EVALUATION OF THE ROLE OF FUSARIC ACID IN FUSARIUM WILT DEVELOPMENT

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ABSTRACT

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by Mong-shang Kuo

Fusarium wilt studies have led to much controversy over the factors involved in causing the several symptoms. Wilting of infected plants has generally been attributed either to plugging of the vessels or to toxins acting on leaf blades. Recent evidence leaves little doubt that the wilting symptom is correlated with inability of affected xylem, especially in petioles, to conduct water. However, this does not rule out toxins as causal factors, because toxins could be inducing xylem dysfunction.

Fusaric acid is one of toxic metabolites produced by Fusarium oxysporum f. lycopersici (Sacc.) Snyd. and Hans. Several types of experiments were designed to evaluate fusaric acid as a factor in disease development. The ability of fusaric acid to interfere with water movement in plants was tested by the use of non-toxic dye solutions. Fusaric acid was found to interfere with water movement in stems, but this appeared to result from decreased transpiration. It did not cause plugging of the xylem as is found in infected plants.

Biochemical effects of infection include increased gas exchange by tissues and increased mitochondrial activity. The ability of fusaric acid to produce such changes was examined by isolating mitochondria from treated cuttings and comparing the succinoxidase activity with that of preparations from non-treated controls. Concentrations which caused visible symptoms in cuttings inhibited succinoxidase activity in all

cases. Thus fusaric acid produces the opposite effect to that found in diseased plants.

Possible correlations between the ability to produce fusaric acid and the ability to cause disease were tested by the use of UV induced mutants of the causal fungus. Mutants were found which produced very little fusaric acid in vitro, yet were highly pathogenic. Other mutants produced much fusaric acid but had lost almost all virulence to plants. Thus there was no correlation between fusaric acid production and pathogenicity.

The claims in the literature for a role of fusaric acid are based on detection of the substance in diseased plants. I was able to detect, by bioassay, inhibitory substance in roots and occasionally in lower stems of infected plants. The inhibitory substance could be fusaric acid. However, no inhibitory substance was detected in affected upper stems and leaves. This and all the other results described above indicate that fusaric acid has no significant role in Fusarium wilt development in tomato plants.

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By

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INTRODUCTION

Fusaric acid and other metabolites are produced by various Fusaria causing vascular wilt diseases of plants. For example, fusaric acid, lycomarasmin, and vasinfuscurin have been found in culture filtrates of Fusarium oxysporum f. lycopersici (Sacc.) Snyd. and Hans. Roles of these substances in disease development have been postulated (4, 7, 8), but there is no direct evidence for their involvement. The role of lycomarasmin has been seriously questioned (3, 22) and in the absence of supporting data it can not be given a significant part in disease. Vasinfuscurin (6) has not been completely purified, but it is said to be an enzyme. Like the two other substances, it is non-specific in its toxin action. Still other, unidentified toxins have been postulated as being involved in disease development (2, 27). Each claim should be evaluated carefully.

Infection of tomato plants by F. oxysporum f. lycopersici causes vein clearing, epinasty, stunting, progressive yellowing and wilting of leaves, vascular browning, and death of plants. The external symptoms are accompanied by increased respiration of leaf and stem tissue (2, 14, 27). There has been much work on and considerable controversy over the factors involved in causing the several symptoms. Wilting of infected plants generally has been attributed either to plugging of the vessels or to toxins acting on leaf blades. Mycelium of the pathogen, its metabolic products within the plants, tyloses, and breakdown products of host cells have been considered as factors in impeding water movement in xylem vessels. The toxin hypothesis is based on the assumption that the pathogen secretes metabolites which are carried with the transpiration stream and affect the leaves

directly. Recent evidence leaves little doubt that the wilting symptom is correlated with inability of affected xylem to conduct water. However, this does not rule out toxins as causal factors, because toxins could induce xylem dysfunction.

This study was made to evaluate any possible role for fusaric acid in disease development. Tomato plants were chosen for experimental purposes, and several approaches to the problem were made. Certain metabolic effects of fusaric acid on plants and on mitochondrial preparations were compared with the changes induced by infection. A number of UV induced mutants of F. oxysporum f. lycopersici were examined for any possible correlations between pathogenicity and ability to produce fusaric acid. The effect of fusaric acid on water-conducting ability of xylem was studied.

REVIEW OF LITERATURE

Literature with direct application to the present work is summarized below. Complete coverage of the literature is not necessary because detailed reviews of fusaric acid have been published (7, 8).

Fusaric acid (5n-butyl pyridine-2-carboxylic acid) was first considered as a "wilt toxin" in Fusarium-infected tomato plants by Gäumann et al. (5). Several facts indicate that fusaric acid could be involved in disease development. Fusaric acid is produced in culture by many species of Fusarium which cause wilting or damping-off of various plants. Other Fusarium spp. do not produce fusaric acid (16, 17). The compound was detected in Fusarium infected cotton (11) and watermelon plants (15). There are no good data showing that fusaric acid is present in Fusarium-infected tomato plants, although tomato cuttings have been used in many experiments with the pure compound.

There are several reasons to be skeptical of a significant role of fusaric acid in disease development. Most of the symptoms of fusaric acid injury to tomato plants (7) differ in kind from those of diseased plants. Fusaric acid is a respiration inhibitor (2, 13). In contrast, Fusarium-infected tomato plants have increased oxygen uptake by stems and leaves from the early stages of infections until advanced symptoms appear, as compared to healthy tissue (2, 14, 27). In stems this increased gas exchange is apparent even after many leaves have collapsed. Respiration in individual leaves will decrease, of course, as the tissue collapses. The latter observation has been used as confirmation of a role of fusaric acid in disease (14).

Paquin and Waygood (18) tested the effect of fusaric acid on mitochondria isolated from the hypocotyls of tomato seedlings. Results showed that succinoxidase activity was greatly inhibited by 10^{-3} M

fusaric acid. The inhibition was overcome by adding cytochrome c (10^{-5}M) to the reaction mixture. Unfortunately, the experiment did not contain a control with cytochrome c but without fusaric acid. Later work has shown that addition of cytochrome c to reaction mixtures will increase the activity of mitochondria from tomato (27). Particles from diseased and healthy plants responded equally to addition of cytochrome c (27). Nevertheless, Paquin and Waygood concluded that fusaric acid affected the succinoxidase system directly by interfering with the cytochrome system or indirectly by its action on the structural integrity of the particle.

