Immunoassay for Naphthazarin Phytotoxins Produced by *Fusarium solani*

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ABSTRACT

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An enzyme-linked immunosorbent assay (ELISA) for isomarticin, a naphthazarin toxin produced by *Fusarium solani*, was developed. A carbodiimide procedure was used to couple the hapten isomarticin to bovine serum albumin for the immunogen and to alkaline phosphatase for the enzyme-linked tracer. The resulting assay had a detection limit of 2 ng/ml for isomarticin; other naphthazarin toxins were detectable at less than 10 ng per well in ELISA plates. The assay was specific for naphthazarins. The cross-reactivity for a number of phenolic compounds, including the closely related naphthoquinones, was three orders of magnitude less sensitive.

Additional keywords: citrus, dihydrofusarubin, root rot.

Fusarium solani (Mart.) Appel & Wr. emend. Snyd. & Hans. is a common soil fungus which causes root rot in peas, beans, lentils, and lettuce (5,6,8,9,11). It has been associated with fibrous and scaffold root rot symptoms as well as cankers in citrus (15,17,18). Some of the naphthazarin toxins produced by *F. solani* can cause veinal chlorosis, leaf wilt, and vessel plugging in rough lemon seedlings (15) and are toxic to tomatoes, rice, and radishes (1,6,9–11).

There are correlations between virulence and disease symptoms

and specific toxin production by the fungus (3,8,10). However, this relationship is not universal (13,15). The fact that *F. solani* can produce at least 11 structurally related toxins (24) may account in part for this discrepancy. All share a core structure of either 2-methoxynaphthazarin or, in the case of dihydrofusarubins, reduced 2-methoxynaphthazarin (Fig. 1). The naphthazarin toxins that can affect higher plants include isomarticin and marticin (9,10,13), fusarubin and javanicin (1,9), 8-0-methyl-javanicin (11), and dihydrofusarubin (24). Other naphthazarin toxins, such as norjavanicin and novarubin, are more toxic to fungi and bacteria (9,10). The most efficacious toxins to plants are marticin and isomarticin (9,10), which are also the most water soluble. Marticin, isomarticin, and fusarubin could be extracted from infected pea roots (9,10) and stems of rough lemon seedlings incubated in the toxins (16).

Production of specific toxins by a given strain of *F. solani* is dependent on the culture conditions (9,24). Hence, whether the fungus can produce the specific toxins after it invades the plant (1,18) may depend on the condition of the plant (12,14,15,20). It has been suggested that early infection may not involve toxin production (19). However, initiation of disease symptoms by a strain of *F. solani* once infection is established may be a result of toxin production within the infected plant (13,16). Hence, further studies of the relationship of fungal infection, toxin production, and symptomatology require a reliable assay of minute quantities of toxin within plant tissue.

Immunoassay techniques are capable of determining nanogram quantities of specific compounds in complex mixtures of plant extracts. Although the naphthazarin toxins themselves are too small to be antigenic, coupling them to a protein resulted in a functional immunogen (25). Two of the toxins, marticin and isomarticin, contain a free carboxyl group. Coupling via this free carboxyl group, which is attached to an extra ring (compare marticin and isomarticin, Fig. 1), should leave the 2-methoxynaphthazarin core exposed on the hapten-protein conjugate and result in an assay specific for this structure. In this study, the development of an immunoassay using polyclonal antibodies specific for the naphthazarin structure of the toxins produced by *F. solani* is reported.

MATERIALS AND METHODS

Reagents. Alkaline phosphatase (Type VII-S), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), *p*-nitrophenyl phosphate, and complete Freund's adjuvant were purchased from Sigma Chemical Co., St. Louis, MO. Marticin, isomarticin, dihydrofusarubin, and fusarubin were prepared according to Baker et al (1,2) and Tatum and Baker (24). Other reagents were of American Chemical Society grade.

