Glycosidase Activity and Flavonoid Accumulation in Alfalfa Infected by Ascochyta imperfecta

Arthur F. Olah and Robert T. Sherwood

Graduate Assistant, Department of Plant Pathology, North Carolina State University, Raleigh 27607; and Plant Pathologist, Crops Research Division, ARS, USDA, respectively. The present address of the senior author: Department of Biology, Frostburg State College, Frostburg, Maryland 21532, and of the junior author: U. S. Regional Pasture Research Laboratory, University Park, Pennsylvania 16802.

Cooperative investigations of the Crops Research Division, ARS, USDA, and the North Carolina Agricultural Experiment Station, Raleigh. Journal Series Paper No. 3181 of the North Carolina Agricultural Experiment Station.

Accepted for publication 20 December 1972.

ABSTRACT

When *Medicago sativa* leaves were inoculated with *Ascochyta imperfecta* β-glucosidase activity increased steadily, beginning 2 days after inoculation. This increase was paralleled by the appearance and accumulation of the aglycones of certain flavonoid glycosides which are constituents of healthy alfalfa leaves. Flavonoid glycosides isolated from healthy plants were hydrolysed by glycosidase preparations from healthy leaves, infected leaves, or cultures of *A. imperfecta*. The pH optimum of β-glucosidase from healthy leaves was 6.5, whereas that from diseased leaves or from pure cultures of *A. imperfecta* was 5.0 to 5.5. In disk-gel electrophoresis, β-glucosidase enzyme bands from diseased leaves corresponded to those from *A. imperfecta*. It is suggested that glycosidases, possibly of fungal origin, may partly account for pathogen-induced accumulation of flavonoid aglycones in alfalfa.

Phytopathology 63:739-742

Additional key words: phytoalexins, coumestans, disease resistance, estrogens, disk electrophoresis.

The flavonoid aglycones (sugar-free flavonoid compounds) of leguminous crops are of interest because certain of them are involved in disease resistance as phytoalexins (9, 15, 19), whereas certain others impart estrogenicity to leguminous hay or pasture (1, 2, 6). The aglycones are absent or barely detectable in healthy tissues but accumulate locally in infected or injured tissue (1, 14, 18).

Healthy tissues contain appreciable quantities of the glycosides of the same, or related, flavonoid compounds (1, 6, 13, 14). Some of the flavonoid glycosides of healthy alfalfa are apparently β-glucosides. Daidzin is a known β-glucoside of alfalfa (13, 14), and the 7,4′-dihydroxyisoflavone-7-monoglycoside of alfalfa appears to be a β-glucoside on the basis of spectral and chromatographic comparisons (13) with published values (11). These and several additional alfalfa flavonoid glycosides have been shown to serve as substrates for a commercial β-glucosidase. Accordingly, we were interested in studying β-glucosidase activity in healthy and infected alfalfa in order to explore the possibility that accumulation of flavonoid aglycones during infection might result from hydrolysis of the flavonoid glycosides by plant or fungal enzymes.

MATERIALS AND METHODS.—Alfalfa plants (*Medicago sativa* L. 'Atlantic') were grown in environmental chambers and inoculated with conidia of *Ascochyta imperfecta* Pk. as previously described (14).

Cultures of *A. imperfecta* used for extracting fungal enzyme were grown on a glucose-nitrate medium (17) supplemented with 3 g of yeast extract and 1 mg of thiamine-HCl per liter. Stationary cultures were grown on 25 ml medium in 250 ml flasks for 15 days at 24 to 26 C. The entire culture (fungus + fluid) was used in making enzyme preparations.

To obtain enzyme preparations, healthy leaves, healthy roots, inoculated leaves, or fungal cultures were processed into acetone powders which were then extracted in a buffer solution. Healthy roots were included because they differ from the leaves in flavonoid composition (14). Samples of 1 to 3 g of plant tissue or fungal culture were ground with acetone at a constant -18 C for 1 min in a Sorvall Omni-Mixer and washed with acetone (-18 C) on filter paper in a Büchner funnel. Grinding and washing were repeated three times. The powders were dried in vacuo and stored in N_2 gas at -18 C. Enzymes were extracted by incubating 100 mg of the acetone powder in 10 ml of a phosphate, citrate, borate, veronal universal buffer (3) at pH 5.5, plus one drop of toluene, for 2 hr at 4 C. The extracts were centrifuged at 10,000 *g* for 20 min. The supernatant liquids were passed through a 0.45-μ Millipore filter. The filtrate constituted the enzyme preparation. Protein content was determined by the method of Lowry et al. (10).

