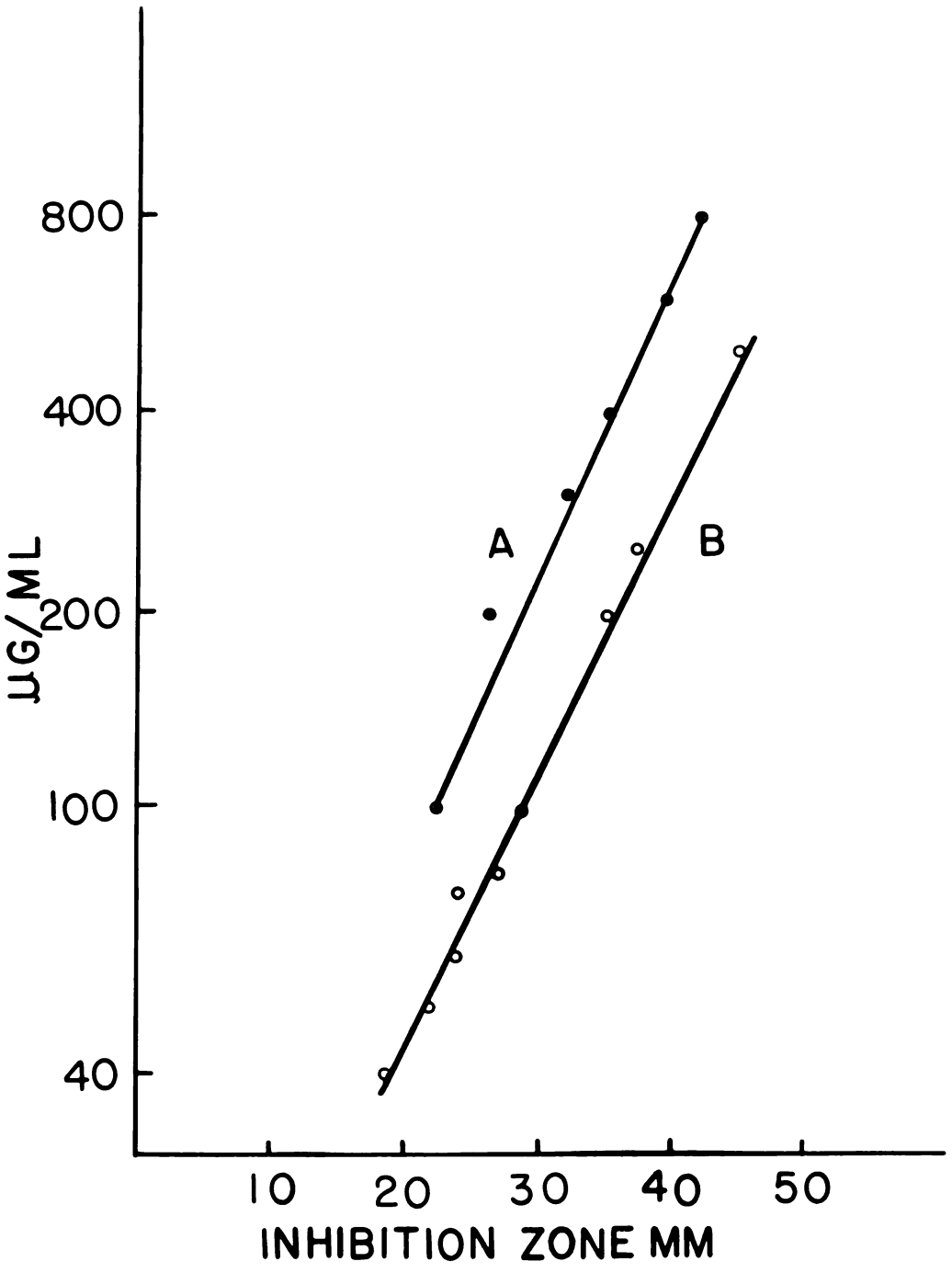

Figure 1.--Standard curves of fusaric acid as determined by bioassay using known concentrations of fusaric acid.

