Effects of Dihydrofusarubin and Isomarticin from *Fusarium solani* on Carbohydrate Status and Metabolism of Rough Lemon Seedlings

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We wish to recognize the support of Mr. Joe Pagano, research technician, Orlando, FL, on this project.
Accepted for publication 16 February 1989.

ABSTRACT

Nemec, S., Phelps, D., and Baker, R. 1989. Effects of dihydrofusarubin and isomarticin from *Fusarium solani* on carbohydrate status and metabolism of rough lemon seedlings. Phytopathology 79:700-705.

Rough lemon citrus seedlings inoculated with *Fusarium solani* and seedlings suspended with their roots in solutions of the *F. solani*-produced naphthoquinones dihydrofusarubin (DHF, 100 mg L^{-1}) and isomarticin (50 mg L^{-1}) developed similar wilt symptoms. Only those seedlings treated with DHF developed chlorosis symptoms. Total soluble and reducing sugars and starch were reduced in inoculated plants, but only starch was consistently reduced in naphthoquinone-treated plants. Total protein did not differ between inoculated plants, naphthoquinone-treated plants, and their controls. DHF-treated plants were evaluated for chlorophylls a and b and ATPase activity, and no differences were present in the treated versus the control plants. In general, naphthoquinone-treated plants, as well as inoculated plants, accumulated more minerals in leaves than did control plants. Respiration increased in DHF-treated plants both in a greenhouse and in a growth chamber. This respiration response was studied in growth chamber experiments with the inhibitors dinitrophenol, sodium azide, salicylhydroxamic acid, and sodium fluoride. These studies suggested that respiration is stimulated via mitochondrial oxidases.

Additional keywords: *Citrus limon*, carbohydrates, phytotoxins.

Fusarium solani (Mart.) Appel & Wr. emend. Snyd. & Hans. is an opportunistic pathogen of citrus causing fibrous (23) and scaffold root rot symptoms (6,15,21) and cankers on trunks and branches (22). The most common symptom on a wide range of hosts is a cortical rot (25). Although *F. solani* is not generally considered a vascular pathogen (25), it readily invades the xylem of citrus stems from inoculated root systems (23). *F. solani* elaborates a large number of phytotoxic naphthoquinone pigments (4), and some isolates have been shown to produce trichothecenes (11) and fusaric acid (9). Limited information is available on the effects of naphthoquinones on plant metabolism. Six naphthoquinones inhibited anaerobic decarboxylation of pyruvate in vitro and the decarboxylation of α-ketoglutaric acid (17). Of these six, the marticins inhibited glutamine synthetase, diminished the semipermeability of pea leaf tissue, and disrupted chloroplast membranes of algae (17). In recent studies, both isomarticin and dihydrofusarubin (DHF) affected the semipermeability of citrus leaf tissue, reduced water uptake in intact citrus seedlings, and stimulated vessel plugging in seedlings maintained in solution cultures of the toxins (24). However, only DHF caused veinal chlorosis in citrus leaves.

This study was undertaken to examine further the effects of *F. solani* on the metabolic activities of citrus. These studies were conducted with citrus inoculated with *F. solani* or treated with DHF and isomarticin, two naphthoquinones produced by this fungus. The production of large-scale quantities of certain of these naphthoquinones (4) has made it possible to study the effects of these toxins in whole plant systems.

MATERIALS AND METHODS

Plant production and naphthoquinone preparation. Rough lemon (*Citrus limon* (L.) Burm. f.) seedlings were grown from seed to 30–40 cm tall in steam-pasteurized Astatula fine sand subsoil (hyperthermic, uncoated typic quartzipsamments) in flats in a greenhouse. Seedlings were carefully removed, and, after their roots were washed, they were transferred to 14-L cylindrical polypropylene containers containing solutions of the naphthoquinones.

The containers were fitted with covers perforated with eight 4.5-cm-diameter holes. The seedlings were supported, one per hole, with soft foam plugs, and their roots were immersed in the solutions aerated with an air pump.