Glycosidase assay reaction mixtures contained 1 ml of enzyme preparation (about 1 mg of protein), 8 ml of universal buffer at pH 5.5, and 1 ml 0.01 M *p*-nitrophenyl-β-glucoside (Sigma Chemical Co.). Reaction mixtures were incubated at 37 C. At various times, 1-ml samples were removed and placed in 1 ml of 1 M Na_2CO_3. This was diluted with 4 ml of water and read at 420 nm (12). Specific activity was expressed as μmoles *p*-nitrophenol liberated/μg protein/hr. Tests on pH optima were conducted as above, with the universal buffer adjusted to the various pH values.

In one test, the substrate consisted of an aqueous

solution containing a mixture of coumestrol-7-glycoside, formononetin-monoglycoside, and 7-hydroxy-11,12-dimethoxycoumestan-glucoside extracted from healthy alfalfa roots (13, 14). The root glycosides were incubated with enzymes in buffer and toluene as described above for 0 to 24 hr. The released aglycones were extracted from the reaction mixture with diethyl ether. The ether was evaporated, and the aglycones were dissolved in methanol for spectrophotometry and for identification by thin-layer chromatography (TLC) as previously described (14). Relative activity was measured as increase in absorbance at 300 nm/50 μg protein/hr; however, the results are of qualitative value only.

Disk electrophoresis on polyacrylamide gel was carried out by the methods of Davis (4). Proteins were extracted from acetone powders in the running buffer (pH 8.3 Tris-glycine with 40% sucrose) without toluene and clarified as described above. About 200 μg of protein, in 0.1 ml of running buffer, was layered on the large-pore gel. After electrophoresis, gels were fixed in 7% acetic acid for 2 hr at 4 C, rinsed briefly in distilled water, and then incubated in 4 ml of substrate solution for 4 hr at 20 C. The substrate solution was prepared by dissolving 10 mg of 6-bromo-2-naphthol-β-glucopyranoside (Dajac Labs, Philadelphia, Pa.), in 10 ml of 2-methoxyethanol and diluting to 50 ml with 0.01 M citric acid-sodium phosphate buffer at pH 5.5. The gels were then rinsed in distilled water and post-fixed in 4 ml of a fresh solution of 10 mg/ml naphthanil diazo blue B (Dajac Labs) made to pH 7.8 with solid $NaHCO_3$. Enzyme sites were pink to dark red (7). The E_f values reported here represent the ratio of the movement of the enzyme bands to the movement of the bromophenol blue tracking dye, with the latter considered as 100.

RESULTS.—The specific activity of β-glucosidase from infected leaves (7 days after inoculation) was 7 times greater than that from healthy leaves, and the specific activity of fungal β-glucosidase preparations was 14 times greater than of healthy leaf preparations (Table 1). Roots had 2 times greater β-glucosidase activity than healthy leaves.

The same extracts were able to hydrolyse a mixture of formononetin-monoglycoside, coumestrol-7-glycoside, and 7-hydroxy-11,12-dimethoxy-coumestan-glycoside which had been isolated from alfalfa roots. TLC procedures showed that all the expected aglycones were released by all four enzyme preparations. The relative hydrolytic activities against the mixture of plant flavonoid glycosides as indicated by increase in absorbance at 300 nm were: healthy leaves 1X, healthy roots 3X, diseased leaves 3X, and fungal cultures 4X.

The effect of pH upon β-glucosidase activity was tested. Extracts from healthy leaves and roots hydrolysed *p*-nitrophenyl-β-glucoside most rapidly at pH 6 to 6.5 (Fig. 1-A). Extracts from infected leaves (7 days) and fungus cultures were most active at pH 5 to 5.5.

In electrophoretic separations upon polyacrylamide gel columns, bands of β-glucosidase activity from diseased leaves corresponded with those from *A. imperfecta* cultures (Fig. 1-B). These were located at E_f 10 and E_f 18. The healthy root extract had a band at E_f 10 and an additional band at E_f 35. Glucosidase bands were not detected from healthy leaves.

