Effect of Disease and Injury on Accumulation of a Flavonoid Estrogen, Coumestrol, in Alfalfa

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ABSTRACT

Coumestrol accumulated in alfalfa in response to infection by all the pathogenic fungi tested: *Ascochyta imperfecta; Cylindrocladium scoparium; Colletotrichum trifolii;* and *Uromyces striatus.* The time course of accumulation paralleled the development of infection, and the concentration of coumestrol was related to the degree of infection. Final steps of coumestrol synthesis occurred only in the infected parts of the plant. Coumestrol was not translocated from the infected areas to other parts of the plant. Very little coumestrol accumulated in leaves inoculated with nonpathogenic fungi or with a pathogenic bacterium, *Xanthomonas alfalfae,* but coumestrol did accumulate when nonpathogenic fungi were incubated on detached leaves. Coumestrol did not accumulate in plants inoculated with the alfalfa mosaic virus or the stem nematode (*Ditylenchus dipsaci*) or in leaves injured by mechanical means, atmospheric oxidants, or desiccation. Treatment with a Cu++ solution resulted in accumulation of coumestrol in roots, but not in shoots. *Ascochyta imperfecta* did not synthesize coumestrol when it was grown on media prepared from alfalfa. Hypotheses concerning the nature of pathogen-induced flavonoid biosynthesis and its relation to phytoalexin production are presented. Phytopathology 60:684-688.

Coumestrol is of interest because (i) it is the principal estrogenic constituent of alfalfa (3, 11); (ii) it accumulates in alfalfa in response to infection by fungi (3, 11, 15); and (iii) it resembles structurally the flavonoid phytoalexins pisatin (20), phaseollin (20), and alfalfa phytoalexin (12). Unlike the phytoalexins, coumestrol apparently does not have significant antimicrobial activity (18, Sherwood & Olah, *unpublished data*). The biochemical and physiological events responsible for flavonoid accumulation during fungal pathogenesis are not well defined.

The purpose of the present investigation was to study the effect of disease and other stress on coumestrol accumulation in alfalfa in order to gain insight into the nature of pathogen-induced synthesis of flavonoid compounds.

MATERIALS AND METHODS.—Alfalfa plants (*Medicago sativa* L. 'Atlantic') were grown in quartz sand in 10-cm polyethylene pots in environmental chambers that provided light of 10,000 lux for 13 hr at 24 C and dark for 11 hr at 20-22 C daily. The plants were watered daily with half-strength Hoagland's solution. Only vegetatively growing plants, 6 to 12 weeks old, were used.

Leaves were inoculated with *Ascochyta imperfecta* Pk. or *Colletotrichum trifolii* Bain & Essary by spraying the plants with suspensions of conidia in sterile tap water containing a few drops of orange juice and Tween 80 (polyoxyethylene sorbitan monooleate). Leaves were inoculated with *Uromyces striatus* Schroet. var. *medicaginis* (Pass.) Arth. by brushing on freshly collected uredospores. The inoculated plants were incubated in a Percival dew chamber at 19 C for 48 hr and then were returned to the environmental chambers.

Inoculum of the root pathogen, *Cylindrocladium scoparium* Morgan, was grown in a cornmeal:sand mixture. Roots were inoculated by carefully unpotting the plants and then repotting them in a mixture of 200 cc inoculum and 400 cc sand. Nematode and virus inoculations were carried out by routine procedures.

Coumestrol was extracted by allowing the fresh plant tissue (1 g) to stand for 3 days in 15 ml of 80% methanol. The methanol solution was removed, and the extracted tissues were washed with two 15-ml portions of 80% methanol. The original extract and the two methanol rinses were combined, concentrated to near dryness in vacuo at 45 C, and quantitatively transferred to 2-ml volumetric flasks. A 25-100 µliter portion of the concentrated extract was removed for chromatography on Whatman No. 1 paper in a solvent system of acetic acid:water:HCl (50:35:15, v/v). The amount of coumestrol extracted from various plant tissues was measured fluorometrically on dried chromatograms according to the procedures of Livingston et al. (13). With this procedure it was possible to detect 5 ppm coumestrol. Coumestrol content is reported as ppm on a dry wt basis.