The naphthoquinones, isomarticin and DHF (Rel-[3R,4aR,10aR]-5,10-dioxo-3,4,4a,5,10,10a-hexahydro-7-methoxy-3-methyl-3,6,9-trihydroxy-1-H-naphtho [2,3-c]pyran), were produced by *F. solani* in a sucrose-mineral salts liquid medium (5), and purified as previously described (5,28). Concentrations of 50 mg of isomarticin per liter and 100 mg of DHF per liter were used per container in these studies. The naphthoquinones were first solubilized in 125 ml of 95% ethanol. Each toxin solution was diluted to 12 L with deionized water in each container; the final ethanol concentration was 1% for each rate. The control was a 1% ethanol solution.

Plants were maintained under greenhouse conditions and observed daily for up to 7 days. Plant wilt ratings based on a scale of 0–5 were made the final day of each test. Zero represented no wilt and 5 indicated severe wilt.

Inoculum production and greenhouse inoculation methods. A known pathogenic isolate of *F. solani*, isolated by Nemec from the stele of root-rot diseased fibrous roots on blight-diseased trees in Florida, was selected for this study. Inoculum was produced by first growing the culture on Fries-medium slants (30) for 4 days and then transferring an aqueous suspension of conidia from the culture to a liquid Fries medium. After being shaken for 4 to 5 days, the mycelial colonies were collected by filtration through Whatman No. 1 filter paper and were resuspended in water. Seedling root systems were dipped in this suspension for about 2 min. The plants were then potted in steam-pasteurized Astatula fine sand in 15-cm clay pots and placed on a greenhouse bench. Wilt was rated 7 days after inoculation as described above. Wilt was a consequence of extensive fibrous root rot caused by the fungus common in tests of this type (23).

Carbohydrate analysis. Leaf tissue for carbohydrate analysis was collected from rough lemon seedlings incubated for 5 days in DHF, from seedlings incubated for 7 days in isomarticin, and from seedlings 7 days after inoculation with *F. solani*. In the toxin studies, leaves were composited from each replicate hydroponic tank, three tanks per treatment. Three control tanks were set up with each test. Leaves were composited from four plants per replicate from each of four replicates in the inoculations with *F. solani*. Four control replications were processed in the same way.

Leaf tissue was dried at 90 C for 90 min, then at 70 C for 22 hr. The tissue was ground in a Wiley mill to a 40-mesh particle size. One-half gram samples were extracted with 76% ethanol for 6 hr in a Soxhlet apparatus. The ethanol extract was evaporated to near dryness in a rotary evaporator, diluted with water, and then filtered through glass wool on a Whatman No. 1 filter paper disk. The filtrate was mixed with a 1:1 mixture of Dowex-50 and Dowex-1 cation and anion resins (one volume of resin mixture per one volume of filtrate), shaken for 1 hr, and then filtered through Whatman No. 1 filter paper. The filtrate was analyzed for total soluble sugars by the anthrone method (16) and reducing sugars by the Somogyi-Nelson method (16).

The residue from the extraction in the Soxhlet was analyzed for starch by the method of Carter et al (8). The residue was oven-dried to remove ethanol and then brought to a simmer in 10 ml of distilled water, cooled, and amended with 10 ml of 0.5% amyloglucosidase in 0.1 M citrate buffer, pH 4.5. This mixture was incubated at 55 C for 2 hr with shaking and then filtered through Whatman No. 1 filter paper. The resulting glucose was determined by the glucose oxidase procedure (Sigma Tech. Bull. 635, Sigma Chemical Co.)

Protein analysis. Soluble proteins were determined in samples of the same leaf tissue analyzed for carbohydrates. One-half gram of dry leaf tissue was ground in a mortar with liquid N_2 to a fine powder. An extraction buffer, 50 mM Tris-HCl, pH 8.4, containing 1 mM EDTA, 30 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 2% polyvinylpyrrolidone (PVP), was added to the powder and the mixture reground in the mortar. The mixture was centrifuged at 3,620 *g* for 30 min. The pellet was resuspended in 3 ml of 0.1 N NaOH and incubated at 60 C for 1 hr. The mixture was recentrifuged at 2,000 rpm for 15 min in a tabletop centrifuge and the supernatant analyzed for soluble proteins by the Lowry procedure (19).