Equipment. The enzyme-linked immunosorbent assays (ELISAs) were performed in 96-well, flat-bottomed polystyrene plates (Dynatech Immulon 2, Dynatech Laboratories, Inc.,

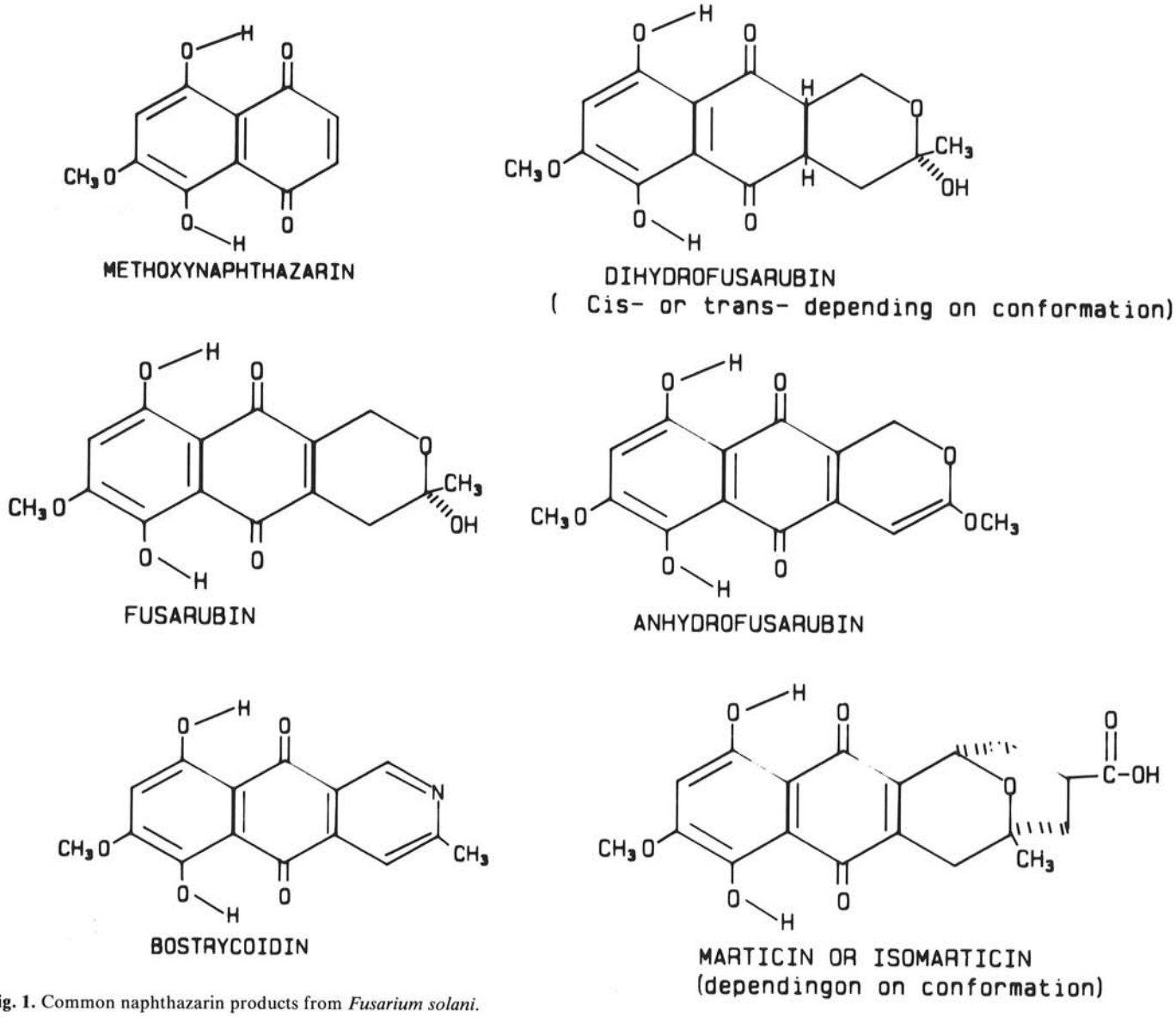

Fig. 1. Common naphthazarin products from *Fusarium solani*.

Chantilly, VA) and read with an Automated Microplate Reader Model EL309 (Bio-Tek Instruments, Inc., Winooski, VT). Silica gel GF thin-layer chromatography (TLC) plates (Analtech, Newark, DE) were used to monitor the synthesis of the immunogen and tracer.

