Flavones, Isoflavones, and Coumestans in Alfalfa Infected by Ascochyta imperfecta

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ABSTRACT

Leaves and roots of healthy, chamber-grown Atlantic alfalfa plants, and of plants harvested daily for 7 days after inoculation with *Ascochyta imperfecta*, were assayed for flavonoid compounds. Glycosides of the following flavonoid compounds occurred in both healthy and infected leaves: formononetin, daidzein, 7,4′-dihydroxyflavone, 7,3′,4′-trihydroxyflavone, and tricin. As the leaf spots developed, the concentration of the flavonoid glycosides increased or remained unchanged. Flavonoid aglycones were not detected in healthy leaves. Aglycones were first detected in leaves 3-4 days after inoculation, and increased in concentration through the 7th day. These included formononetin, daidzein, 7,4′-dihydroxyflavone, 7,3′,4′-trihydroxyflavone, coumestrol, 7-hydroxy-11,12-dimethoxycoumestrol, 4′-0-methylcoumestrol, sativol, and medicagol. Several additional fluorescent compounds, apparently flavonoid glycosides, were detected in healthy and infected leaves, but were not identified.

Roots contained principally formononetin-glycoside and coumestrol-glycoside. Fewer flavonoid glycosides and aglycones were detected in roots than leaves. Infection of leaves by *A. imperfecta* did not alter the flavonoid contents of the roots.

The results suggest that flavonoid pathways and constituents which occur in normal leaves may be involved in the formation of flavonoid estrogens and phytoalexins during pathogenesis. Phytopathology 61:65-69.

Several flavonoid compounds accumulate as aglycones (sugar-free phenolic moieties) in alfalfa leaves infected by fungi (3, 20). The flavonoid aglycones are not detectable, or are present only as traces in healthy leaves. One of them, a partly characterized pterocarpan, has been implicated in disease resistance (11, and R. L. Millar, *personal communication*), and has properties similar to the pterocarpan phytoalexins (16) of peas and beans; e.g., pisatin and phaseollin, respectively. Another aglycone, coumestrol, is the principal estrogenic component of alfalfa (10). Thus, the mechanism of fungal-induced accumulation of flavonoid aglycones in alfalfa is of practical interest.

Flavonoid compounds, in the form of glycosides, are normal leaf constituents of many leguminous species (6, 14, 18). It seems possible that fungal-induced accumulation of flavonoid aglycones may involve conversions of preexisting flavonoid glycosides, or flavonoid pathways, of the host. This contrasts with the proposal (9, 13) that the induced accumulation of pisatin involves a pathway, not active in normal plants, beginning at the level of phenylalanine.

The present paper describes the occurrence of flavonoid glycosides in healthy alfalfa leaves and changes in the flavonoid composition during pathogenesis. A companion paper describes the role of glycosidases in the pathogen induced accumulation of aglycones (17).

MATERIALS AND METHODS.—Alfalfa plants (*Medicago sativa* L. 'Atlantic') were grown in sand watered with half-strength Hoagland's solution (20). The growth chambers were programmed for 13-hr light periods (10,000 lux) at 24 C, and 11-hr dark periods at 20 C. The leaves of 6-12-week old plants were inoculated with conidial suspensions of *Ascochyta imperfecta* Pk. Inoculated plants were kept in a dew chamber at 19 C for 2 days and then were returned to the growth chambers. Symptoms (slight chlorosis) first appeared 3 days after inoculation, and typical black leafspots formed by the 6th day.

Flavonoid compounds were extracted from the leaves and roots of plants harvested 0, 1, 2, 3, 4, 5, and 7 days after inoculation. To extract the flavonoids with min alteration by plant enzymes, weighed samples of entire leaves or roots were ground in liquid nitrogen in a mortar with a pestle. When the frozen material warmed to about 0 C it was quickly plunged into a ×100 volume of boiling 50% methanol and boiled for 1 min. The preparations were kept at room temp for 2 days and filtered through glass fiber filter pads. The filters were rinsed with 95% methanol, and the combined filtrate was evaporated to dryness in vacuo at 55 C. The dry extract was taken up in 20% methanol (1 ml/g fresh tissue) and stored at 4 C.