Chlorophyll assay. Chlorophyll was extracted from leaves of rough lemon rootstock in a separate test in which the seedlings were incubated in 100 mg of DHF per liter. Three hydroponic containers were prepared with solutions of DHF, and three were controls. Seven days after the test was established, five to seven leaves of each plant in each container were cut, pooled, and weighed. Chlorophyll was isolated from 5 g of leaf tissue from each container replicate, according to a modification of the procedure by Goldberg and Brakke (13). The leaves were cut into small pieces with a pair of scissors and ground in a mortar with a pestle in 80% acetone three times. Between each grinding, the acetone extract was filtered through Whatman No. 50 filter paper. The residue in the mortar after the third grinding was no longer green. All combined filtrates were diluted to 200 ml with 80% acetone. Aliquots of each sample were diluted 10-fold with 80% acetone and read at both 663 nm (chlorophyll a) and 645 nm (chlorophyll b) versus 80% acetone. The concentrations of chlorophyll a and b were converted to milligrams of chlorophyll per gram of leaf tissue (2).

Chloroplast isolation and ATPase determination. Leaves (10 g) from the same hydroponic containers used for chlorophyll analyses were cut into small pieces with a pair of scissors and placed in a nylon bag. Ten milliliters of isolation medium (0.25 M sucrose, 10 mM 3-(*N*-morpholino)propane-sulfonic acid, in 5% PVP, pH 7.2) was added to the bag in a mortar, and the contents of the bag were ground with a pestle for a minute. The extract was decanted and centrifuged at 3,000 *g* for 10 min. The resultant green pellet was suspended in isolation medium and stored at −70 C until used.

ATPase activity was determined in the chloroplast fractions from the above procedure. Ca-ATP was used as the ATPase substrate. The Ca-ATPase activity was measured according to the procedure of Lien and Racker (18), using the phosphate assay of Taussky and Shor (29).

Leaf mineral analysis. Leaf tissue from plants treated with isomarticin (50 mg L^{-1}) and DHF (100 mg L^{-1}) was collected 7 days after exposure of plants to the toxins. Leaves were analyzed for N, P, K, Ca, Mg, S, and B. Nitrogen was determined by digesting leaves in H_2SO_4 and measuring released ammonia N colorimetrically with basic phenol and NaOCl solution (7). Phosphorus, K, Ca, Mg, S, and B were extracted from leaf tissue digested by nitric-perchloric acid (wet-ashed). Phosphorus was determined colorimetrically with ammonium molybdate-ascorbic acid solutions. Sulfur was determined turbidimetrically with barium chloride, and B was determined colorimetrically with curcumin solution. The other elements were determined by atomic absorption (3). All analyses were done by Agroservices International of Orange City, FL.

Metabolism. Oxygen uptake was determined first in leaves from DHF-treated and control plants maintained for 192 hr in a growth chamber (34 C day, 12 hr; 30 C night, 12 hr; 300 μE m^{-2} sec^{-1}) and in plants maintained in a greenhouse (32–36.3 C, day; 20 C minimum, night; 450 μE m^{-2} sec^{-1}). In this test, oxygen uptake was determined at 48-hr intervals after seedlings were incubated in 100 mg of DHF and control solutions per liter in the growth chamber and greenhouse. Forty 5-mm-diameter leaf disks per treatment replicate (five disks per plant from eight plants) were

placed in a Gilson Differential Respirometer flask and dark respiration determined. The flasks were suspended in a 30 C water bath with the shaker set at 100 rpm. The center well of the flask contained 0.2 ml of 10% KOH and a filter paper disk. Oxygen uptake was calculated as mean microliters per milligram of dry leaf weight per hour from three replicates per treatment.

The effects of dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, sodium azide, salicylhydroxamic acid (SHAM), and NaF were evaluated in other tests on oxygen uptake, using leaf disks from the control and from treatments with DHF at 100 mg L^{-1}. Forty leaf disks per treatment were infiltrated with 5 mM DNP, 10 mM sodium azide, 10 mM SHAM, or 20 mM NaF by laying the disks stomate side down on filter paper impregnated with the compound solubilized in 67 mM potassium phosphate buffer, pH 6.7. The leaf disks were exposed for 3 hr at 27 C in closed petri plates sealed with Parafilm. Plants in this study were maintained in a growth chamber set to the same environmental parameters as described in the first metabolism test. Data were reported as mean microliters of O_2 per milligram of dry leaf weight from two replicates per treatment. Oxygen uptake data were collected at 15-min intervals for 60 min. Oxygen uptake slopes generated by each treatment were analyzed by linear regression and beta values of each slope separated by Duncan's multiple range test at $P \leq 0.05$.