A comparison of respiration in diseased and healthy plants could serve as the basis for evaluating the role of fusaric acid. Wu and Scheffer (27) showed that diseased tissue containing only traces of the causal fungus yielded more mitochondria per unit weight, with higher activity per unit of mitochondrial protein. No evidence was found for changes in oxidative pathways with disease. Phosphorylation was as efficient by mitochondria from diseased plants as by those from healthy plants. These effects of disease differ in kind from those of fusaric acid as outlined above. However, a diffusible toxin, not previously described, is postulated because metabolic responses occur in host cells not in contact with the fungus (2).

Some plant tissues appear to have the ability to break down fusaric acid. Cabbage cuttings were allowed to take up a toxic dose of fusaric acid (100-200 mg/kg green weight) and were later analyzed for residual toxin. No fusaric acid was demonstrated in the plant tissues (9). Decomposition of fusaric acid was similarly rapid in tomato plants; 48 hours after treatment to tomato cuttings, less than 30 per cent of the applied fusaric acid was recovered (21). It was shown that about 10 per cent of the fusaric acid was decarboxylated when tomato cuttings were

allowed to take up C^{14}-labelled fusaric acid. One decarboxylated product, 3n-butyl-pyridine, was suggested as an important factor in disease development, since this compound is 100 times more toxic than fusaric acid. However, no butyl-pyridine was found when fusaric acid was broken down by plant tissues. All of the intermediates identified were less toxic than fusaric acid (1).

In a study of pathogenicity of UV induced mutant strains of Fusarium oxysporum f. vasinfectum Atk., Venkata Ram was unable to show a correlation between fusaric acid synthetic ability of the strains in vitro and their pathogenicity to cotton plants (24). Some of the strains did not produce fusaric acid in culture, according to the assay used, yet they caused disease as well as did the high fusaric acid producing strains. There are similar data from naturally occurring strains. Two Fusaria, F. lateritium Nees and F. scirpi Laub and Fautr., are known to infect tomato plants and cause typical wilt disease (20), but there have been no indications of fusaric acid production by these fungi (7).

MATERIAL AND METHODS

Host material, fungus material and inoculation method:

The tomato variety Bonny Best was used in all experiments. Plants were grown in the greenhouse in peat-sand mixture and were watered with a soluble commercial fertilizer on alternate days. When cuttings were required, they were taken from plants about 6 inches tall. Stems were cut under water with a razor blade and the cuttings were placed in test solutions.

F. oxysporum f. lycopersici strain R5-6 was used for production of fusaric acid. The fungus was grown in 1 liter Roux bottles, placed on the side to give greater surface exposure to the air. Each bottle contained 200 ml culture fluid. The following modified Richard's solution, in g per liter, was used: NH_4NO_3 10; KH_2PO_4 5; Mg $SO_4 . 7H_2O$ 2.5; Fe $Cl_3 . 6H_2O$ 0.02; and glucose, 50. Cultures were seeded with small pieces of mycelial mat, and were grown in still culture at 28°C. for 3 weeks.

To inoculate, plants were uprooted and the roots were washed in tap water and dipped directly into a Fusarium bud-cell suspension, produced by growing the fungus in shake culture (22). Plants were then reset in a peat-sand mixture and kept under greenhouse conditions. Plants treated in the same manner but without inoculation were kept as controls.

Method for testing xylem conductivity:

Leaves were allowed to take up fusaric acid solution through the cut petioles for 24 hrs., then the bases of petioles were placed in eosin solution (1 g Bacto eosin in 100 ml water). Leaves were allowed to take

up dye until the color showed in the uppermost leaflet of untreated leaves. Under the conditions of this experiment, dye reached the top leaflets in about 5 minutes. Freehand sections were then cut to determine dye distribution in treated and control leaves (22).

In another experiment cuttings from healthy plants were allowed to take up fusaric acid solution for 24 hrs. Control cuttings were allowed to take up water. Equal length of stem sections were then taken from these cuttings, and dye was forced through by vacuum. The sections were hooked in parallel to a vacuum pump to insure equal suction. After drops of dye appeared on the ends of nontreated sections the vacuum was removed, and freehand sections were made to determine dye distribution (22).

Induction of fungus mutants:

The stable, virulent, monoconidial strain R5-6 (25) was grown on potato sucrose agar. Spores were washed from 8 days old cultures, filtered through cheesecloth and cotton, and centrifuged. Concentration of the spore suspension was adjusted to approximately 5600 spores per ml. A shallow layer of the spore suspension in sterilized distilled water in petri dishes was irradiated for 1 or 2 minutes at a distance of 7 inches from an ultraviolet lamp. The lamp used for irradiation was a Mineralight laboratory model V 41, which has 89.8 per cent of its output at the 2540 Å. wavelength. A 3 minute exposure under these conditions killed all spores. Non-irradiated controls were maintained.

After irradiation, 1 ml of the spore suspension was diluted with 9 ml of sterilized water to give a concentration of 560 spores per ml. Then 0.1 ml of the suspension was added to 10 ml melted potato sucrose agar and plated in petri dishes for incubation at 28°C. Ten replicates were used. Growth of all colonies was somewhat retarded by irradiation.

A total of 84 individual colonies developing from the spores were chosen mostly at random (some colonies were chosen because of unusual appearance), and transferred to potato sucrose agar slants. Pathogenicity and fusaric acid producing abilities were tested as described above and below.

Bioassay of fusaric acid:

A strain of Bacillus subtilis (Cohn) Prazmowski was used as a bioassay test organism. The bacteria were grown on potato sucrose agar for 20 hours before use. Standard antibiotic assay paper discs (13 mm in diameter) were dipped in Fusarium culture filtrates and dried under a hair drier. The treated discs were then placed on agar plates freshly seeded with B. subtilis. Two drops of sterilized water was added to each dried disc. The petri dishes were kept at 4° C. for 10 to 12 hours, then were removed to room temperature for 1 hour, and finally were incubated at 28° C. The diameters of inhibition zones were measured after 24 hours at 28° C.

A similar bioassay was used to detect fusaric acid in diseased plants which had been inoculated 28 days previously. Small pieces (2-3 cm long) were cut from roots, stems, petioles, and leaf blades. The tissue sections were crushed between sterile glass plates and plated on agar seeded with B. subtilis. The plates were then held overnight at 4° C. and moved to the 28°C incubator for 24 hours. The development of inhibition zones around the crushed tissue was taken as an indication of fusaric acid production in infected plants. Healthy controls were used. This method is similar to the one used by Kalyanasundaram et al. (11).