All experiments were repeated twice with two or more replications in each experiment.

RESULTS.—*Accumulation in response to pathogenic fungi.*—The amount of coumestrol in healthy Atlantic alfalfa plants was usually below the level of detection (5 ppm), but coumestrol accumulated markedly in response to infection by each of the pathogenic fungi tested (Table 1). Differences in amounts of coumestrol observed between diseases appeared to be related to differences in the amounts of infection occurring in these particular inoculations, and did not necessarily indicate differences between the abilities of the fungi to induce synthesis. Other pathogenic fungi which have been reported to induce coumestrol accumulation in pure culture inoculations are *Pseudopeziza medicaginis* (3,

TABLE 1. Coumestrol content of various tissues of alfalfa plants infected by pathogenic fungi and bacteria

Fungus	Part of plant infected	Part of plant assayed	Coumestrol ppm, dry wt
Noninoculated plants		roots	0[a]
		stems	0
		leaves	0
Ascochyta imperfecta			
isolate LC—test 1	leaves	roots	0
isolate LC—test 1	leaves	leaves	542
isolate LC—test 2	leaves	leaves	174
isolate 17	leaves	leaves	140
isolate 59	leaves	leaves	132
Cylindrocladium scoparium			
isolate Y-4	roots	roots	247
isolate 8	roots	roots	362
isolate 8 + Y-4	stem base	roots	0
isolate 8 + Y-4	stem base	stem base	88
isolate 8 + Y-4	stem base	leaves	0
Colletotrichum trifolii	leaves	leaves	76
Uromyces striatus	leaves	leaves	115
Xanthomonas alfalfae			
isolate N. C.	leaves	leaves	22
isolate Kansas	leaves	leaves	40

[a] Minimum level of detection = 5 ppm.

15), *Leptosphaerulina briosiana* (11), and *Stemphylium botryosum* (11). The maximum amounts of coumestrol reported for these fungi (219, 84.9, and 41.5 ppm, respectively) are of the same magnitude as those presented in Table 1.

Localization of accumulation.—When leaves, stems, or roots were infected separately, coumestrol accumulated in the infected tissues but not in uninfected parts of the same plant (Table 1). Examination of different parts of a single plant inoculated with *U. striatus* showed that noninfected leaves did not contain detectable coumestrol, but infected leaves contained 115 ppm, pustules contained 168 ppm, and the rust uredospores contained 746 ppm coumestrol.

Time course of accumulation.—The amount of coumestrol in plants inoculated with *A. imperfecta* remained below the level of detection for 2 days after inoculation, but began to increase by the end of the 3rd day (Fig. 1). The first appearance of small chlorotic areas on the leaves also occurred on the 3rd day after inoculation. Four days after inoculation, small, dark lesions on the leaves were apparent and the concentration of coumestrol increased nearly 3-fold. By the end of 8 days, the leaf lesions were fully expanded and the concentration of coumestrol had reached a maximum. Coumestrol synthesis appeared to parallel the growth curve of microorganisms.

Accumulation in response to nonpathogenic fungi.—Since phytoalexins accumulate when spore suspensions of nonpathogenic fungi are incubated on detached leaves, pods, or stems (2, 4, 6, 12), we inoculated detached leaflets with some fungi which are not pathogenic to alfalfa. Conidia of *Cochliobolus carbonum* Nelson, *Aspergillus ochraceus* Wilhelm, and *Alternaria tenuis*