RESULTS

Plant wilt occurred in seedlings treated with both naphthoquinones, with optimum expression occurring in 7 days. Plant wilt was scored as 2.8 and 3.0 for plants treated with isomarticin and DHF, respectively. No wilt developed in control plants. Only the isomarticin treatment resulted in a decrease in total soluble sugars compared to that of the controls (Table 1). Starch also decreased in plants treated with both toxins. In the isomarticin treatment, reducing sugars were 31.1 mg g^{-1} and total protein 291 mg g^{-1} compared with control values of 33.3 and 295 mg g^{-1}, respectively; these comparisons were not significantly different. In the DHF treatment, reducing sugars were 28.1 mg g^{-1} and total protein 268 mg g^{-1} relative to control values of 21.4 and 250 mg g^{-1}, respectively, comparisons that were also not significantly different.

Although DHF caused a wilt rating of 4.3 compared to no wilt in treated plants, changes in chlorophyll a or b between treatments were too small to be significant. Chlorophyll a in the DHF treatment was 5.1 mg g^{-1} versus 5.7 in the control, and chlorophyll b was 2.2 mg g^{-1} in the DHF treatment versus 2.3 in the control. The a/b ratio, however, was significantly lower in treated plants (Table 1). ATPase in the DHF treatment (6.8 μm hr^{-1} mg^{-1} of chlorophyll) was not significantly different from activity in the control (4.7 μm hr^{-1} mg^{-1} of chlorophyll).

Inoculated plants contained significantly less total soluble and reducing sugars and less starch compared to controls, but total protein did not differ between the treatments (Table 2).

In general, naphthoquinone-treated plants, as well as inoculated plants, accumulated more minerals in leaves than did the controls. All minerals except S accumulated in leaves of plants treated with DHF, while N, Ca, and Mg accumulated in leaves of isomarticin-treated plants (Table 3).

Respiration rates of DHF-treated plants in both the greenhouse and the growth chamber increased significantly during the first 48 hr compared to those of controls, and they subsided more rapidly with time in DHF-treated plants maintained in the growth chamber than in those maintained in the greenhouse (Fig. 1). Respiration increased in greenhouse control plants after 96 hr, and by the end of the test respiration in this treatment did not

TABLE 1. Total soluble sugar, starch, and chlorophyll a/b ratio in leaves of rough lemon rootstock seedlings incubated in solutions of isomarticin and dihydrofusarubin[a]

Treatment	Total soluble sugar[b,c] (mg/g)	Starch[b,c] (mg/g)	Chorophyll[b] a/b
Isomarticin (50 mg L^{-1})	54.3*	27.8***	–
Control	86.7	285.8	–
Dihydrofusarubin, (100 mg L^{-1})	60.5[ns]	67.5***	2.3
Control	53.4	742.2	2.5*

[a]All data are means from three hydroponic tanks per treatment, each containing eight seedlings. Sugar and starch values are reported on a dry weight basis. Treatment means compared with control, by Student's t test: * = $P \leq 0.05$; *** = $P \leq 0.001$; ns = not significant, – indicates no test was made.
[b]Dihydrofusarubin: leaf samples taken 5 days after exposure to toxin for sugar and starch analysis and 7 days after exposure to toxin for chlorophyll analysis.
[c]Isomarticin: leaf samples taken 7 days after exposure to toxin.

TABLE 2. Plant wilt, total soluble and reducing sugars, starch, and total protein in leaves of rough lemon seedlings inoculated with *Fusarium solani*[a]

Treatment	Plant wilt[b,c] (0–5)	Total soluble sugar[c] (mg/g)	Total reducing sugar[c] (mg/g)	Starch (mg/g)	Total protein (mg/g)
Inoculated	3.0	51.3*	22.1**	70.9***	285[ns]
Control	0.0	84.5	35.7	307.1	297

[a]All sugar and protein values are reported on a dry weight basis. Treatment means compared with control, by Student's t test: * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; ns = not significant.
[b]Wilt rated on a scale of 0–5: 0 = no wilt, 5 = severe wilt and leaf desiccation. Leaf wilt and leaf samples taken 7 days after inoculation.
[c]All data are means of four samples per treatment; four plants were pooled to comprise each sample.