Methods of isolation and identification of fusaric acid:

Standard methods for isolation of fusaric acid (7, 21) were followed, with slight modification. Culture filtrate (10 l) was brought to pH 4.0 with 5 N hydrochloric acid, then extracted 3 times with ethyl acetate (1.5 l per extraction). The acetate extract was evaporated to about 100 ml. The remaining solution was then mixed with a little water (about 50 ml), adjusted to pH 8.0-8.5 with 30 per cent NaOH, and extracted two times with half its volume of ether. Then the water fraction was acidified again with 5 N hydrochloric acid and thoroughly extracted with 150 ml ether in a liquid-liquid continuous extractor. The ether extract (100 ml) was dried with 20 g anhydrous Na_2SO_4, and distilled. The residue was extracted 5 times with 100 ml hot ligroin (B.P., 60-90° C) using a refluxing condensor. The ligroin extracts were combined and evaporated. About 800 mg of crystals were obtained from 10 liters of culture filtrate.

The product was identified by UV spectrum, paper chromatography and bioassay. A small sample of the unknown crystals was dissolved in two ml glass distilled water. The UV spectrum was determined with a Beckman DU spectrophotometer, with special attention to the 220-300 mμ range. Two absorption peaks, at 226 and 271 mμ, were obtained from the unknown (Fig. 1). This spectrum is characteristic of fusaric acid and closely related substances (19). Paper chromatography was used for some indication of relative purity of the preparation. The unknown substance (22.4 mg), dissolved in 1 ml ethyl alcohol, was spotted on a strip (12 x 45 cm) of Whatman No. 1 filter paper, and developed with butyl alcohol: acetic acid: water (4:1:1). After development the filter paper was dried and examined under UV light. Only one spot with R_f 0.85-0.88 was found; this is the proper R_f for fusaric acid (11, 15). The spot was eluted and bioassayed for antibiotic activity against B. subtilis, as described above. Inhibition zones characteristic of fusaric acid were obtained (28).

Preparation of mitochondria:

Pierpoint's method, as modified by Wu and Scheffer (26) was used. Forty g tomato stem tips were cut into pieces about 1 cm long and were chilled at 2° C. for one hour. The pieces were then ground vigorously for 2 minutes with a mortar and pestle, with 20 g sea sand and 80 ml extraction fluid per batch. Material was then filtered through 4 layers of cheesecloth. The filtrate was centrifuged for 10 minutes at 1000 G, and the pellet was discarded. The supernatant was recentrifuged for 20 minutes at 10,000 G and the pellets containing the mitochondria were suspended in 40 ml washing medium. After a second centrifugation at 10,000 G for 20 minutes, the final washed pellet was suspended in 2.5 ml 0.25M sucrose solution for enzymatic study.

Respiratory determinations:

The Warburg apparatus was used to determine oxygen uptake, following standard procedures (23). In each experiment, 4 flasks contained mitochondria from fusaric acid treated cuttings, 4 contained mitochondria from healthy cuttings, and one served as a thermobarometer. Each flask contained 2.0 ml total fluid (pH 7.3) including 0.4 ml mitochondrial suspension. Reaction vessels contained the following in μ moles per flask: potassium succinate 50; KH_2PO_4 140; sucrose 300; $MgSO_4$ 15; and ATP 1. The mixture was chilled before addition of mitochondrial suspension. The flasks and their contents were allowed to equilibrate for 10 minutes at 30° C., after which the stopcocks were closed. Four readings were taken at 10 minute intervals. Activity was expressed as oxygen uptake per mg protein nitrogen or as oxygen uptake per flask.

Protein nitrogen analysis of mitochrondrial samples:

Protein nitrogen in mitochondrial preparation was determined by Ma and Zuazaga's micro Kjeldahl method (12) slightly modified by Wu and Scheffer (26). One ml mitochondrial preparation was dried at 100° C and washed by centrifugation with 10 ml of 15 per cent (w/v) trichloroacetic acid (TCA), then with 10 ml 7.5 per cent TCA. Centrifugation was at 15,000 G for 15 minutes at room temperature. The pellet was dissolved in 1 ml of 1 N NaOH, to which was added 1 ml water. Samples (0.2 ml) of this solution were digested in a tube with 3 mg selenium, 5 mg potassium sulfate-copper sulfate mixture, and 1 ml concentrated sulfuric acid for 20 minutes. The reaction mixture was distilled into a flask containing 5 ml 2 per cent boric acid, 4 drops of mixed indicator was added to the boric acid solution, and the contents were titrated with 0.01 N hydrochloric acid to measure ammonium released.

EXPERIMENTAL RESULTS

Effect of fusaric acid on conducting ability of the xylem:

Vascular browning and vessel plugging are characteristic of Fusarium infected tomato plants, and inability of vessels in petioles to conduct fluid is correlated with wilting (22). Since fusaric acid is one of the toxic metabolites isolated from culture filtrates, an attempt was made to determine whether or not fusaric acid can cause the plugging and vascular browning characteristic of diseased plants.

Ten leaves from healthy plants were allowed to take up fusaric acid solution (about 10^{-2} M of partially purified fusaric acid). After 24 hours, bases of petioles were placed in eosin solution to determine ability to conduct the dye. Petioles were exposed until dye reached the uppermost leaflet of untreated leaves, which took about 5 minutes. Freehand sections were cut to determine dye distribution in treated and control leaves. The dye moved very slowly in the fusaric acid treated leaves. Such results would be obtained if vessels were blocked or if transpiration from the leaves were stopped. Fusaric acid is known to give the latter effect (8). However, this experiment does not tell us whether or not plugging occurs.

In a second experiment, two cuttings were treated with fusaric acid and two others were left as controls. A relatively pure preparation of fusaric acid was used in a 1.7×10^{-3} M solution. After 24 hours, mild damage was seen on leaves of the treated cuttings. Stem sections 10 cm long were then taken from the cuttings and dyes were drawn through the sections, as described earlier, to determine whether or not plugging had occurred. Microscopic examination of freehand sections cut from these stem sections showed that dye passed through

the fusaric acid treated stems as freely as it did through the non-treated stems. Thus the toxin damages leaf blades but does not affect conductive ability of the xylem. These results are in contrast to the situation in diseased plants, where xylem malfunction appears before leaf injury is apparent (22).