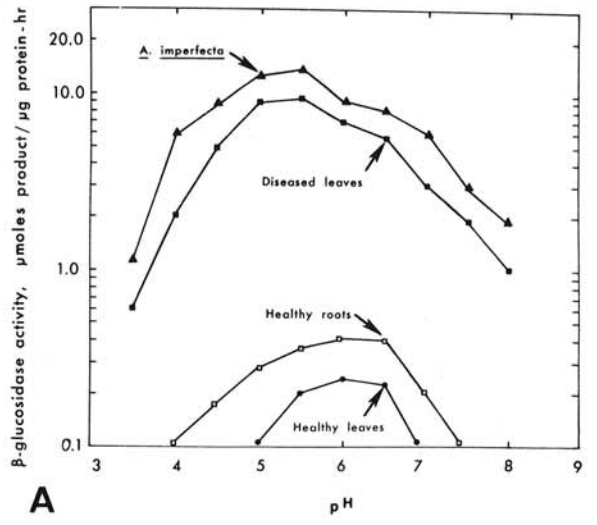
Leaves were assayed for β-glucosidase activity and for concentrations of flavonoid glycosides and their corresponding aglycones at various times up to 7 days after inoculation with *A. imperfecta*. The healthy controls consisted of samples taken immediately after inoculation. The experiment was run at two different times, and the two trials gave similar results. The β-glucosidase activity of infected leaves was approximately 80% greater than that of control leaves 2 days after inoculation. The β-glucosidase activity increased steadily up to the termination of the experiment (Fig. 1-C). Earlier reports (13, 14) gave the relative flavonoid contents of these samples.

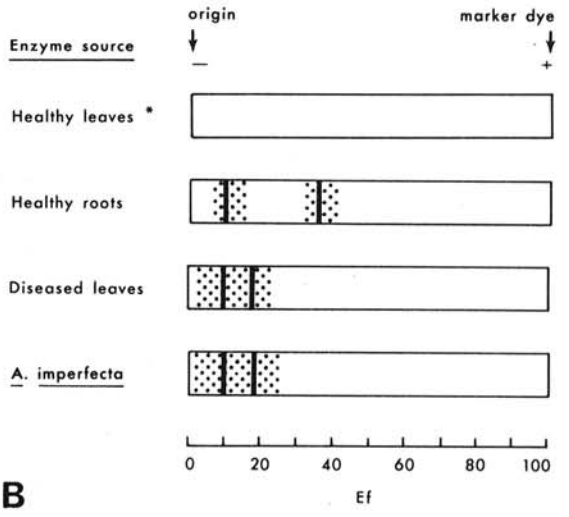
DISCUSSION.—Earlier reports (13, 14) on the flavonoid composition of the same leaf samples used in this study showed that healthy control leaves contained at least 7 flavonoid glycosides. Among them were included daidzein-7-monoglucoside, 7,4′-dihydroxyflavone-7-monoglucoside, formononetin-monoglycoside, and 7,3′,4-trihydroxyflavone-diglycoside. Flavonoid aglycones were not detected in healthy leaves nor in leaves harvested 1 or 2 days after inoculation. The aglycones of the above mentioned glycosides were detected 3 days after inoculation and increased in concentration thereafter. Chromatographically pure samples of these glycosides could be hydrolysed to the aglycones by a commercial β-glucosidase preparation (13) of unspecified purity. The present study shows that there is a low level of β-glucosidase activity in healthy leaves, and that β-glucosidase activity increases by 2 days after inoculation and continues to increase thereafter. These results suggest that the accumulation of certain flavonoid aglycones in response to infection may result, at least in part, from

TABLE 1. β-glucosidase activity in protein extracts from acetone powders of healthy alfalfa leaves, healthy roots, leaves infected by *Ascochyta imperfecta*, and pure cultures of *A. imperfecta*

Enzyme source	β-glucosidase activity[a] (μmoles product/μg protein/hr)
Healthy leaves	0.55
Healthy roots	1.20
Infected leaves	4.50
A. imperfecta culture	7.80

[a]Reaction mixtures contained ca. 0.01% protein (w/v), and 0.001 M *p*-nitrophenyl-β-glucoside in buffer at pH 5.5, and were incubated at 37 C.