TABLE 3. Mineral analyses of leaves from rough lemon rootstock seedlings incubated in solutions of isomarticin and dihydrofusarubin and of leaves of seedlings inoculated with *Fusarium solani*

Treatment	Percent composition, dry wt[a] N	P	K	Ca	Mg	S	B[a] (ppm)
Isomarticin[b] (50 mg L^{-1})	3.27	0.25	1.31	2.61	0.26	0.20	58
Control	3.04*	0.22[ns]	1.28[ns]	2.21*	0.22*	0.18[ns]	52[ns]
Dihydrofusarubin (100 mg L^{-1})	4.06	0.32	1.66	2.36	0.24	0.22	70
Control	3.27**	0.24**	1.23***	2.06*	0.18*	0.18[ns]	53*
Inoculated	3.84	0.22	1.31	2.48	0.21	0.20	74
Control	3.21[ns]	0.15[ns]	1.12[ns]	2.04***	0.20[ns]	0.13*	70[ns]

[a]All toxin data are means from three hydroponic tanks per treatment, each containing eight seedlings. Data from inoculation test are means of four samples per treatment; four pooled plants comprised each sample. Treatment means compared with control, by Student's t test: * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; ns = not significant.
[b]Isomarticin: leaf samples taken 7 days after exposure to toxin; dihydrofusarubin: leaf samples taken 5 days after treatment.

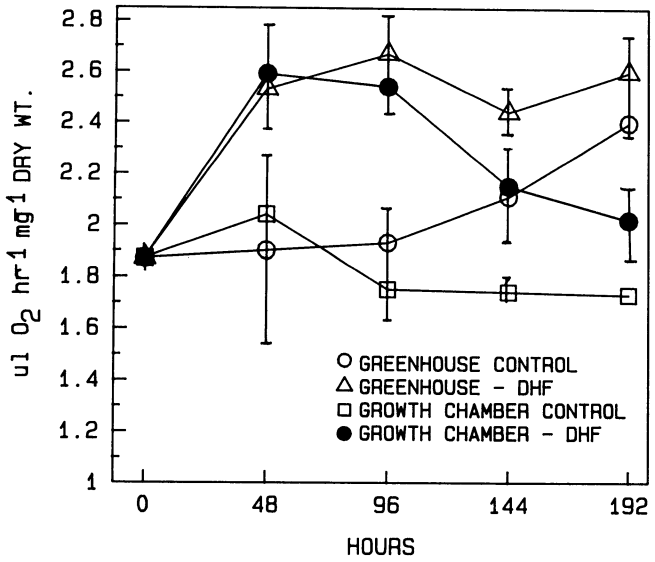

Fig. 1. Respiration of rough lemon seedlings treated with 100 mg of dihydrofusarubin per liter and of controls in a greenhouse and a growth chamber for 192 hr.

differ significantly from respiration in the DHF-treated greenhouse-maintained plants (Fig. 1). Lowering daytime growth-chamber air temperature from 34 to 23 C reduced symptom development in toxin-treated plants (data not shown).

DNP significantly enhanced respiration in control plants and had a slight but significant stimulatory effect on respiration of DHF-treated seedlings (Fig. 2A). Beta values (data not shown) of all slopes in Figure 2A were significantly different from one another. Beta values of all slopes in Figure 2B were also significantly different from one another, indicating that sodium azide (cytochrome c oxidase inhibitor) inhibited respiration in both control and DHF-treated tissue. Beta values for slopes generated by the control and DHF-treated tissue were not significantly different in Figure 2C, but sodium fluoride significantly inhibited respiration independent of treatment. Beta values for slopes generated by the control and DHF-treated tissue were not significantly different in Figure 2D, but sodium azide in combination with SHAM (alternate pathway inhibitor) significantly inhibited respiration in all tissue and also repressed any DHF-related stimulation of respiration.