Coupling of isomarticin to bovine serum albumin. Isomarticin was coupled to bovine serum albumin (BSA) by means of EDC activation of the toxin's free carboxyl group. Isomarticin was dissolved in 50 mM Tris-HCl (1 mg/ml) and titrated to pH 6 with NaOH. BSA was added (4.4 mg/ml), followed by EDC (1.2 mg/ml). The reaction proceeded overnight at room temperature and then was stopped by the addition of 0.5 ml of 0.5 M ammonium acetate per milliliter of reaction mixture. The mixture was dialyzed in the cold (4 C) versus several changes of water and then lyophilized. During all steps in this procedure, the reaction mixture was protected from exposure to light to prevent photodegradation of the toxin. The coupling ratio was determined by measuring the absorption of a solution of conjugate (1.0 mg/ml) in 50 mM Tris-HCl, pH 8, at 575 nm ($\epsilon = 2.7 \times 10^4\ M^{-1}cm^{-1}$). The ratio was found to be 1.2 moles isomarticin/mole BSA. If the coupling was not stopped by the addition of ammonium acetate, a nearly insoluble precipitate containing both the BSA and isomarticin formed during the dialysis step.

Immunization procedure. Female and male New Zealand white rabbits were used for immunization. Conjugates, 0.4 mg in phosphate-buffered saline (PBS) emulsified in complete Freund's adjuvant, were administered intradermally every week for the first 4 wk and every 4 wk thereafter until the rabbit was sacrificed. Bleedings were made at various intervals after the last injection. The immunoglobin G (IgG) fraction was isolated from the serum in the cold by precipitation with half-saturated ammonium sulfate (pH 7.0) at 4 C. The fraction was precipitated further with 40% saturated ammonium sulfate and dialyzed against PBS. The antibodies were stored lyophilized at −10 C.

Preparation of tracer. The carboxyl group of isomarticin was coupled to free amino groups of alkaline phosphatase with carbodiimide as the carboxyl-activating agent. The solution for this procedure was prepared with 9 mg of isomarticin (acid form) dissolved in 0.3 ml of a solution containing 0.15 M NaCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7. This mixture was titrated with NaOH to pH 6.0 for complete solubilization. Added to this mixture were 9 mg of EDC and 5,000 units of alkaline phosphatase in 0.7 M Tris-HCl that previously had been dialyzed overnight in the Tris-HCl buffer. The reaction proceeded overnight at room temperature in the dark and then was stopped by the addition of 1 ml of 0.5 M ammonium acetate. Unbound isomarticin products were removed by dialysis against a solution containing 0.15 M NaCl, 10 mM Mes [2-(*N*-morpholino) ethanesulfonic acid] Na, pH 6.0, and 1 mM $MgCl_2$ for 3 days at 4 C. Any alkaline phosphatase that precipitated during the conjugation step was removed by centrifugation at 600 *g* for 15 min. The volume of the tracer stock solution was adjusted to give a final alkaline phosphatase concentration of approximately 1,000 units/ml. Proof that conjugation had occurred was determined by eluting samples of conjugate through Sephadex G-25 medium-grade gel columns with water and by developing the conjugate fraction on Silica gel GF TLC plates with benzene:ethyl acetate:1-butanol (16:3:1).

Coating plate wells with antibodies. Anti-isomarticin IgG and the IgG of a preimmunized rabbit were dissolved separately in 50 mM sodium bicarbonate buffer, pH 9.5, to concentrations of 5 μg/ml unless otherwise indicated. Wells in a microtiter plate were filled with 200 μl of one or the other of these solutions or left empty. The wells receiving the nonimmunized rabbit IgG and four that were untreated served as controls. The plates were wrapped in plastic and incubated overnight at 4 C. The antibody solutions were decanted, and the wells were washed three times with a buffer containing 0.15 M NaCl, 25 mM Tris-HCl, pH 8.5, and 0.02% NaN_3 (TBS). Any remaining sites were blocked by incubation at room temperature for 0.5 hr with 200 μl of 0.05% BSA in TBS. This solution was decanted. The wells were washed once with TBS and this also was decanted. The plates were used immediately with various dilutions of the tracer.

Preparation of toxin standards and plant material. The immunological specificity of the assay was determined by its ability to detect related compounds and components of citrus tissue. Eight naphthazarins, various phenolic acids, quinonic compounds, psoralen, coumarin, limonin, and flavonoids that can be found in citrus (23) were included in the tests. Most phenolics were dissolved in water with the initial aid of a small amount of concentrated NaOH in methanol. The naphthazarin toxins were dissolved in either water, methanol, or chloroform, titrated as needed, and diluted at least 10-fold with TBS. Sap from leaves of rough lemon (*Citrus jambhiri* Lush.) was expressed in a press and clarified by centrifugation at 600 *g* for 30 min.