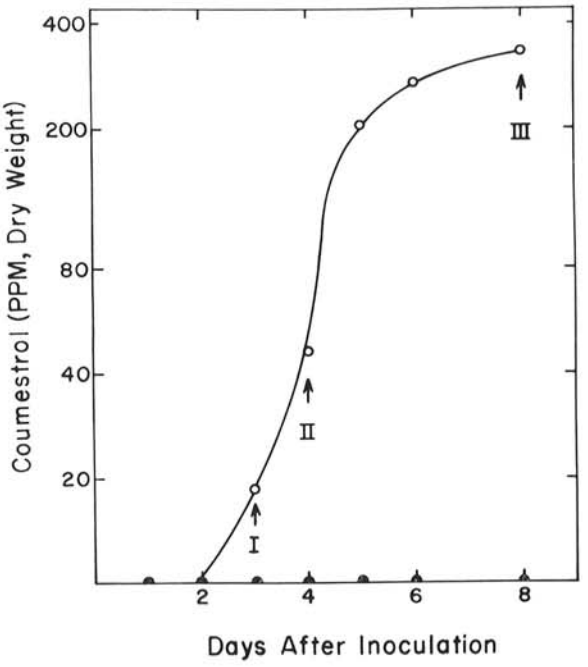

Fig. 1. Time course of coumestrol accumulation in Atlantic alfalfa following inoculation with *Ascochyta imperfecta*. All leaves from four replicate pots were harvested every 24 hr for a period of 8 days. I = Small chlorotic areas first become visible; II = small, dark lesions on leaves were present; III = size of leaf lesions had reached a maximum. Open circles = inoculated with *A. imperfecta*; closed circles = noninoculated plants. Minimum level of detection = 5 ppm.

Nees were brushed on the leaflets with a cotton swab. The leaflets were floated on 3% sucrose in sterile tap water in petri dishes kept at 20 C under fluorescent light of 700-1,000 lux. After 10 days, noninoculated, detached leaflets remained green and showed no detectable coumestrol. Leaflets inoculated with *C. carbonum*, *Aspergillus ochraceus*, and *Alternaria tenuis* showed little or no visible damage, but contained 750, 308, and 253 ppm coumestrol, respectively. Detached leaflets inoculated with pathogenic fungi (*Ascochyta imperfecta* and *U. striatus*) developed characteristic symptoms and also accumulated coumestrol.

Leaves of intact plants inoculated with the same nonpathogenic fungi showed no obvious symptoms, but at 7 days there was a trace of coumestrol in leaves inoculated with *C. carbonum* and 38 ppm in leaves inoculated with *A. tenuis*.

Effect of bacterial infection.—Infection by a leaf spot bacterium, *Xanthomonas alfalfae* (Riker, Jones, & Davis) Dowson, resulted in the accumulation of low amounts of coumestrol (Table 1). Infected leaves showed small, brown lesions surrounded by water-soaked, chlorotic margins. Leaves tested 15 days after inoculation (when the lesions were expanded almost fully) contained 2.8 and 10.5 ppm coumestrol for the two isolates tested. At 21 days, the leaves contained 22 and 40 ppm coumestrol, respectively. The bacterial infection occupied approximately one-fourth as much of

the leaf area as *Ascochyta imperfecta*-infected leaves in the same test, but yielded less than one-tenth as much coumestrol.

Effect of virus and nematode infection.—No accumulation of coumestrol was detected in young alfalfa leaves that showed mottling and stunting due to alfalfa mosaic virus. These results are in harmony with those of Hanson et al. (11), who observed that coumestrol did not accumulate in plants infected by yellow mosaic virus.

Accumulation of coumestrol was not observed in seedling buds showing swelling and stunting caused by the stem nematode (*Ditylenchus dipsaci* [Kühn] Filipjev) or in roots galled by the root knot nematode (*Meloidogyne incognita* [Kofoid & White] Chitwood).

Effect of wounding and a nonpathogen.—Beck (1) reported that nearly complete release of free isoflavones was obtained within 1 min after the leaves of subterranean clover (*Trifolium subterraneum*) were crushed in water. The mechanism involved was considered to be an enzymatic hydrolysis of isoflavone glycosides. Phytoalexins are not formed in response to mechanical wounding (4, 5).

In the present studies, no coumestrol was detected when alfalfa leaves were ground with water and incubated at 25 or 37 C up to 40 min. Intact alfalfa leaves punctured with pin lugs or crushed gently between forceps and incubated for 10 to 15 days showed only trace quantities of coumestrol.