Fusaric acid production and pathogenicity of mutant strains:

Fungus strains isolated after UV exposure of the virulent parent strain (R5-6) were tested for fusaric acid production in vitro and for pathogenicity to tomato plants. Of 84 isolates, recovered after exposure to UV, 37 lost or decreased their potentials for fusaric acid production in vitro, while 13 strains produced more fusaric acid than did the parent strain (Table 1). For clear-cut results, strains which produced no fusaric acid were desired. Therefore, further tests were necessary to be sure of this point, because very low levels would not be detected by the bioassay method. Seven isolates were selected from the low producers for further tests. The strains were cultured in modified Richard's solution for 3 weeks at 28° C. A 150 ml portion of each filtrate was adjusted to pH 4.0 and extracted 5 times with ethyl acetate (40 ml per extraction). All ethyl acetate extracts for any one strain were combined, then completely dried in vacuo. Ten ml water was added to dissolve the residue. The antibiotic activity of each solution was then tested by the paper disc bioassay method. The isolates which gave no antibiotic activity when crude culture filtrates were assayed, now gave a positive assay as a result of extraction and concentration of the filtrate (Table 2). We can not be sure that fusaric acid is responsible for all the inhibition. With the present data, however, we cannot conclude that any of the isolates completely lacked the ability to form fusaric acid.

Table 1. Fusaric acid production *in vitro* by mutant strains of *F. oxysporum* f. *lycopersici*, as determined by bioassay.

Mutant Type	No. of Strains
Low producer[1]	37
Medium producer[2]	35
High producer[3]	13

[1]Low producing mutants gave less fusaric acid than did the parent strain. Fusaric acid was not detected from some of these mutants by the method used in this experiment. Four assay discs were used for each assay.

[2]Medium producers were approximately equal to the parent strain in fusaric acid production.

[3]High producers exceeded the parent strain in production of fusaric acid.

Table 2. Pathogenicity tests and *in vitro* fusaric acid production by selected isolates of *F. oxysporum* f. *lycopersici*.

Mutant Strain	Inhibition zone[1] produced by Crude Filtrate	Inhibition zone[1] produced by Ethyl Acetate Extract	Pathogenicity[2]
1	0	2	1
2	0	3	2
3	0	2	3
4	0	3	1
5	0	2	1
6	4	5	1
32	0	3	-
33	0	3	-
Control (R5-6)	2	4	3

[1]The following scale was used to rate the relative size of inhibition zone: 0 = no inhibition zone; 1 = trace of inhibition; 2, 3, 4, and 5 had, respectively, inhibition zones approximately 20, 25, 30 and 30+ mm in diameter.

[2]Pathogenicity was compared with that of the parent strain by the following scale of disease development in inoculated plants: 1 = barely detectable symptoms; 2 = mild disease symptoms; 3 = moderate symptoms equal to those of the parent (R5-6) strain; 4 = symptoms somewhat more severe than those of the parent strain; 5 = symptoms definitely more severe than those caused by the parent strain. Values are determined from 8 plants inoculated with each strain. Strains 32 and 33 were not tested.

Thirty-one of the 84 mutants were chosen for pathogenicity tests. This group included low and high producers and two morphological mutants, M-15 and M-18, which were medium producers. Because of greenhouse space limitations, the selected mutants were divided into two groups for pathogenicity tests. In each group, R5-6 was included along with non-inoculated controls. In each experiment, 8 plants were inoculated with an individual strain, and the experiment was repeated. Disease development was somewhat slow and erratic because of temperature fluctuations in the greenhouse. The night temperature sometimes fell below 21° C. During daytime, temperature was between 21° C. and 28° C. The results (Table 3) allow for the following conclusions: 1) There is no correlation between pathogenicity and fusaric acid producing ability of mutants. Some of the high producers caused almost no symptoms, while others were highly pathogenic. Likewise, some low producers were highly pathogenic while others were not. 2) Variable results were obtained with some isolates, so the genetic stability of these isolates is questionable. A possible aneuploid-condition is suggested (10). 3) Several mutant strains appeared to be more virulent than the parent strain. However, such a conclusion should be made with caution, because of the small number of plants inoculated. Again, chromosom[e] abberations may be involved (10).

Bioassay of fusaric acid in Fusarium infected plants:

Tomato plants were inoculated with several mutants varying in relative pathogenicity. After 28 days, sections of roots, stems, petioles and leaves were cut from the infected plants, crushed between sterilized glass plates, and assayed for antibiotic activity against B. subtilis. If we assume that inhibition zones are caused by fusaric acid, as others have done (11), then fusaric acid was detected in roots and lower stems (Table 4). No fusaric acid was found in petioles, or in leaves. This could

Table 3. Pathogenicity tests and fusaric acid producing ability of mutants of strain R5-6.

Mutant	Fusaric acid[1] producing ability	Pathogenicity[2] Experiment 1	Pathogenicity[2] Experiment 2
1	L	1	2
2	L	2	2
3	L	3	3
4	L	1	1
5	L	1	1
6	H	1	1
7	L	3	3
8	L	4	4
9	L	2	2
10	H	5	5
11	L	4	4
12	H	1	1
13	L	2	4
14	H	2	2
15	M	1	1
16	L	4	4
17	H	2	5
18	M	1	1
19	H	3	3
20	L	3	3
21	L	3	3
22	L	4	2
23	L	3	3
24	L	2	4
25	L	3	3
26	L	3	5
27	H	5	2
28	H	5	5
29	L	2	2
30	L	3	3
31	H	3	3
Control (R5-6)	M	3	3

[1]L = low producer; M = medium producer; H = higher producer of fusaric acid.

[2]Pathogenicity was compared with that of the parent strain by the following scale of disease development in inoculated plants: 1 = barely detectable symptoms; 2 = mild disease symptoms; 3 = moderate symptoms equal to those of the parent (R5-6) strain; 4 = symptoms somewhat more severe than those of the parent strain; 5 = symptoms definitely more severe than those caused by the parent strain. Values are determined from 8 plants inoculated with each strain.

Table 4. Detection of fusaric acid in infected plant tissues by bioassay.