The flavonoid components of the extracts were separated by two-dimensional (2D) paper chromatography. Either 60 µliters of leaf extract or 100 µliters of root extract was applied near one corner of 46 × 57 cm Whatman 3 MM paper. The chromatograms were developed by using 30% acetic acid (ascending) for the first dimension and *tert*-butanol:acetic acid:H_2O (3:1:1, v/v) (descending) for the second dimension. By this procedure, the flavonoid aglycones migrated to the lower right-hand area of the chromatogram while their corresponding glycosides migrated to the upper left-hand area (14, Fig. 1). The chromatograms were viewed under ultraviolet light (258 nm) in the pres-

ence of NH_3 vapor in a hood. Each fluorescent spot was delineated and given a four-digit number for identification. The first two digits referred to the R_F in the first solvent, and the last two digits referred to the R_F in the second solvent.

To measure the relative fluorescence intensity of each spot, 2D chromatograms were brushed with 0.2 N NaOH, dried for 1 hr, and scanned on a Bailey (1) fluorometer. The galvanometer was adjusted to give a reading of 100 for a spot containing 0.8 μg coumestrol.

The fluorescent compounds were identified by spectral and chromatographic comparison with authentic standards as follows. Corresponding spots were cut from replicated papers (not brushed with NaOH) and pooled. The compounds were eluted with 95% methanol, brought to dryness, and dissolved in 100% methanol for spectroscopy. When flavonoid aglycones overlapped, or when purity was questionable, the eluted compounds were separated prior to spectroscopy by thin-layer chromatography (TLC) on Silica Gel G with chloroform:methanol (190:10, v/v), or benzene:ethyl acetate (4:1, v/v).

The ultraviolet spectra of the compounds dissolved in 100% methanol were recorded. Positions of the functional groups were determined by addition of sodium acetate, sodium methylate, boric acid-sodium acetate, or aluminum chloride to the methanol solutions as described by Jurd (12) and Bickoff et al. (4).

After spectroscopy, the eluates of suspected glycosides were evaporated to dryness, then hydrolyzed at 37 C for 12 hr with 2 ml of a 5 mg/ml solution of a commercial β-glucosidase, emulsin (Sigma Chemical Co.), plus one drop of toluene. Alternatively, the glycosides were hydrolyzed in a covered test tube of 2 N HCl in a boiling water bath for 2 hr. The aglycones released by hydrolysis were extracted from the hydrolysates with three 5-ml aliquots of diethyl ether, brought to dryness, then separated by TLC for identification.

RESULTS.—*Flavonoid content of healthy leaves.*—The following glycosides were detected in healthy leaves: 7,4′-dihydroxyflavone-7-monoglycoside (Compound No. 54-39), 7,4′-dihydroxyflavone-7-diglycoside (67-10), 7,3′,4′-trihydroxyflavone-diglycoside (52-14), daidzein-7-monoglycoside (66-65 = daidzin), formononetin-monoglycoside (72-63), tricin-monoglycoside (35-21), and tricin-diglycoside (48-07) (Table 1; Fig. 1-A). These compounds, except 72-63, 35-21, and 48-07, were identified by comparing the R_F values and ultraviolet absorption spectra to published values (14) for known glycosides, and by determining the identity of the aglycones (released by hydrolysis) in ultraviolet and chromatographic comparisons to authentic aglycones. Formononetin-glycoside (72-63) was identified by comparison to an authentic sample. The tricin glycosides were tentatively identified in partly purified extracts from alfalfa leaves by showing that enzyme hydrolysis gave a loss of 35-21 and 48-07 with the corresponding appearance of a compound identical to authentic tricin, and by inference from positions on the 2D chromatograms (17).

Several additional, unidentified, fluorescent or quenching compounds also occurred in the area of the chromatograms where flavonoid glycosides would be expected. Some of these had ultraviolet spectra characteristic of the coumestans (17). Many were degraded by NH_3 or were difficult to recover. Two unidentified compounds (33-75 and 20-82) appeared in the area where flavonoid aglycones would be expected.

Flavonoid content of infected leaves.—During the 7 days of disease development, the abovementioned flavonoid glycosides and unidentified compounds found in healthy leaves either remained at the concn found in healthy leaves or increased in concn (Table 1). Compound 20-82 was no longer observable at 3 days.

Flavonoid aglycones were first detected on the 3rd and 4th day after inoculation and increased in concn thereafter (Table 1). These included 7,4′-dihydroxyflavone (40-85), 7,3′,4′-trihydroxyflavone (27-65), daidzein (61-85), formononetin (68-87), coumestrol (25-80), 7-hydroxy-11,12-dimethoxy-coumestan (12-63), 4′-0-methylcoumestrol (29-85), sativol (15-58), and medicagol (26-82). These aglycones were purified by TLC and were found to have ultraviolet spectra and chromatographic properties identical to those of authentic samples.