In field-grown alfalfa, portions of leaves killed by nonpathogenic agents often support saprophytic fungi. Large amounts of coumestrol were found in these leaves, particularly those damaged by alfalfa weevils.

To provide a laboratory situation in which a nonpathogenic fungus could proliferate on injured tissue adjacent to uninjured tissue, portions of intact leaves were wounded by pinching gently with forceps and were then inoculated with *Cochliobolus carbonum*. The fungus grew in the wounded areas. The amount of coumestrol in wounded, inoculated leaves was 41 ppm 4 days after inoculation, and did not increase further upon prolonged incubation. Control leaves that were wounded only and control leaves that were inoculated without wounding, contained trace quantities of coumestrol.

Effect of Cu^{++}.—Phytoalexin production has been induced by application of dilute solutions of Cu^{++} or Hg^{++} to plant surfaces (2, 12, 17). In our studies, an aqueous solution of 10^{-3} M $CuCl_2$ taken up in the transpiration stream of excised alfalfa shoots and leaves or applied topically to detached leaflets did not induce coumestrol synthesis in 24 hr, even though phytotoxicity was evident as manifested by light green leaf margins. Young detached roots of alfalfa plants immersed in 10^{-3} M $CuCl_2$ solutions for 24 hr contained 57 ppm coumestrol, while detached roots in distilled water showed only a trace of coumestrol.

Detached leaves and shoots treated with 10^{-3} M $CuCl_2$ solutions accumulated one principal fluorescent compound which was identified as 7,4′-dihydroxyflavone (*unpublished data*).

Effect of other abiotic factors.—Leaves incubated in an atmosphere of dilute formaldehyde gas developed dead, bleached spots 1 to 3 mm broad, but these did not accumulate coumestrol.

Plants were not watered until they showed moderately severe wilting and were then watered normally. After 4 to 6 days, the partially desiccated leaves developed interveinal discoloration, but accumulation of coumestrol could not be detected in these leaves.

Wilting, freezing, and desiccation reportedly do not induce coumestrol accumulation (14). Loper & Hanson (15) found only small amounts of coumestrol (0 to 2.1 ppm) in leaves, stems, and stem tips at four stages of growth under various temperature and phosphorus treatments. Senescing leaves in the green seedpod stage contained 14 ppm coumestrol (14).

Production in culture.—Since high concentrations of coumestrol were localized in lesions and spores, the question arose as to whether the plant or the plant pathogen synthesized the estrogen. Various attempts to demonstrate the synthesis of coumestrol by growing *A. imperfecta* on media prepared with extracts or homogenates of alfalfa plants failed to result in coumestrol production.

Discussion.—Bickoff et al. (3) and Loper & Hanson (15) showed that infection of alfalfa by pathogenic fungi induces a striking accumulation of coumestrol and related flavonoid compounds. We have shown that coumestrol accumulation is also induced by inoculation with pathogenic bacteria or nonpathogenic fungi, and by treatment with Cu^{++}, and that coumestrol does not accumulate in response to infection by alfalfa mosaic virus or pathogenic nematodes. Any treatment which resulted in coumestrol accumulation also gave an increase in other fluorescent spots which were presumably flavonoid compounds (*unpublished data*). Usually, no coumestrol or only traces of coumestrol were detected in tissues which appeared to be free from infection or injury. This raised the question as to whether the coumestrol in alfalfa is principally, or solely, a product of the metabolism of the plant, or a product of associated microorganisms.

Fungi or bacteria have never been shown to produce flavonoid compounds (8); in agreement with this, we did not detect flavonoid synthesis by *A. imperfecta* cultured on media prepared from alfalfa. High levels of coumestrol were found in rust uredospores by us and by Bickoff et al. (3). The coumestrol detected in this fungus could have been absorbed from surrounding host tissue and accumulated by the spores.