DISCUSSION

The production of large-scale quantities of certain naphtho-

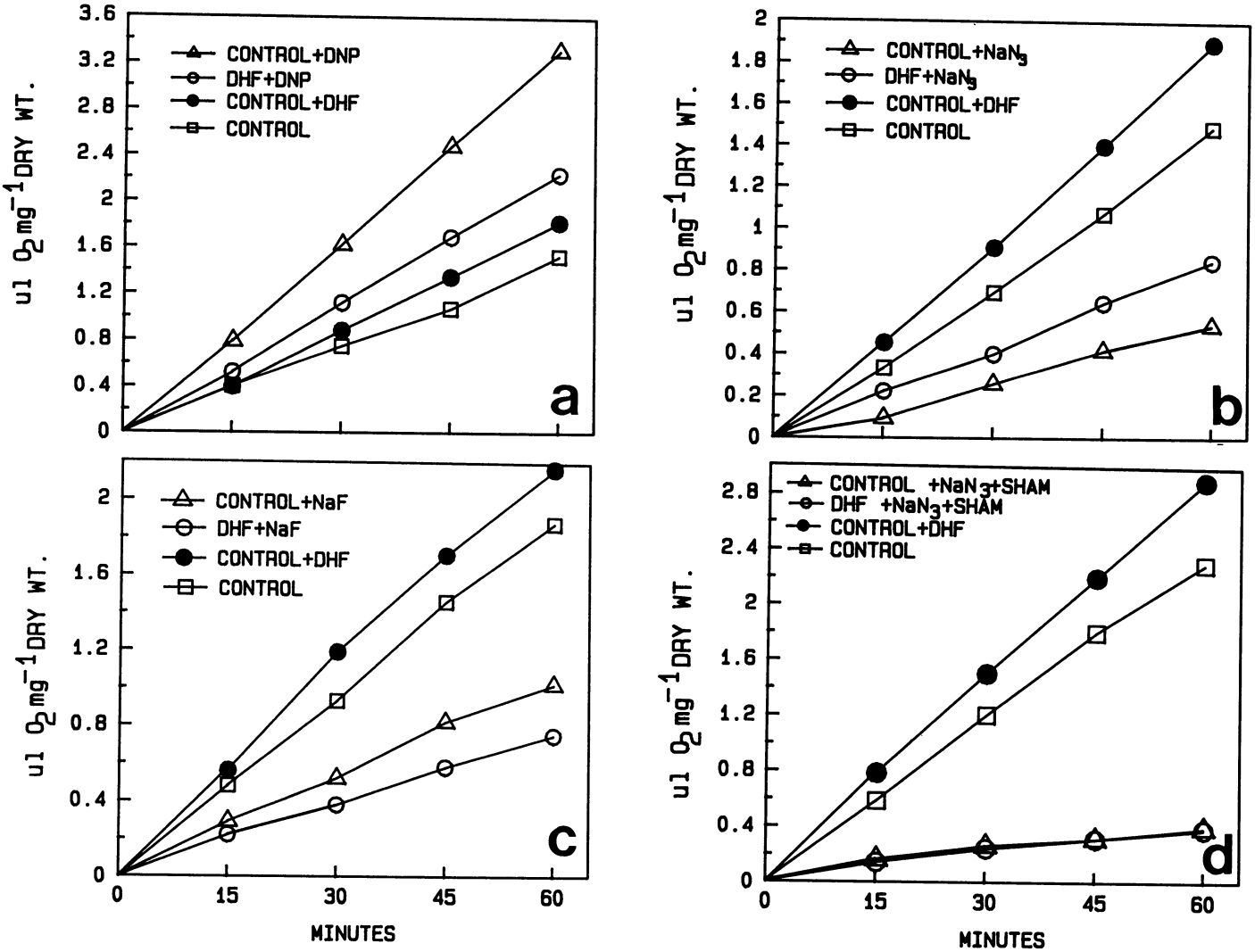

Fig. 2. Effects of inhibitors on respiration of rough lemon seedling leaf disks from plants maintained in solutions of 100 mg of dihydrofusarubin (DHF) per liter and in control solutions. Oxygen uptake values were recorded at 15-min intervals for 60 min. **A,** Effect of dinitrophenol (5 mM) on respiration of rough lemon leaf tissue from plants treated with DHF and from control plants. **B,** Respiration of leaf disks from DHF-treated plants and control plants, both treated with sodium azide (10 mM). **C,** Respiration of leaf disks from DHF-treated plants and control plants, both inoculated with NaF (20 mM). **D,** Respiration of leaf disks from DHF-treated plants and control plants, both incubated with 10 mM sodium azide and 10 mM salicylhydroxamic acid (SHAM).

quinones has made it possible to evaluate effects of these toxins on whole plant systems (4). The first expression by the plant of toxin uptake in these studies was a significant increase in respiration (Fig. 1A). This coincides with the first irreversible wilt symptoms that appear 24–48 hr after toxin treatment (24). Increases in respiration usually are characteristic of most diseased plant tissues (31) and are nonspecific reactions in plants (14). Increased respiration caused by DHF partially depleted starch reserves, which were also depleted in plants in the isomarticin and inoculation treatments.