Isolate	F. A. in vitro [1]	Symptoms [2]	Tissue tested [3]			
			Root	Stem	Petiole	Leaf
3	L	mild	+	+	-	-
5	L	trace	-	-	-	-
6	H	trace	+	-	-	-
12	H	trace	+	-	-	-
28	H	severe	+	+	*	*
R5-6	M	moderate	+	+	-	-
Ck	0	none	-	-	-	-

[1] L = low producer; M = medium producer; H = high producer of fusaric acid.

[2] Symptoms at the time of assay, 28 days after inoculation.

[3] + = an inhibition zone; - = no inhibition zone; * = no test because all petioles and leaves were killed. Four plant pieces were used for each determination.

mean that no fusaric acid is reaching these tissues, or that the concentration is too low to detect, or that fusaric acid is being inactivated by the tissues. In any case the results lead to doubt as to the importance of fusaric acid. B. subtilis assay is said to be capable of detecting 20 μg per ml of fusaric acid (9).

Succinoxidase activity of mitochondria from fusaric acid treated and control cuttings:

A preliminary experiment was necessary to compare activities of mitochondria from cuttings and freshly harvested plants. There was no significant difference in succinoxidase activity of mitochondria from fresh plants and from cuttings made 24 hours previously (Fig. 2).

Tomato cuttings were allowed to take up fusaric acid solution (pH 4.5) while control cuttings took up water. Mitochondrial samples were prepared from the treated and non-treated cuttings, and succinoxidase activity was determined manometrically at 30°C. Two separate experiments were run using different concentrations of fusaric acid. In the first experiment a partially purified preparation of fusaric acid was used at a concentration of 358 mg per liter (10^{-2} M, if the product was 100 per cent pure). In the second experiment a more highly purified preparation was used at 1.7×10^{-3} M. Results (Figs. 3 and 4) showed that fusaric acid treated cuttings gave a mitochondrial preparation which had lost most of its succinoxidase activity. In contrast, preparations from diseased stems have higher succinoxidase activity than preparations from healthy stems (27).

DISCUSSION AND CONCLUSION

Many metabolites are produced by Fusaria in vitro, and some of these can accumulate to high levels. Fusaric acid is one of the toxic metabolites of F. oxysporum f. lycopersici, which can accumulate in cultures. There is no evidence in the literature to show that fusaric acid is responsible for the wilting of diseased plants or is a significant factor in disease development. There are many conclusive experiments with purified fusaric acid, but these do not establish a causal role in disease. The hypothetical role of fusaric acid is based entirely on isolation of the compound from culture filtrates and detection of it in infected plants. Since substances can accumulate in dying tissue, just as they can in culture fluid, further proof of a causal role is necessary.

There are indications in the literature that fusaric acid is of little or no significance in disease development. Symptoms of fusaric acid injury (7) are not at all similar to symptoms usually developed by infected plants. These symptoms include both external and internal symptoms, and respiratory responses or "biochemical symptoms." Fusaric acid is not specific to plants which serve as hosts of the various Fusaria; it is toxic to many plant species (5). There is no correlation between fusaric acid producing ability and pathogenicity (24). These considerations are enough to indicate the need of a critical examination of fusaric acid as a causal factor in disease development.

The low relative toxicity of fusaric acid adds further doubt of a significant role in disease. A toxic treatment with fusaric acid requires 150 mg per kg fresh weight (5). Yet the highest estimated level of fusaric acid in diseased plants was 17.2 mg per kg fresh weight, and this level was found for cotton plants which were inoculated 2 to 3 weeks

before being assayed (11). Furthermore, estimates of the amount of fungus present in diseased plants (27) leads to doubt as to whether or not a toxic amount could be produced.

Several types of experiments were designed in an attempt to evaluate fusaric acid as a factor in disease development. Because wilt in Fusarium infected tomato plants is correlated with xylem dysfunction, especially in the petioles, the ability of fusaric acid to interfere with water movement in plants was tested by the use of non-toxic dyes. Fusaric acid does interfere with water movement in stems, because transpiration is decreased drastically (7). It does not lead to plugging of the xylem. This is not comparable to infected plants, where wilt is always correlated with inability of the petiole to conduct water (27).

A consideration of the respiratory response of tissue after infection was used for a further evaluation of fusaric acid in disease development. Respiration of leaves of infected plants is known to increase gradually soon after infection, remain at a relatively high level in relation to healthy tissue, and decrease after tissues begin to collapse (2, 14). In stems, respiration increases as a result of infection, and remains at a high level even after many of the leaves have collapsed (Table 5) (27). The decrease in respiration in advanced stages of disease is of no help in evaluation, because all tissue will lose activity as it dies, regardless of the cause.

In contrast to the pattern in diseased plants, uptake of fusaric acid by cuttings never leads to increased respiration, regardless of the concentration used. If concentrations are high enough respiration is inhibited (2, 14). Concentrations which do not affect respiration cause no visible symptoms. These considerations support only one conclusion: fusaric acid does not have a major role in disease development.

Studies with active mitochondria were used for still further evaluation of the role of fusaric acid. The experiments of Wu and Scheffer

Table 5. Effect of infection on respiration of stem tissue (after Wu and Scheffer, 1962).

Days after inoculation	Symptoms of inoculated plants	% Stimulation of oxygen uptake
7	none	0
9	slight	2
11	slight	51
13	slight	46
16	severe	73
17[1]	moderate	59

[1]Not same group of plants.

showed that preparations from diseased tissue contained more mitochondria, per unit green weight, than did preparations from healthy tissue. Activity per unit mitochondrial protein was higher for the preparation from diseased plants. Addition of cytochrome c to the reaction mixtures stimulated succinoxidase activity equally in both diseased and healthy preparations (27). Fusaric acid, on the other hand, inhibits succinoxidase activity when it is added to mitochondrial preparations (18). The final step in this approach was to allow tomato shoots to take up fusaric acid and then isolate the mitochondria. Results of this experiment showed that succinoxidase activity was inhibited greatly, although the amount of mitochondrial protein was not affected. The conclusion from the mitochondrial experiments is that fusaric acid has no significant role in disease.