On the other hand, coumestrol and other flavonoids have been reported as constituents of several *Medicago* species and related Leguminosae (7, 14). Stuthman et al. (19) and Hanson et al. (11) reported 1-25 ppm coumestrol in leaves or shoots of greenhouse-grown alfalfa free from visual evidence of aphids, foliar disease, or virus infection. Thus, it would appear that certain higher plants contain the complete mechanism for coumestrol biosynthesis.

Although the intermediates and enzymes in the biosynthesis of coumestrol remain unknown, the synthesis

of coumestrol in alfalfa appears to proceed by the flavone-isoflavone pathway. Grisebach (9) and Grisebach & Barz (10) showed that radioactive acetate, cinnamate, and 4,2′,4′-trihydroxychalcone glucoside were incorporated into coumestrol in alfalfa in the same manner as they were into isoflavones in other legumes. These authors, however, did not eliminate the possibility of pathogenic infection in the tissues used in their experiments.

The accumulation of coumestrol appears to be correlated closely with the metabolic activity of the fungal pathogens. Thus the accumulation in leaves following infection by *A. imperfecta* paralleled the increase of the fungal colonies. Loper et al. (16) showed that the coumestrol content is positively associated with size and number of lesions. The increased accumulation of coumestrol in plants following the onset of infection by microorganisms may be a result of a key enzyme(s) or flavonoid precursor(s) being supplied by the microorganism. The introduction of an intermediate in the flavonoid pathway could activate existing plant enzymes or lead to the induction of plant enzymes involved in coumestrol biosynthesis. Fungi produce phenylpropane derivatives which, conceivably, could contribute to flavonoid synthesis (8). It is also known that the aglycones of certain phenolic compounds are released from plant glycosides through the action of glycosidases produced by plant pathogens (20).

Because wounding results in the release of isoflavone aglycones in clover (1), it seemed possible that pathogen-induced accumulation of coumestrol in alfalfa might be due to mechanical damage by the pathogen. However, this possibility is unlikely, since coumestrol did not accumulate when alfalfa leaf cells were injured or killed by mechanical crushing or puncturing.

All the diseases in which coumestrol accumulation has been observed are characterized by a necrotic response of the host tissue. Coumestrol did not accumulate in tissues infected by viruses or nematodes; these tissues were not necrotic. When detached leaflets were inoculated, coumestrol accumulated in the absence of obvious infection; however, the nonpathogens grew in the sucrose solution on which the leaves were floated. These results suggest that enzymes or metabolites formed by the saprophytically growing fungi entered the leaflets and caused coumestrol to be released. Consistent with this possibility was the observation that coumestrol accumulated when a nonpathogenic fungus, *H. carbonum,* proliferated in wounded tissue adjacent to healthy tissue.

Translocation or diffusion of coumestrol in appreciable amounts from the site of infection was not detected. If coumestrol left the site of accumulation, it was metabolized rapidly by processes not operating at the site of accumulation. Evidence indicating that translocation did not occur was provided by the observation that the amounts of coumestrol found in inoculated, detached leaflets were similar to those occurring in infected leaves still attached to the plants. This latter observation and the fact that coumestrol accumulated in Cu++-treated, detached roots, indicate that roots and leaves can carry out the final steps of coumestrol synthesis independently of one another.

Induction of coumestrol synthesis in alfalfa shows several important similarities to the induction of pisatin and phaseollin: coumestrol is produced in response to pathogens, nonpathogens, and Cu++; the accumulation is localized, and wounding or injury does not result in accumulation.

From the evidence discussed above, we postulate that the pathways involved in the disease-induced accumulation of coumestrol and related flavonoids in alfalfa have the following characteristics: (i) the host plant is the principal, or sole, contributor of flavonoid precursors and enzymes involved in flavonoid biosynthesis; (ii) the mechanism of induction is related to metabolism of the microorganisms rather than to mechanical disruption of host cells; (iii) the accumulation is associated with catalytic processes leading to cell necrosis and tissue degradation; (iv) the biosynthesis of coumestrol, at least in its final steps, takes place in the tissue infected; (v) the coumestans, flavones, and isoflavones are biosynthetically related to one another through common intermediates and pathways. The pterocarpan phytoalexins may also be derived from the same precursors.

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