Total protein was unaffected in DHF- and isomarticin-treated seedlings as well as in inoculated plants. The general early increase in protein levels that occurs in fungus-infected plants (14) did not develop in this study, and plant health had not deteriorated enough for the marked decreases in protein apparent in late stages of disease development caused by fungus pathogens (14). Both DHF and isomarticin caused small but significant increases in leaf N, an effect seen in fungus-infected plants (14); in this study such increases appeared unrelated to protein synthesis.

These toxins are known to exert an effect on chloroplasts. Isomarticin disrupted chloroplast structure in *Spyrogyra* (17) and in citrus mesophyll cells (D. Achor, *personal communication*). Veinal chlorosis, a symptom caused by DHF (24), occurred on plants treated in DHF in this study but was not related to a general chloroplast degradation in this tissue (D. Achor, *personal communication*). Furthermore, no changes in chlorophylls a and b were detected between treatments in our study. However, in another study, reduction in photosynthesis without a change in leaf chlorophyll content did occur in cotton affected by Verticillium wilt (20). DHF effected no change in ATPase. Other possible changes in chloroplast functions were not investigated.

Isomarticin is known to interfere with respiration by inhibiting the anaerobic decarboxylation of pyruvate in vitro and the oxidative decarboxylation of α-ketoglutarate (17). This toxin was the most toxic of the naphthoquinones tested on peas (10,17). At a lower dosage than that of DHF, isomarticin was equally effective in altering the physiological parameters reported here, as well as water relations of citrus in another study (24). Isomarticin may have been the preferred toxin for evaluating inhibition of respiration in citrus, but unfortunately our fungal cultures did not produce enough of the compound for this type of test.

Experiments with DHF in combination with sodium azide, sodium azide and SHAM, and NaF were conducted, in which DHF was expected to have a maximal effect on respiration (Fig. 1). The studies suggest that the cause of the stimulation of respiration is to be found in the pathways using the mitochondrial terminal oxidases (Fig. 2A and D) and not in the pentose phosphate pathway (Fig. 2C). Sites in the affected pathways at which the stimulation occurs have yet to be determined. Inhibition of the mitochondrial cytochrome c oxidase alone did not significantly affect the DHF-related stimulation of respiration (Fig. 2B).

The one effect of DHF on respiration that is obvious is the ability of DHF both to stimulate respiration and at the same time to retard or block the stimulation of respiration by DNP (Fig. 2A). Such an effect has been reported in studies on organotin compounds in mitochondria (1). The proposed site of interaction of organotin with the mitochondrial oxidative phosphorylation system is the membrane-bound ATP synthetase complex (12). Also, other quinolic compounds are known to interact with the mitochondrial electron transport at various sites (26,27). Therefore, it is likely that DHF does affect oxidative phosphorylation in mitochondria.

It is not known to what extent these toxins are produced by *F. solani* in naturally infected roots. The fungus is systemic in xylem of fibrous roots and wood of scaffold roots on field trees and thus may synthesize these toxins in this tissue. Conventional extraction methods have not detected naphthoquinones in naturally infected citrus tissue; however, DHF can be extracted (principally as fusarubin) from stems of toxin-treated plants and reisolated on thin-layer chromatography plates (24). If toxins of *F. solani* are active in pathogenesis in citrus, the naphthoquinones may be the responsible agents. A continuation of metabolic studies with inoculated plants may be a means to clarify their involvement in pathogenesis. Other toxins reported from *F. solani*, such as fusaric acid (S. Nemec, *unpublished data*) and trichothecenes (Osamu Kawamura, *personal communication*) have not been detected in cultures of *F. solani* from citrus tissue.

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