Enzyme-linked immunoassay procedure. Tracer was diluted to a 1:1,000 final volume with TBS containing 0.05% BSA. Isomarticin standards, the plant chemicals listed in Table 1, or expressed leaf were added to this incubation buffer containing the tracer. Concentrations of isomarticin standards were prepared before the isomarticin was mixed with the tracer solution; final concentrations reported were based on a mixing of equal ratios of the toxin and tracer solution. Aliquots of 100 μl were transferred to coated wells in a microtiter plate. After a 0.5-hr incubation at room temperature, the solutions were decanted, and the wells were washed three times with TBS. Each well then received 200 μl of a solution of 0.1 M diethanolamine, pH 9.8, 1 mM $MgCl_2$, and 15 mM *p*-nitrophenyl phosphate. The plates were incubated at 37 C for 30–50 min, and absorbance was read at 405 nm. When necessary, the reactions were stopped by the addition of 50 μl of 3 M NaOH. Standard isomarticin curves were determined over pH values ranging from 7.0 to 8.5.

RESULTS

Tracer and antigen synthesis. Isomarticin was coupled successfully to alkaline phosphatase and BSA with carbodiimide as an activating reagent. Both toxin and protein were retained in the dialysis bag during exhaustive dialysis. The conjugate migrated in the void volume on a Sephadex G-25 column, but traces of free toxin were retained by the Sephadex. On TLC plates, the protein-bound toxin no longer migrated with the organic phase. Isomarticin was protected from light during conjugation steps to prevent it from inactivation.

In preparing hapten-carrier conjugates, the best yields of soluble product were obtained if the synthesis was stopped by the addition of ammonium acetate. Otherwise, most of the product could not be resolubilized after lyophilization. The ammonium acetate pre-

TABLE 1. Specificity of anti-isomarticin serum

Compound	Amount of compound per milliliter required to displace 50% of tracer
cis-Dihydrofusarubin	2 ng
Methyl dihydrofusarubin	30 ng
Fusarubin	30 ng
Anhydrofusarubin	10 ng
Javanicin	30 ng
Marticin	100 ng
Isomarticin	2 ng
Bostrycoidin	10 ng
Cinnamic acid	>1 mg
p-Coumaric acid	>1 mg
Caffeic acid	30 μg
1,4-Benzoquinone	>0.3 mg
Hydroquinone	1 mg
2-Hydroxy-1,4-naphthoquinone	>0.1 mg
5-Hydroxy-1,4-naphthoquinone	0.3 mg
Limonin	>0.1 mg
Naringin	>1 mg
Coumarin	100 μg
2,5-Dihydroxybenzoic acid	30 μg
2,4-Dihydroxybenzoic acid	>1 mg
Psoralen	>100 μg
Seslin	>1 mg

sumably inactivated carbodiimide-activated protein carboxyl groups which could then cross-link the protein molecules.

The pH of the dialysis medium to remove unbound isomarticin was critical. Unbound isomarticin precipitated on the dialysis tubing walls when the dialysis medium pH was neutral to slightly alkaline (pH 7.5), and this interfered with further dialysis. When the dialysis medium was adjusted to pH 6.0, this problem was prevented.

Absorption of antibodies to polystyrene. Dilutions of 1:500 to 1:16,000 of the tracer added to various dilutions of the anti-isomarticin IgG indicated that maximum antibody coating was reached at 4 μg protein/ml (Fig. 2). This concentration of IgG was used in subsequent experiments. For an absorption reading of at least 1.0 to be reached at an incubation of 37 C, the tracer could not be diluted more than 1:2,000 (500 units/ml). The IgG of the nonimmunized rabbit and control wells reacted with absorption values of less than 0.1 when treated with tracer dilutions (data not shown in Fig. 2).

Standard curve. Figure 3 shows that the presence of free isomarticin decreased the amount of bound enzyme. A logit plot of the data was linear (insert, Fig. 3). Best sigmoidal standard curves were obtained when the pH of the TBS was adjusted to 8.0 to 8.5. The basis for this assay is competition between tracer and free toxin for a limited number of antibody sites on the polystyrene. The resulting assay had a detection limit of 2 ng/ml for isomarticin.