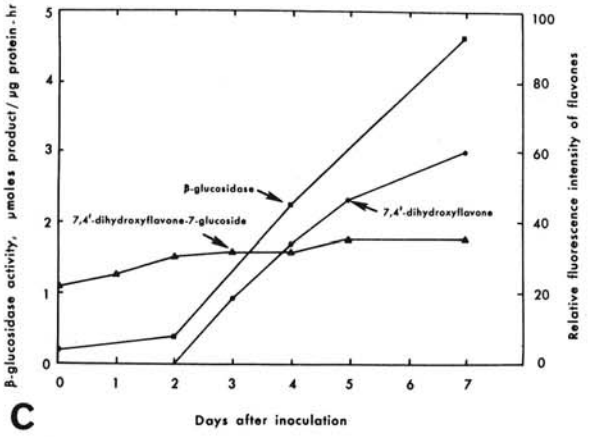


Fig. 1. **A)** Effect of pH of the reaction mixture on β-glucosidase activity of extracts from acetone powders of healthy alfalfa leaves and roots, alfalfa leaves infected by *Ascochyta imperfecta*, and pure cultures of *A. imperfecta*. Reaction mixtures contained ca. 0.01% (w/v) protein and 0.001 M *p*-nitrophenyl-β-glucoside in a buffer and were incubated at 37 C. **B)** Electrophoretic separation of β-glucosidase in extracts of the same acetone powders. Separations were carried out on polyacrylamide gels with pH 8.3 Tris-glycine-40% sucrose running buffer, and glucosidases were detected with 6-bromo-2-naphthol-β-glucopyranoside followed by naphthanil diazo blue B. * = no band detected. **C)** β-glucosidase activity in extracts from acetone powders of alfalfa leaves at various times after inoculation with *Ascochyta imperfecta*, and relative fluorescence intensity of 7, 4′-dihydroxyflavone-7-glucoside and 7, and 7,4′ dihydroxyflavone in the same leaves. Enzyme reaction conditions were as in 1-A and data on flavones were from (14).

hydrolysis of flavonoid glycosides which occur as natural constituents of leaves. Further support for this possibility was provided by the observation that at least three flavonoid glycosides which occur naturally in alfalfa could be hydrolysed by enzyme preparations from alfalfa leaves or roots, and from infected leaves or fungal cultures, with the release of the corresponding aglycones. Other glycosidases in addition to β-glucosidase, could be involved in hydrolysis of flavonoids during pathogenesis. Low levels of β-galactosidase activity have been found in healthy leaves, infected leaves, and *A. imperfecta* cultures (13).

The results suggest, but do not prove, that the β-glucosidase in diseased leaves may be produced, at least partly, by the fungal pathogen. The pH response and electrophoretic properties of β-glucosidase from diseased leaves corresponded with those from fungal cultures. These differed from the pH response of the enzyme from healthy leaves or roots. Unfortunately, no glucosidase bands were detected in gel columns of healthy leaf preparations, perhaps because of the low specific activities of the preparation. Therefore, we did not exclude the possibility that β-glucosidase in diseased leaves was of plant origin.

Contrary to what might be expected, there was not a decline in the flavonoid glycosides during the time when the aglycones accumulated. Formononetin-monoglycoside and 7,3′,4′-trihydroxyflavone-diglycoside remained at about the same concentration in infected leaves as in healthy controls (14). Daidzein-7-monoglucoside and 7,4′-dihydroxyflavone-7-monoglucoside increased in concentration approximately 160% and 60%, respectively, over the levels measured in the controls (14). Stimulated synthesis of phenolic compounds is a frequent response of plants to infection (20). The pathways of flavonoid synthesis in alfalfa appear to be similar to those in other leguminous plants, but these are only partly known (8). It is possible that infection of alfalfa leaves stimulates normal pathways of flavonoid glycoside synthesis, thus accounting for the increased levels of glycosides found in infected leaves. At the same time, however, there is an increase

in glycosidase activity during infection resulting from glycosidase production by the pathogen or from stimulated host metabolism. Furthermore, the compartmentation which separates glycosides from glycosidases in healthy tissue is probably disrupted by activities of the invading fungus, thus permitting a mixing of enzyme and substrate in infected cells. The released aglycones accumulate in the lesion area. A portion of the aglycones may be converted to other flavonoid aglycones which are also detected in infected tissue, such as the estrogenic coumestans (2, 18) and the alfalfa phytoalexin (9, 19). Obviously, there are other possible pathways for the production of flavonoid aglycones in diseased alfalfa that are compatible with the data presented here. In alfalfa (18), unlike subterranean clover (1, 6), mechanical crushing of healthy leaves to mix glycosides with glycosidases of the healthy plants does not result in a release of flavonoid aglycones. Thus, the release of aglycones during fungal infection probably involves some mechanism in addition to cell disruption and increased enzyme activity.