Four compounds first appeared in the glycoside area of the chromatogram at 4 days. These included coumestrol-glycoside (57-65) which was identified as follows. The ultraviolet spectrum was max (MeOH): 245, 255, 306, 344 nm. Treatment with sodium acetate showed no change in the peak at 344 nm, indicating that there was no free 7-hydroxyl group. The spectral shift in 0.1 N NaOH (max 282, 312, 350 nm) indicated a free 4′-hydroxyl. Acid or β-glycosidase hydrolysis of spot 57-65 released coumestrol, whose identity was confirmed by chromatography and ultraviolet and mass spectrum analysis. Compound 57-65 was deduced to be coumestrol-7-monoglycoside from the position on 2D papers, and susceptibility to hydrolysis by β-glycosidase. There is no previous report of this compound.

Flavonoid content of roots.—Healthy roots contained large amounts of formononetin-glycoside and coumestrol-glycoside. Most of the flavonoid glycosides and unidentified fluorescent compounds found in healthy leaves were not detected in roots (Fig. 1). Root samples contained low levels of free coumestrol, 7-hydroxy-11,12-dimethoxy-coumestrol, and formononetin. These may have been artifacts formed by hydrolysis during isolation, since it took several minutes to collect, weigh, and kill root tissue. There was no quantitative or qualitative change in the flavonoids found in roots during the 7 days of disease development on the leaves.

DISCUSSION.—This study shows that flavone and isoflavone glycosides are normal constituents of healthy alfalfa leaves. These results were expected, since flavonoid glycosides have been reported in leaves of other leguminous species (5, 6, 14, 18).

Labeling experiments by Grisebach & Barz (8) indicate that flavonoid biosynthesis in alfalfa, like that of other plants, is as follows: Phenylpropane inter-