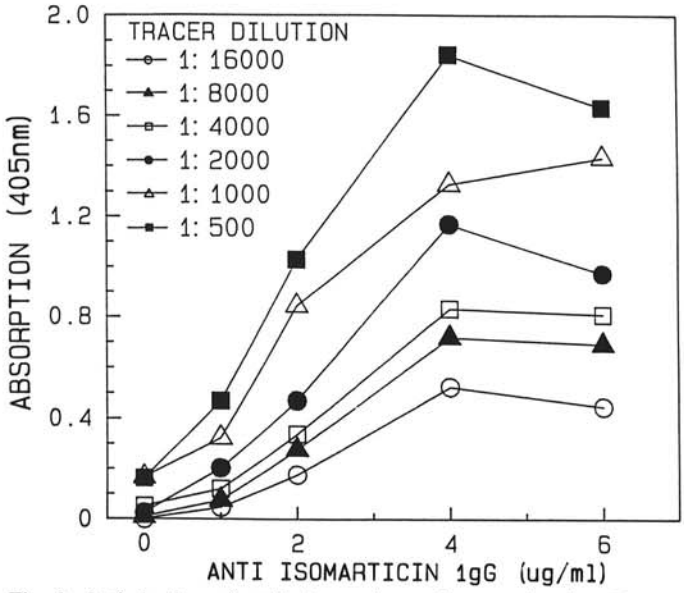

Fig. 2. Optimization of antibody coating and tracer titration. Except for IgG and tracer concentrations, the assays were done as indicated in the Materials and Methods section. The microtiter plate was incubated for 60 min at 37 C; then absorbance was read at 405 nm.

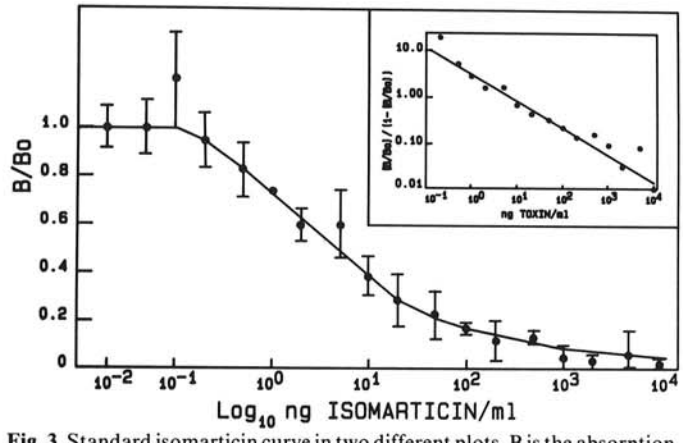

Fig. 3. Standard isomarticin curve in two different plots. B is the absorption with isomarticin present at 405 nm after a 50-min incubation corrected for unspecific tracer binding. B_o is the absorption in the absence of isomarticin. Vertical bars are standard deviations. Logit = $\log x/1 - x$.

Specificity. The *Fusarium* toxins displaced 50% of the enzyme tracer from coated antisera in concentrations as low as 2 ng/ml (Table 1). The other compounds did not interfere in the ELISA, required between 0.3 and 1 mg/ml to reduce enzyme activity bound by 50%, or, in the case of morin (not reported in Table 1), interfered at concentrations of 1 mg/ml by increasing the activity of bound alkaline phosphatase. Both 5-hydroxy-1,4-naphthoquinone (juglone) and 2-hydroxy-1,4-naphthoquinone, with structures similar to 2-methoxynaphthazarin, decreased tracer binding by at least 50% at 1 mg/ml.

Studies with various dilutions of rough lemon seedling leaf sap indicate that factors in the leaf sap may inhibit alkaline phosphatase activity at high concentrations of sap but do not compete significantly with the tracer for antibody binding sites (Table 2). When 2 μl of leaf sap was added to a 1.0-ml assay of alkaline phosphatase in 0.1 M diethanolamine, 1 mM $MgCl_2$, and 15 mM *p*-nitrophenyl phosphate, pH 9.8, the activity was inhibited by 17% (data not shown).

DISCUSSION

We have developed an enzyme-linked immunoassay for a number of naphthazarins produced by *F. solani*. These toxins are derivatives of 2-methoxynaphthazarin. That is, they are derivatives of 2-methoxy-*p*-dihydroxy-1,4-naphthoquinone (6). Much of this core structure is necessary for the sensitivity of the assay. If a monohydroxy-1,4-naphthoquinone is substituted, the sensitivity of the assay drops by at least three orders of magnitude. The assay is even less sensitive to other phenolic compounds.

