Control of Fusarium Wilt of Safflower by Mangiferin

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Appreciation is expressed to the Council of Scientific and Industrial Research and to the University Grants Commission, New Delhi, for award of research fellowships to K. B. and D. K. C., respectively.

Accepted for publication 29 September 1976.

ABSTRACT

GHOSAL, S., K. BISWAS, D. K. CHAKRABARTI, and K. C. BASU CHAUDHARY. 1977. Control of Fusarium wilt of safflower by mangiferin. Phytopathology 67: 548-550.

Antifungal activity of mangiferin, a naturally occurring xanthone-*C*-glucoside from *Canscora decussata,* against *Fusarium oxysporum* f. *carthami* was evaluated. Safflower seedlings grown from mangiferin-treated seeds in infested potting soil were protected for up to 2 wk from infection by three strains of the fungus known to cause wilting in safflower. Mangiferin caused lysis of hyphal cells in vitro and significantly retarded mycelial growth of the fungus. Production of fusaric acid, a normal metabolic product of this fungus, was prevented completely by mangiferin and a different amino acid that was produced was isolated and partially characterized.

Additional key words: *C*-glucoside, fungicide.

Fusarium oxysporum Schlecht. f. *carthami* Klisiewizc and Houston, the causal agent of wilt of safflower (6, 9), previously was shown to produce a number of toxic substances (viz., fusaric acid, lycomarasmin, and 12, 13-epoxytrichothecenes) both in vitro and in vivo (5). The production of trichothecenes by the fungus is a cause for alarm from a public health viewpoint. Although low levels of orally-ingested trichothecenes may not cause mycotoxicosis, the chronic effects of even low doses of these toxins over a long period of time are not known. Also, it was shown (Ghosal et al., *unpublished*) that, in the absence of any other extraneous infection, seeds of safflower could be infected by the fungus during storage by contact of healthy with infected seeds. Thus it was thought worthwhile to search for compounds that could protect safflower from the fungus attack and/or destroy the substances toxic to the host that are secreted by the fungus.

Phenolic substances have been reported to be responsible for the general resistance which higher plants show towards bacteria and fungi (11). These reports prompted us to evaluate the potential of mangiferin (1, 3, 6, 7-tetrahydroxyxanthone-C_2-β-D-glucoside, molecular weight 422), obtained from *Canscora decussata* Schult (Gentianaceae) (4) against *F. oxysporum* f. sp. *carthami.*

MATERIALS AND METHODS

Aqueous sodium carbonate solutions (1%) of mangiferin (1×10^{-5} to 1×10^{-3} M) were used for determining the antifungal activity. Unless stated otherwise, the data given indicate the effect of mangiferin at a concentration of 1×10^{-4} M. In all seed-treatment experiments, 100 seeds (10 seeds in each batch) were used for the nontreated control and the mangiferin-treated groups; the control received only 1% sodium carbonate solution. The experiments were conducted at ambient temperature of 21 ± 2 C. The data (means of ten sets of readings) were analyzed by Student's *t*-test and the chi-square (χ^2) test.

The resistance shown by the mangiferin-treated seeds of safflower against three strains (IMI-186539, IMI-186543, and IMI-186544; cultures have been deposited with the Commonwealth Mycological Institute, Kew, Surrey, England) of the pathogen was evaluated. In the interaction of the three strains of the fungus and mangiferin, no significant qualitative difference in results was discernible; therefore, the reported results were obtained from tests with the most virulent strain (IMI-186539).

The effect of mangiferin on fungal invasion of the seeds of safflower was determined. Surface-sterilized seeds (washed successively with 0.1% aqueous mercuric chloride solution and sterile distilled water) were soaked for 24 hr in the 1% sodium carbonate solution or in the mangiferin solutions. Excess solution was wiped from the outer surfaces of the seeds which were placed on the fungal mat grown on a potato dextrose agar (PDA) medium. After 24 hours, the seeds were recovered, washed successively with aqueous mercuric chloride solution (0.1%) and sterile distilled water, and again placed on PDA plates to determine whether they were infected with *Fusarium.*

The effect of mangiferin on growth of the fungus hyphae grown (48 hr) in sterile Richards' medium (150 ml) was determined. Subsequently, the nature of changes of the chemical constituents produced by the fungus and chemical change of mangiferin were determined by solvent extraction, analytical thin-layer chromatography (TLC), and spectrometric determination of the resulting

compounds. Silica gel G (E. Merck) was used (0.2-mm plate thickness) for the analytical TLC.

Mangiferin, in three different concentrations (1×10^{-5}, 1×10^{-4}, and 1×10^{-3} M, aqueous suspension), was added to Richards' solution (30 ml) to which the fungus was added. The mixtures were incubated for 7 days. Subsequently, the dry weights of mats grown in the control and in mangiferin-treated groups were compared.

To determine the effect of mangiferin on seed germination, \ 100 surface-sterilized (0.1% aqueous mercuric chloride solution) seeds (10 seeds in each batch), soaked in mangiferin solution (1×10^{-3} M), were sown in sterilized sand. Control experiments were conducted with nontreated seeds sown in sterilized sand. Likewise, an equal number of mangiferin-treated and nontreated seeds were kept in moist chamber for 7 days to determine the difference, if any, in the germination of the seeds in the two different conditions. Seeds with 40 to 60% germination capacity were selected for this purpose.

RESULTS

Mangiferin protected safflower seeds against invasion by the fungus. The fungus grew on the surface of all the nontreated control seeds within 72 hr, whereas the mangiferin-treated seeds remained fungus-free. Even after prolonged exposure (96 hr) of mangiferin-treated seeds to the pathogen, nearly 50% of the seeds remained fungus-free.