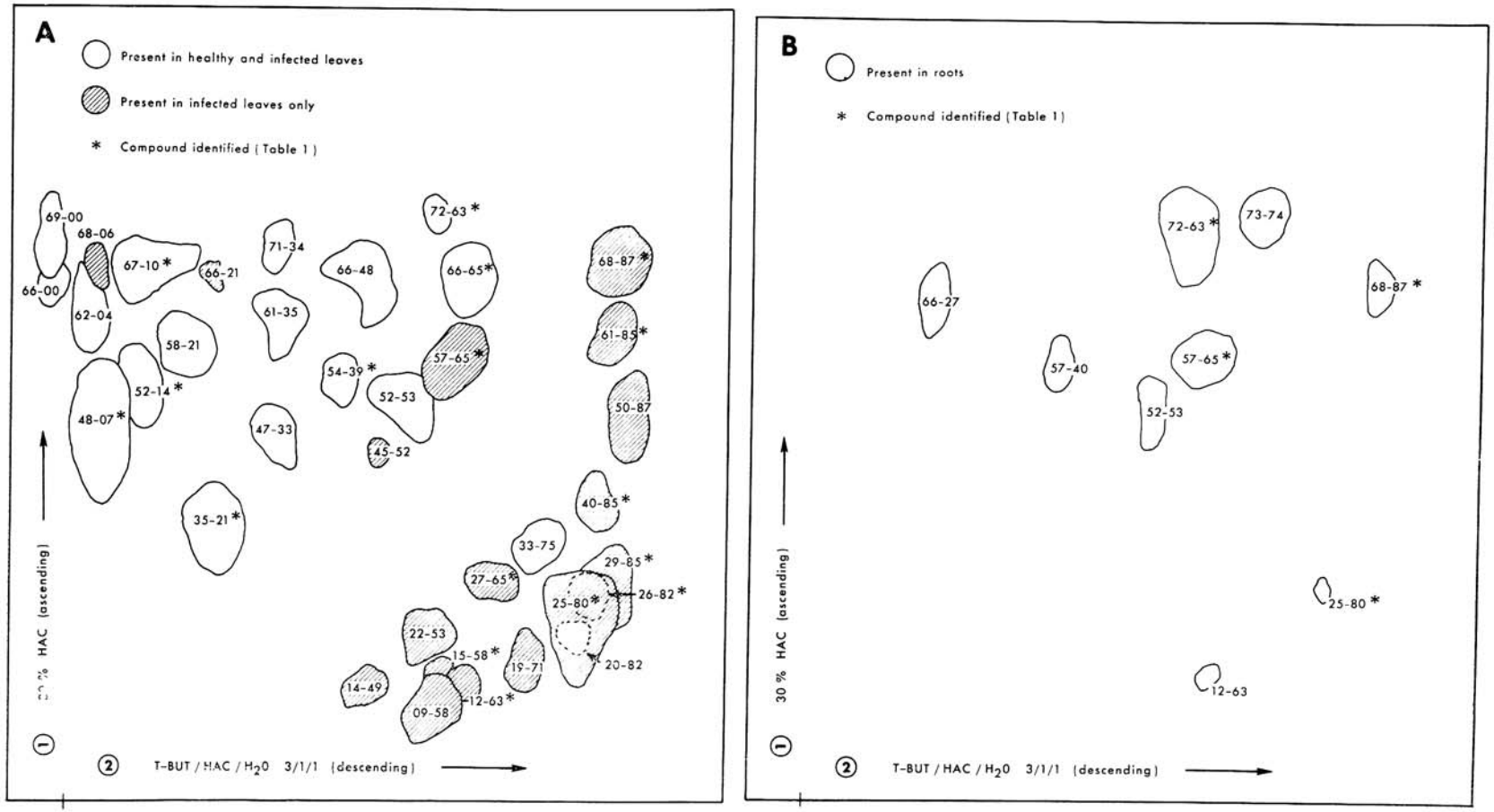

Fig. 1. Diagrammatic representations of the two-dimensional paper chromatographic separation of flavonoid compounds from alfalfa. **A)** Compounds from healthy leaves and from leaves infected by *Ascochyta imperfecta* for 4-7 days. **B)** Compounds from healthy roots of noninoculated or *A. imperfecta* inoculated plants. The first two digits of the identification number represent the R_F in the first dimension with 30% acetic acid, ascending. The last two digits of the identification number represent the R_F in the second dimension with *tert*-butanol: acetic acid: water (3:1:1 v/v), descending. Spots shown are those visible in ultraviolet light (258 nm) or ultraviolet light plus NH_3 vapor. See Table 1 for identification and relative fluorescence intensity.

TABLE 1. R_F values, fluorescence colors, and relative fluorescence of flavonoid and unidentified compounds from leaves of healthy alfalfa and from leaves infected by *Ascochyta imperfecta*

Compound	R_F 30% HAC[a]	R_F TBAW[b]	Color with ultraviolet light + NH_3	Relative fluorescence[c] at indicated day after inoculation[d] 0	1	2	3	4	5	7
Compounds detected in healthy and diseased leaves										
Tricin-monoglycoside	35	21	Yellow	33	42	43	42	46	50	50
Tricin-diglycoside	48	07	Yellow	32	35	42	43	47	55	64
7,3′,4′-trihydroxyflavone-diglycoside	52	14	Green	8	7	7	8	6	7	8
7,4′-dihydroxyflavone-7-monoglycoside	54	39	Green	22	25	30	32	32	35	35
Daidzein-7-monoglycoside	66	65	Blue	10	11	12	17	17	26	26
7,4′-dihydroxyflavone-7-diglycoside	67	10	Blue-green	8	8	9	11	14	16	16
Formononetin-monoglycoside	72	63	Green-white	3	3	4	4	5	5	5
Unidentified	20	82	Orange	5	5	2				
Unidentified	33	75	Green-yellow	10	10	9	11	10	10	10
Unidentified	47	33	Yellow	23	26	29	36	52	57	62
Unidentified	52	53	Orange	6	7	8	9	9	12	12
Unidentified	58	21	Green	16	12	13	14	15	14	15
Unidentified	61	35	Orange	8	10	12	10	12	10	10
Unidentified	62	04	Absorbs			Not readable				
Unidentified	66	00	Absorbs			Not readable				
Unidentified	66	48	Violet	8	10	12	15	15	16	18
Unidentified	69	00	Absorbs			Not readable				
Unidentified	71	34	Yellow	4	3	4	5	7	7	8
Compounds detected only in diseased leaves										
7-hydroxy-11,12-dimethoxycoumestan	12	63	Violet					4	13	17
Sativol	15	58	Pink-white					1	3	5
Coumestrol	25	80	Tan-white				19	69	127	214
Medicagol	26	82				Not readable				
7,3′,4′-trihydroxyflavone	29	85	Violet				8	15	19	19
4′-O-methylcoumestrol	27	65	Green-yellow				6	21	39	70
7,4′-dihydroxyflavone	40	85	Blue-green				19	34	46	60
Coumestrol-glycoside	57	65	Violet					20	26	30
Daidzein	61	85	Blue				3	16	21	25
Formononetin	68	87	Green-white				2	6	9	12
Unidentified	09	58	Blue					10	24	49
Unidentified	14	49	Green				3	5	7	10
Unidentified	19	71	Orange					5	6	9
Unidentified	22	53	Green					1	3	7
Unidentified	45	52	Violet					1	2	2
Unidentified	50	87	Yellow-green				12	21	20	26
Unidentified	66	21	Yellow					4	6	7
Unidentified	68	06	Yellow			Not readable				