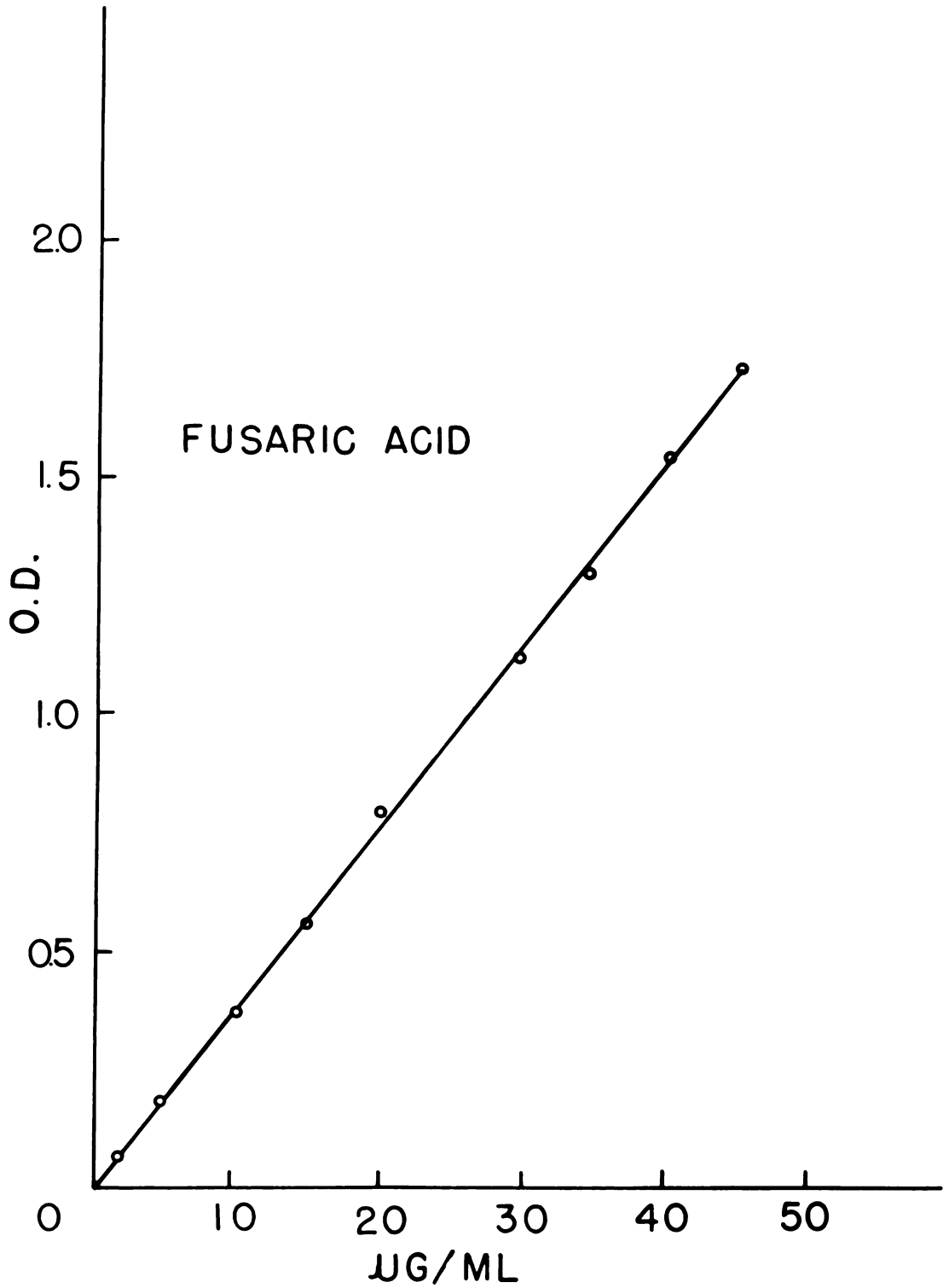

Figure 2.--Standard curve for fusaric acid concentration as determined by absorbancy at 268 mu.