Initial immunoassays were conducted with the more commonly used PBS solution; however, when this buffer was replaced with TBS, the sensitivity of the assay was markedly improved. Another factor affecting the sensitivity of the results involved protection of the isomarticin solutions from light throughout the assays. Isomarticin is bleached by light, and this can affect results of the assays.

One use for this assay is in determining the possible physiological roles that the toxins play in the disease process. The fungus can invade the roots and the lower stem or trunk of the plant (9,12,18,22). In California, *F. solani* invades the trunk through the root system and sporulates on the bark (4). Symptoms apparent in leaves may be due to the dysfunction of roots and lower stem because of the presence of the fungus or its effects

TABLE 2. Effect of rough lemon leaf sap on enzyme-linked immunosorbent assay (ELISA) of isomarticin[a]

Leaf sap dilution[b]	Absorption at 405 nm	
	No toxin	10 ng of isomarticin/ml[c]
No sap control	1.01	0.44
1/1,000	0.66	0.30
1/100	0.50	0.25
1/50	0.32	0.14
1/20	0.22	0.08
1/10	0.15	0.05
Nonspecific antibody control[d]	0.19	...
No antibody control	0.10	...

[a] Assay conducted as described in the Materials and Methods section, except that the incubation time was 50 min.
[b] Final volume of leaf sap was 100 μl.
[c] ELISA reagent: alkaline phosphatase in 0.1 M diethanolamine containing 1 mM $MgCl_2$, and 15 mM *p*-nitrophenyl phosphate, per milliliter, pH 9.8.
[d] IgG from a nonimmunized rabbit.

on host physiology; or symptoms may be due to the direct effects of previously undetectable low levels of toxin transported by the xylem to the canopy. The presence of toxins in upper portions of pea plants with advanced cases of infection of *F. solani* has been reported (9,10). Purified toxins in hydroponic solutions also can be transported from the roots of rough lemon seedlings into the leaves after a period of time (15). This assay should make the detection of nanogram levels of transported toxins feasible.

Strains of *F. solani* can produce different naphthazarin toxins (1,9,21,22). This assay detected as little as 2 ng of naphthazarins per milliliter. Because the current assay does not distinguish the various naphthazarin toxins, its usefulness in determining which of these are elaborated in the host by the pathogen is limited. However, this same property should make this assay useful in detecting fungal infection and mobility of this toxin group. This would be true even though the exact composition of toxins produced is not known or may not be constant. The composition could change because of the progress of disease or changing environmental conditions (9,10). This property also would permit the toxin to be detected, even though some of these toxins are sensitive to pH, and might allow the toxin to be condensed with alcohol during extraction (24).

The usefulness of this type of immunoassay in exploring the relationship between toxin production and disease expression might be extended if another naphthazarin toxin derivative is conjugated to BSA for immunization of the rabbit or is used in tracer synthesis. Because none of the other naphthazarin toxins have a moiety that can be directly coupled to protein, the toxins chosen would have to be modified first. Two procedures tried as an adjunct to this research were to succinylate the free hydroxyl group (7), which is common to the dihydrofusarubins and to fusarubin, and to condense *p*-benzoic acid diazonium salt with any of the naphthazarins at the three position (25). For both reactions, the products were complex mixtures. Usable products, that is, ones that could be linked to BSA via the carbodiimide reaction and ones with a spectrum resembling that of one of the toxins, proved to be minor components in both cases. A usable product of the diazonium salt condensation reaction with dihydrofusarubin was purified despite problems with its stability. This instability would make this product a poor candidate for creating a tracer. However, its uniqueness might make it a desirable alternative antigen. The succinylation reaction produced several potentially useful products. Although work still needs to be done in purifying and characterizing the products, one was used successfully as a hapten.

The immunoassay approach has significant advantages over older techniques where the toxins were extracted from culture fluids and quantitated spectrally (1) or separated by TLC for qualitative analysis. Quantitation by the immunoassay should not be affected by the presence of other chromaphores. Therefore, it should be more readily applicable to plant extracts. The amount of extract required is manageable because of the assay's sensitivity. In addition, the IgG also should be useful for developing an immunohistochemical assay for studying the distribution of the toxins within the plant tissue.

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