A final attempt was made to evaluate the role of fusaric acid by comparing the fusaric acid producing ability of UV induced mutant strains with their ability to cause disease. Similar experiments have been done with the Fusarium causing cotton wilt (24), although the author was not willing to make a definite conclusion from his results. I had hoped to find a number of strains which produced no fusaric acid for comparative pathogenicity studies; unfortunately, all mutants apparently produced at least a trace of the compound. Also, some of the mutants appeared to be genetically unstable, which should be considered in evaluating the experiment. Results of the experiment showed that some strains which produce only a trace of fusaric acid have little or no virulence, while other low producers are highly pathogenic. High fusaric acid producing strains can vary likewise. Again, all the evidence indicates that fusaric acid is not important in disease development.

The fact that no fusaric acid production could be demonstrated from Fusaria causing tomato wilt in south India gives further confirmation in this regard. F. lateritium and F. scirpi are known to cause

tomato wilt which is practically identical to the wilt induced by F. oxysporum f. lycopersici (20) yet these two fungi seem to lack the ability to produce fusaric acid (7).

In diseased plants, fusaric acid was detected only in roots and occasionally in the basal parts of stems (Table 4); none was detected in petioles and leaves. Fusaric acid is known to be metabolized rapidly by host tissue (1, 9, 21) and by the fungus itself (1). It could be produced in low concentrations which were not detected by the methods used. In either case, the evidence supports the conclusion that fusaric acid has no significant role in disease development.

It is still possible that fusaric acid affects metabolic systems other than those studied. Nevertheless, the concentrations which cause visible symptoms also inhibit respiration. If other systems are affected, it will be necessary to identify the systems before fusaric acid can be given a role in disease development.

SUMMARY

Fusaric acid was evaluated as a factor in development of Fusarium wilt of tomato. The causal fungus produces the compound in culture and probably in diseased plants. Fusaric acid caused decreased transpiration but had no effect on conducting ability of the xylem of treated cuttings. These and the external symptoms of fusaric acid injury are not comparable with symptoms of infected plants. Biochemical effects of infection include increased gas exchange and increased mitochondrial activity. In contrast, fusaric acid caused a drastic decrease in succinoxidase activity by mitochondria from treated shoots. In diseased plants, fusaric acid was detected only in roots and occasionally in basal parts of stems. There were no parallels between fusaric acid producing ability and pathogenicity of a number of UV induced mutants of F. oxysporum f. lycopersici.

The evidence all indicates that fusaric acid has no significant role in disease development.

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APPENDIX

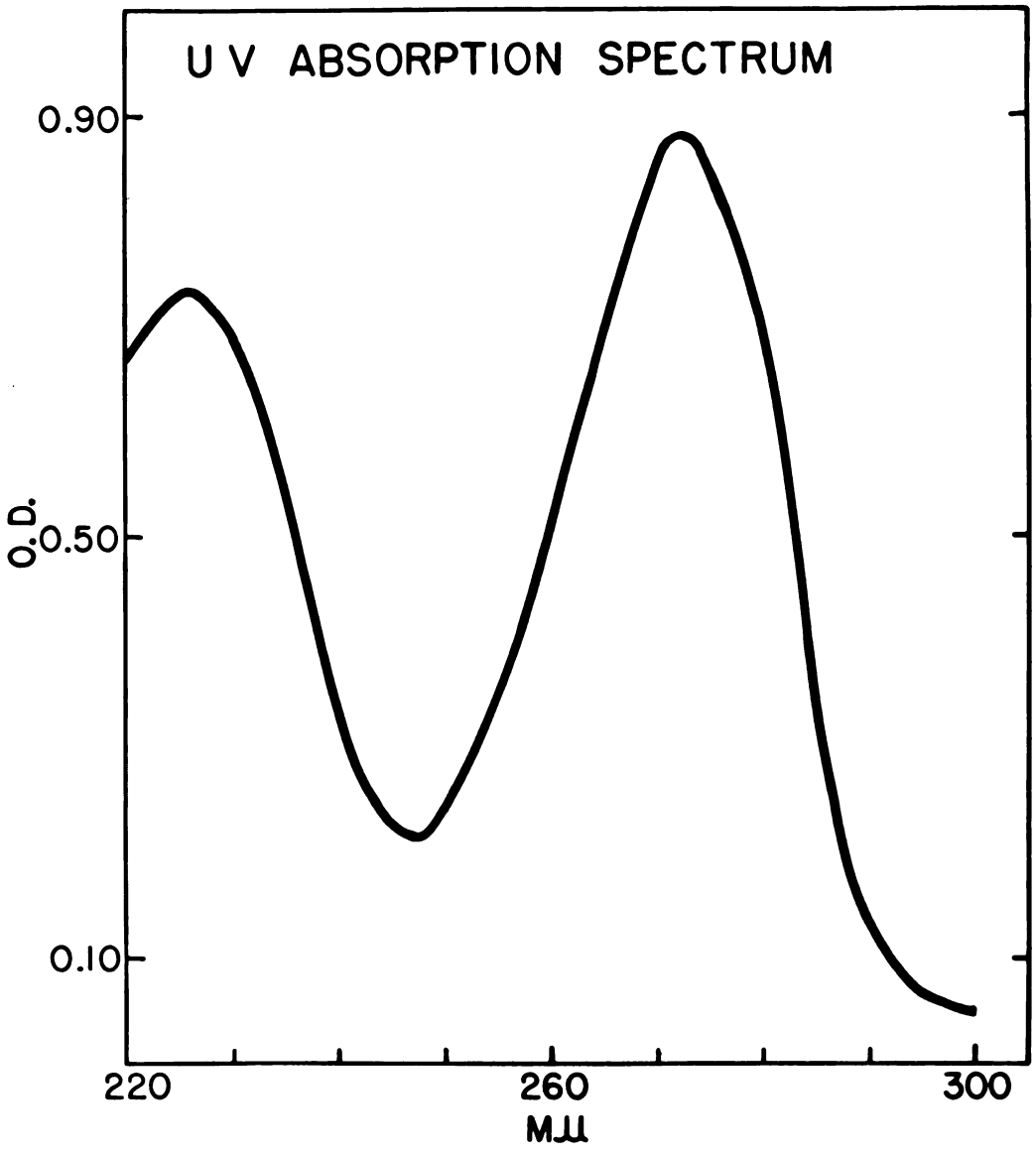

Fig. 1. UV absorption spectrum of the substance isolated from the culture filtrate of F. oxysprorum f. lycopersici and dissolved in glass distilled water.