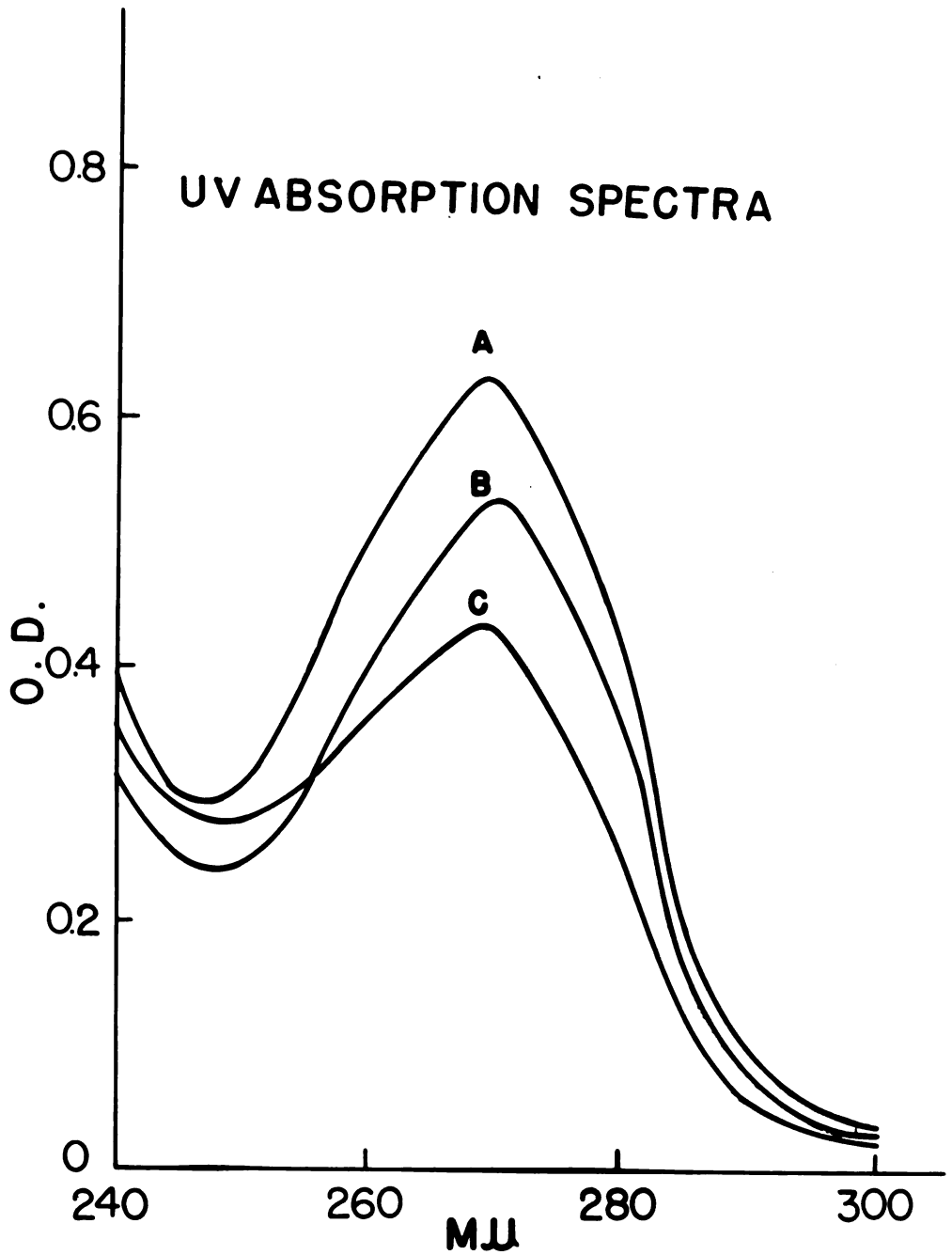

Figure 3.--UV-absorption spectra of cultural filtrate (A), pure fusaric acid (B) and extract from a tomato cutting treated with fusaric acid (C).