Considerable emphasis has been placed on phenolic glycosides and aglycones as factors in disease resistance (20), yet there are few reports which describe glycosidase activity within diseased plants (5, 16). Sequeira (16) found that scopolin (a coumarin glycoside) and scopoletin (the aglycone) increased in tobacco stems infected by a bacterium. In contrast to our results, he did not find an increase in β-glucosidase activity, and he concluded that stimulated synthesis accounted for the increase in both compounds.

LITERATURE CITED

1. BECK, A. B. 1964. The oestrogenic isoflavones of subterranean clover. Australian J. Agr. Res. 15:223-230.
2. BICKOFF, E. M., R. R. SPENCER, S. C. WITT, & B. E. KNUCKLES. 1969. Studies on the chemical and biological properties of coumestrol and related compounds. USDA, ARS Tech. Bull. 1408. 95 p.
3. BRITTON, H. T. S. 1956. Hydrogen ions. Vol. I. Van Nostrand, New York. 452 p.
4. DAVIS, B. J. 1964. Disc electrophoresis–II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121:404-427.
5. DAVIS, D., P. E. WAGGONER, & A. E. DIMOND. 1953. Conjugated phenols in the Fusarium wilt syndrome. Nature 172:959-962.
6. FRANCIS, C. M., A. J. MILLINGTON, & E. T. BAILEY. 1967. The distribution of oestrogenic isoflavones in the genus Trifolium. Australian J. Agr. Res. 18:47-54.
7. GOLDBARG, J. A., K. C. TSOU, S. H. RUTENBURG, A. M. RUTENBURG, & A. M. SELIGMAN. 1958. A method for the colorimetric determination of X-β-glucosidase chromogenic substrate. Arch. Biochem. Biophys. 73:435-439.
8. GRISEBACH, H. 1968. Recent investigations on the biosynthesis of flavonoids. p. 379-406. *In* T. J. Mabry, R. E. Alston, & V. C. Runeckles [ed.]. Recent advances in phytochemistry. Appleton-Century-Crofts, N.Y.
9. HIGGINS, VERNA J., & R. L. MILLAR. 1969. Comparative abilities of Stemphylium botryosum and Helminthosporium turcicum to induce and degrade a phytoalexin from alfalfa. Phytopathology 59: 1493-1499.
10. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, & R. J. RANDALL. 1951. Protein measurement with Folin-phenol reagent. J. Biol. Chem. 193:265-275.
11. MARKHAM, K. R., & T. J. MABRY. 1968. The identification of twenty-three 5-deoxy- and ten 5-hydroxyflavonoids from Baptisia leconti (Leguminosae). Phytochemistry 7:791-801.
12. NORKRANS, B. 1957. Studies of β-glucoside and cellulose splitting enzymes from Polyporus annosus Fr. Physiol. Plantarum 10: 198-214.
13. OLAH, A. F. 1970. Glycosidase and flavonoid composition of fungal-infected alfalfa. Ph.D. Thesis. North Carolina State University, Raleigh. 42 p.
14. OLAH, A. F., & R. T. SHERWOOD. 1971. Flavones, isoflavones, and coumestans in alfalfa infected by Ascochyta imperfecta. Phytopathology 61:65-69.
15. PERRIN, DAWN R., & I. A. M. CRUICKSHANK. 1969. The antifungal activity of pterocarpans towards Monilinia fructicola. Phytochemistry 8:971-978.
16. SEQUEIRA, L. 1969. Synthesis of scopolin and scopoletin in tobacco plants infected by Pseudomonas solanacearum. Phytopathology 59:473-478.
17. SHERWOOD, R. T. 1969. Morphology and physiology in four anastomosis groups of Thanatephorus cucumeris. Phytopathology 59:1924-1929.
18. SHERWOOD, R. T., A. F. OLAH, W. H. OLESON, & E. E. JONES. 1970. Effect of disease and injury on accumulation of a flavonoid estrogen, coumestrol, in alfalfa. Phytopathology 60:684-688.
19. SMITH, D. G., A. G. MC INNES, VERNA J. HIGGINS, & R. L. MILLAR. 1971. Nature of the phytoalexin produced by alfalfa in response to fungal infection. Physiol. Plant Pathol. 1:41-44.
20. WOOD, R. K. S. 1967. Physiological plant pathology. Blackwell Sci. Publ. Ltd., Oxford, England. 570 p.