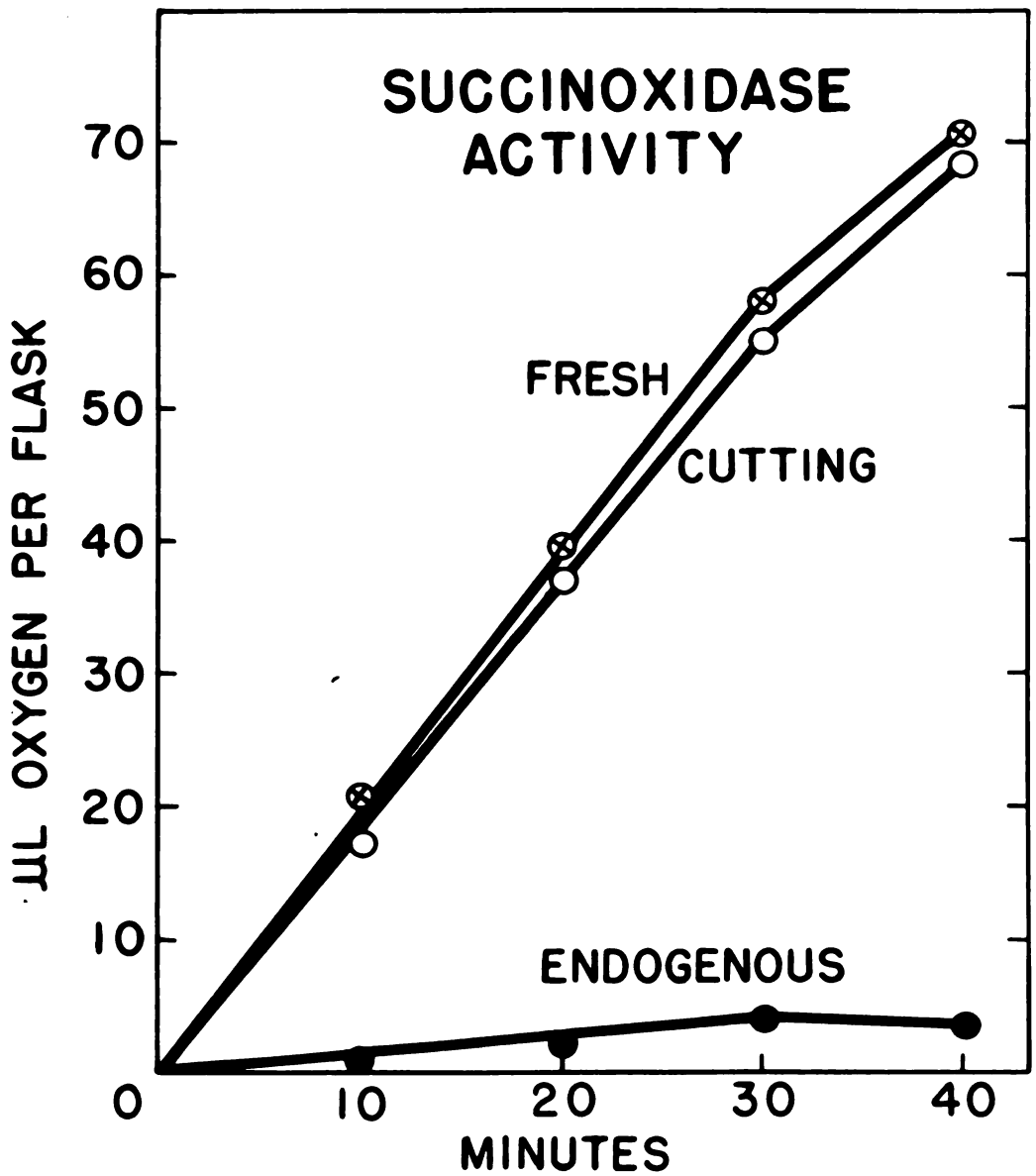

Fig. 2. Comparative activity of mitochondria from cuttings and freshly harvested plants (fresh). Cuttings were made 24 hours before mitochondria were isolated. Reaction mixtures (pH 7.3) contained the following, in μ moles per flask: K-succinate, 50; KH_2PO_4, 140; sucrose, 300; $MgSO_4$ 15; and ATP, 1.