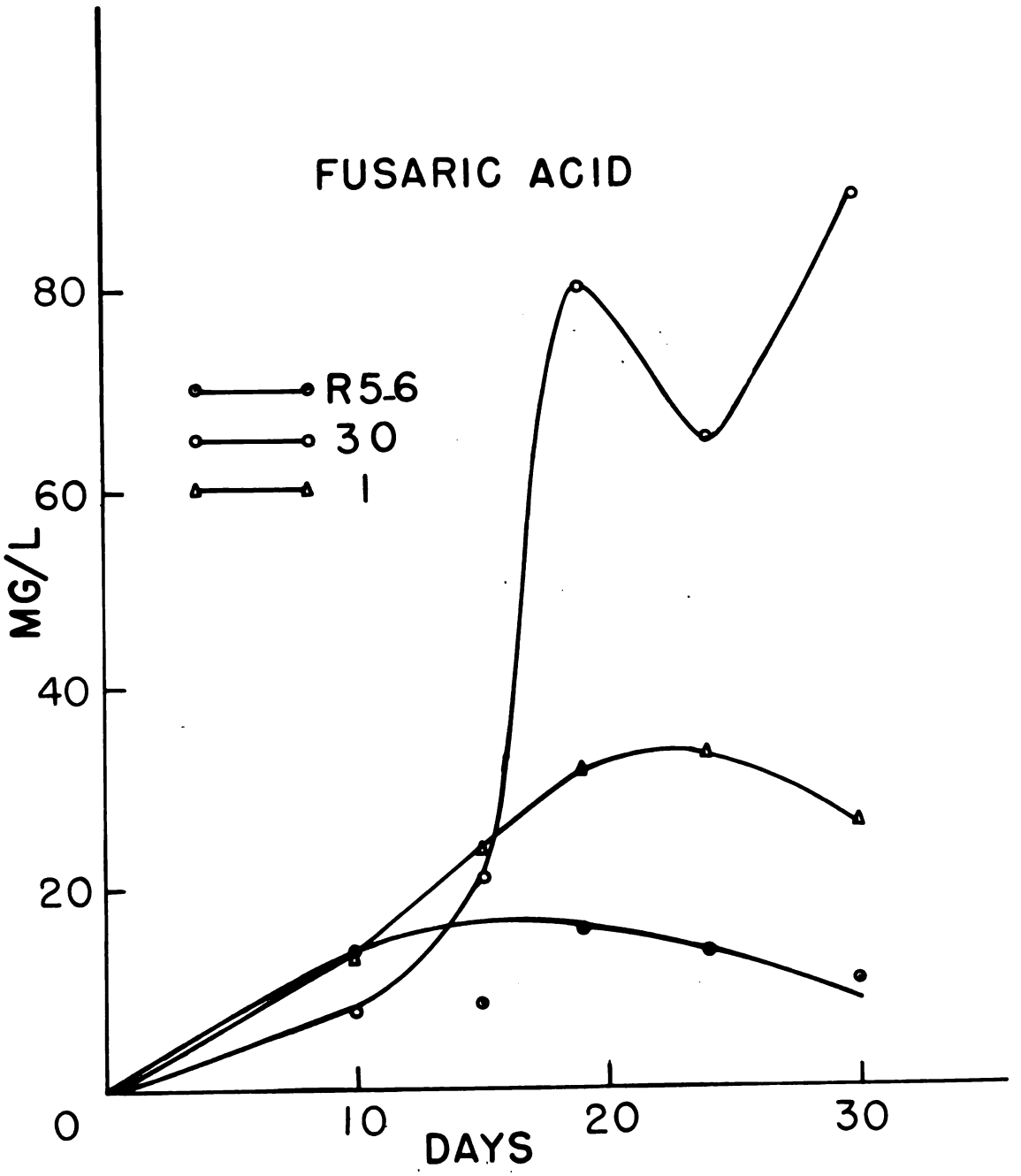

Figure 4.--Fusaric acid production in culture by three selected isolates of Fusarium oxysporum f. lycopersici.