[a] First dimension on paper chromatogram was 30% acetic acid (ascending).
[b] Second dimension on paper chromatogram was *tert*-butanol:acetic acid:water (3:1:1 v/v) (descending).
[c] Deflection of a galvanometer which was set to deflect 100 units for a spot containing 0.8 μg of coumestrol. Average of two determinations in two tests using the extract from 60 mg fresh tissue/spot.
[d] The healthy plants were sampled at 0 days, before inoculation.

mediates from the shikimic acid pathway condense with acetate units to form chalcones; ring closure of the chalcone leads to the flavones; while ring closure plus aryl migration leads to the isoflavones and coumestans. Genetic evidence in other species indicates that final glycosylation patterns are determined late in biosynthesis (7).

Many of the flavonoid glycosides of alfalfa increased in concn during infection. This suggests that infection accelerated the normal pathways of flavonoid biosynthesis of phenolic intermediates (13). Alternatively, accumulation in leaves could result from impaired transport of glycosides from the site of infection to other parts of the plant. Flavonoid transport patterns in plants are not well known (19).

The aglycones of certain of the glycosides which occurred naturally in the leaves (e.g., 7,4′-dihydroxyflavone and daidzein) began to accumulate the 3rd or 4th day after infection. This suggested either that infection released the aglycone from the glycone (presumably by a glycosidase enzyme) or that infection impaired glycosylation at the final steps of flavonoid biosynthesis. In either case, the aglycones accumulated at the infection site. Evidence supporting the gylcosidase alternative is presented in another paper which shows that β-glycosidase, possibly of fungal origin, increases greatly during infection, and that the increase in enzyme activity precedes the appearance of the aglycones (17).

Spectral data indicated that some of the unidentified, fluorescent compounds of healthy leaves were coumestans (17). Coumestan glycosides were not detected with certainty in healthy leaves. Thus, there was no indication of whether the coumestan aglycones

in infected leaves were derived from naturally occurring coumestans by mechanisms similar to those suggested for the flavones and isoflavones, or whether the coumestan aglycones were abnormal products derived by metabolic conversions of the isoflavones. The observations on roots, however, indicate that coumestrol glycoside is a natural constituent of healthy roots. The appearance of coumestrol glycoside in leaves 4 days after inoculation is compatible with the suggestions that disease either stimulates glycone synthesis or impairs transport.

The flavonoid composition of the roots was surprisingly different from that of leaves. Moreover, roots contained higher β-glycosidase activity than healthy leaves (17). Roots, unlike leaves, released coumestrol when they were injured with 1.0×10^{-3} M Cu (20). The importance of these differences in terms of biosynthetic pathways and disease resistance remains to be determined. Since root flavonoids did not change qualitatively or quantitatively when leaves became infected, the roots were apparently not a major source of the flavonoids accumulated in infected leaves.

The above suggestions regarding possible biosynthetic and transport pathways in diseased alfalfa remain purely speculative. The elucidation of those pathways which are actually operational would be most readily accomplished by using radioactive tracers.

The discovery of flavonoid glycosides in normal alfalfa leaves has implications in the problem of estrogenicity of legume forages. Formononetin and daidzein cause infertility in sheep fed on subterranean clover (5, 6). Coumestrol is estrogenic (10). Millington et al. (15) presented evidence that isoflavone glycosides may be hydrolysed to the estrogenically active form within the digestive tract of sheep.

The existence of a flavonoid pool in healthy alfalfa also has implications in understanding the biogenesis of phytoalexins. There is evidence that daidzein is converted to coumestrol by oxidation (2). Coumestrol could theoretically be converted to the pterocarpan phytoalexin of alfalfa by removing a carbonyl function in the B ring and adding a methyl group at the 4′ or 7- position.

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