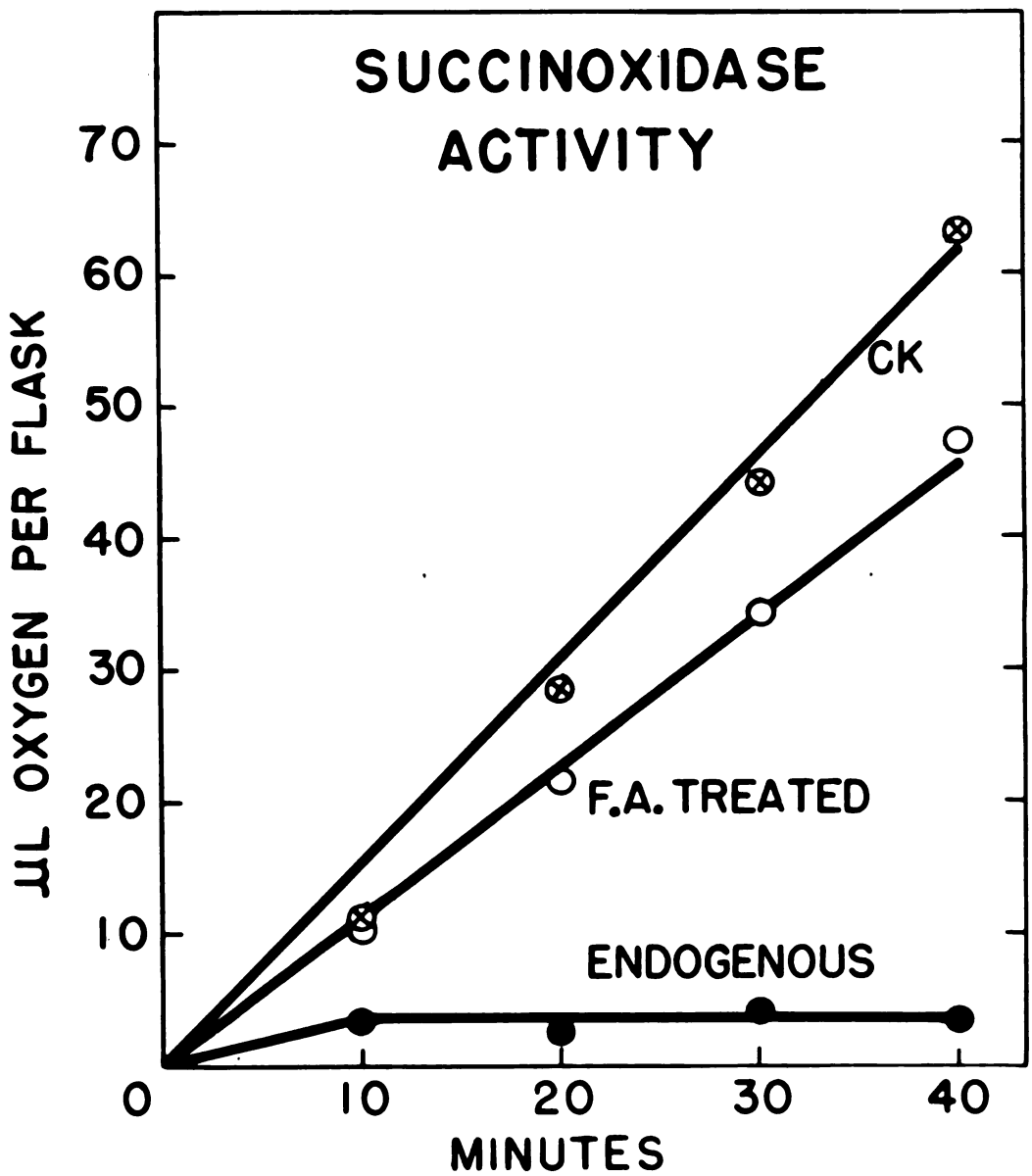

Fig. 3. Effect of partially purified fusaric acid on activity of mitochondria from tomato cuttings. Cuttings were allowed to take up fusaric acid (358 mg/l) for 24 hours before mitochondria were isolated. Controls (Ck) were exposed to water. The reaction mixtures (pH 7.3) contained the following in μ moles/flask: K-succinate, 50; KH_2PO_4, 140; sucrose, 300; $MgSO_4$, 15; and ATP, 1.0.

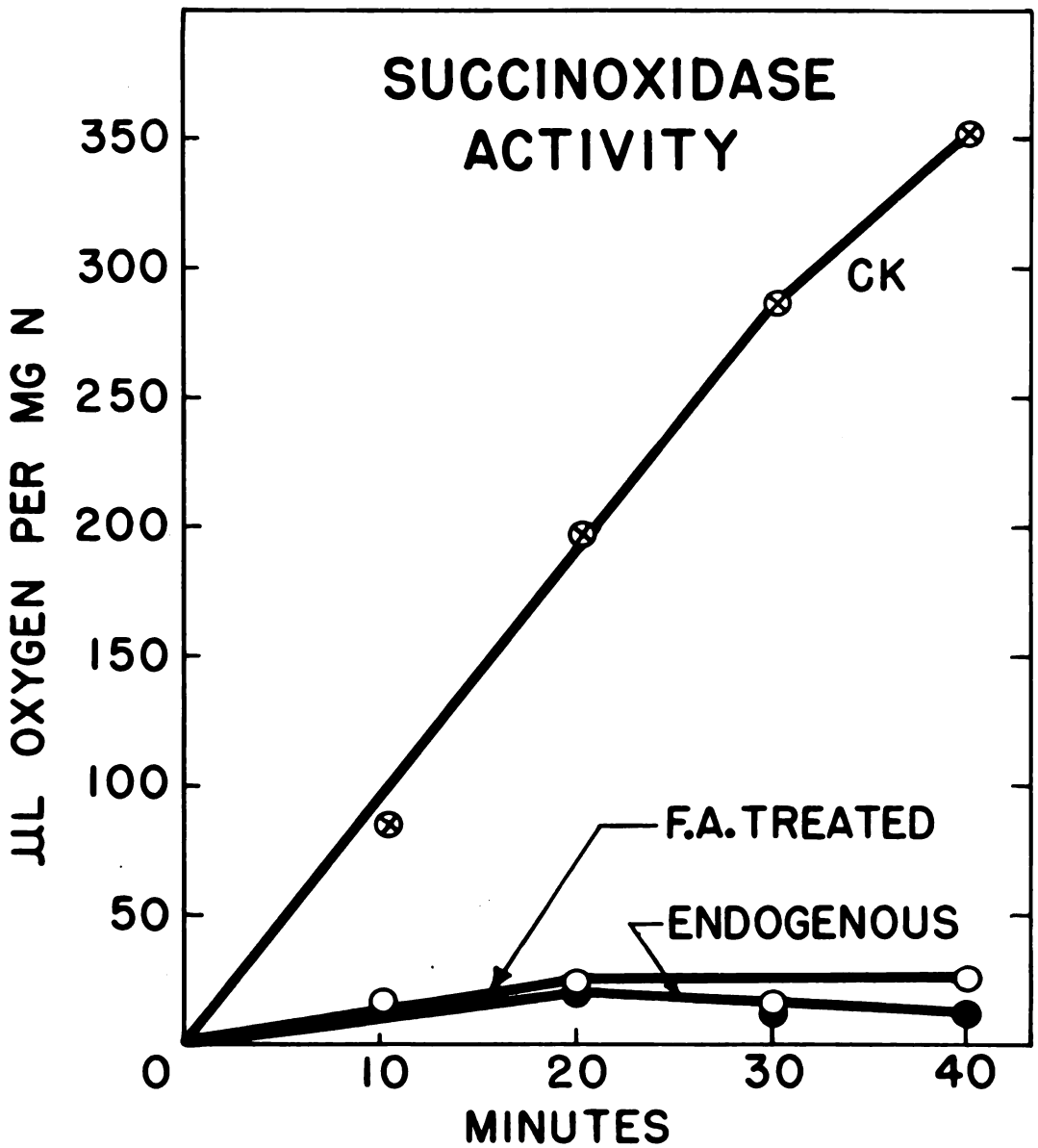

Fig. 4. Effect of highly purified fusaric acid on activity of mitochondria from tomato cuttings. Treated cuttings were allowed to take up fusaric acid (1.7×10^{-3} M) for 24 hours before mitochondria were isolated. Controls (Ck) were exposed to water. Reaction mixtures (pH 7.3) contained the following, in μ moles/flask: K-succinate, 50; KH_2PO_4, 140; sucrose, 300; $MgSO_4$, 15; and ATP, 1.0.

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