The hyphal cells were lysed within 72 hr after addition of mangiferin to the fungal mat grown in Richards' medium; the mycelium, became black, the protoplasts were contracted and detached from the cell wall, and had collected at one corner or in the middle of the cells. After 7 days, only a poor growth of the fungus was observed. At the lowest concentration (1×10^{-5} M), mangiferin apparently promoted growth of the mycelium, whereas at the highest concentration (1×10^{-3} M), the mycelial growth was significantly retarded. The mycelial growth (in grams $\pm$ the standard error of the mean) in the control and mangiferin-treated (1×10^{-3} M) groups were 0.422 ± 0.0023 (0.397-0.443 at 95% fiducial limits) and 0.202 ± 0.0012 (0.190-0.214 at 95% fiducial limits), respectively ($P < 0.01$, Student's t-test). At lower concentrations of mangiferin (1×10^{-4} and 1×10^{-5} M), the mycelial weights were 0.438 g and 0.472 g, respectively, which were statistically insignificant. The absorbed mangiferin also was converted into other compounds, and fusaric acid, the major nitrogenous acid component in the control culture fluid (3), was not detected in the mangiferin-treated culture filtrates or in the corresponding fungal mat extract. In lieu of fusaric acid, an apparently new amino acid, m.p. $> 360°$, R_f 0.4 (*n*-butyl alcohol:acetic acid:water, 4:1:2); ninhydrin color, purple; ultraviolet: λ_{max} (ethyl alcohol) nm (optical density) 210 (0.12), 270 (0.08); infrared: γ_{max} (mineral oil) 3,300 (broad) (γ_{NH}), 1,750 (γ_{CO}), 1,665, 1,642 (monosubstituted "Guanidinium I" and "Guanidinium II" bands) (7), 1.025 cm^{-1} ($\gamma_{OH)NH}$). The positive Sakaguchi test (after addition of sodium hypochlorite to alkaline solution of the compound and α-naphthol, a red color was produced) showed by the compound also suggests the presence of a monosubstituted guanidine group in the amino acid. Mangiferin, on the other hand, was metabolized into a mixture of phenolic carboxylic acids and polyoxygenated xanthones. The major component, R_f 0.8 (chloroform:methanol:acetic acid, 100:5:3), showed a positive ferric test and ultraviolet absorption: λ_{max} (ethyl alcohol) nm (optical density) 225 (0.69), 258 (0.36), 275-280 (0.33), 335 (0.23), typical of 1, 3, 5, 6, 7-penta-oxygenated xanthones (4). Mangiferin and fusaric acid did not interact, as detected by analytical TLC, when mixed in aqueous solution and kept at 28 ± 5 C overnight.

In potting soil infested with the fungus, typical disease symptoms appeared in all seedlings of the nontreated control group. In the mangiferin-treated groups all seedlings appeared healthy on emergence and no disease symptoms developed until about 10 days after germination. At this stage, when the seedlings were sprayed a second time with mangiferin (1×10^{-4} M) solution, a significant number (65%, $P < 0.01$, χ^2 significance in relation to the nontreated control group) of seedlings remained healthy until the fruiting stage when some of these plants (10-15%) again showed the disease symptoms. During a study of pathogenicity of this fungus against safflower, in fields and in potting soil, a waxing and waning of the disease symptoms with the growing of the plants previously were observed (Ghosal et al., *unpublished*). The seedlings which could survive the initial fungal attack regained vitality at the flowering stage and some of them again showed the disease symptoms at the fruiting stage. The nature of the phenolic constituents of safflower at the flowering and fruiting stages were found to be different. Preliminary investigation with the phenolic constituents produced by safflower at the flowering stage, showed significant resistance in safflower seedlings against the fusarial infection. Further work in this direction is in progress.

Another noteworthy observation made was the seed-germination promoting effect of mangiferin (170% over control, $P < 0.01$, χ^2 significance in relation to the control group). There was no significant difference in the rate of germination of the seeds when tested in sand and in a moist chamber.

DISCUSSION

After mangiferin treatment, safflower seeds and seedlings were protected from fungal attack. The experiments were conducted with fungal concentrations which were much higher than those usually found in the field. A recent field survey in the area (Varanasi District) indicated that the rhizosphere soil samples of safflower contained the fungus (*F. oxysporum* f. sp. *carthami*) in the range of 50 to 70 chlamydospores as determined by the soil smear technique (8) and by the standard soil-plate method (10). The number of chlamydospores in infested potting soil was three to four times greater.

Since the pathogen of this disease of safflower is seed-borne (6, 9), and the fungus hyphae are located in the parenchymatous cells of the seed coat of infected seeds, any hyphae present in the apparently healthy seeds must be destroyed prior to sowing and human consumption. This precaution also is necessary for protecting healthy seeds from secondary infection during storage by contact with infected seeds (Ghosal et al., *unpublished*). Our results indicate that mangiferin caused lysis of the hyphal cells and reduced growth, and, presumably, also altered

the metabolism of the fungus since there was no production of fusaric acid. Mangiferin earlier was shown to produce significant monoamine oxidase inhibition (2). This could be the mode of action of mangiferin against *F. oxysporum* f. sp. *carthami*.

Although further investigations will be necessary to assess the practical significance of these results, they suggest that the susceptibility of safflower to *F. oxysporum* f. sp. *carthami* can be reduced by treatment of seeds and seedlings with externally-applied antifungal substances of plant origin. This investigation has demonstrated, for the first time, the potentiality of mangiferin as a fungicidal agent. Another impressive aspect of this finding is the very low order of toxicity of mangiferin. The LD_{50} of mangiferin in albino rats (based on a total of 16 animals) was 365 mg/kg (303 to 416 mg/kg at 95% fiducial limits) (1).

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