Phytochemistry, 1976, Vol. 15, pp 133–134. Pergamon Press. Printed in England.

GENETIC REGULATION OF FLAVONOID CONTENT IN SEEDS AND SEEDLINGS OF *MELILOTUS ALBA**

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(*Revised received* 1 *July* 1975)

Key Word Index—*Melilotus alba*; Leguminosae; sweetclover; biochemical genetics; *C*-glycosylflavonoids; di-*C*-pentosylapigenin.

Abstract—Chemical analysis of seeds and seedlings of the *CC* and *cc* genotypes in *Melilotus alba* indicated that these alleles affect flavonoid biosynthesis. The *CC* seed coats contained orientin and *iso*-orientin, which were absent in the *cc* seed coats. The pigment responsible for the red pigmentation of young seedlings of *CC* genotypes was a cyanidin glycoside. The embryos of seeds of both the *CC* and *cc* genotypes contained a flavonoid tentatively identified as a 6,8-di-*C*-pentosylapigenin. The observation that 3′,4′-dihydroxyflavonoids were absent in the *cc* genotype and that 4′-hydroxyflavonoids were present in both genotypes indicated that the *C/c* alleles controlled the 3′-hydroxylation of flavonoids. The *C/c* alleles did not, however, control 3′-hydroxylation of cinnamic acids since caffeic acid was detected in both genotypes.

INTRODUCTION

In a previous communication [1] it was demonstrated that the presence or absence of brown pigmentation in the seed coats and red pigmentation in young seedlings of white-flowered sweetclover (*Melilotus alba* Desr.) was conditioned by a single pair of alleles, designated *C/c*. Preliminary investigations suggested that the pigments involved were flavonoids.

In this paper, the isolation and identification of several flavonoids found to be under control of the *C/c* locus in sweetclover are described. In addition, various possibilities are discussed with regard to the identity of the specific biosynthetic step affected by the *C/c alleles*.

RESULTS

Chromatographic comparisons of the brown (genotype *CC*) and non-brown (genotype *cc*) seed coat phenotypes revealed that the former contained two prominent constituents that were absent in the latter. These two constituents were isolated by PC and identified by standard procedures as orientin (8-*C*-glucosyl-luteolin) and *iso*-orientin (6-*C*-glucosyl-luteolin). Although *C*-glycosylflavonoids have been reported in other species of legumes [2, 3], this is the first reported occurrence of such compounds in *Melilotus*.

In the chromatographic comparison of embryos of the 2 genotypes, *CC* and *cc*, no differences in composition were detected. However, a constituent in embryo extracts of both genotypes was observed to have properties similar to the two *C*-glucosyl-flavonoids found only in the *CC* seed coats. This constituent was isolated and classified on the basis of spectral analysis as a *C*-glycosyl apigenin derivative. Since prolonged hot acid treatment apparently did not cause hydrolysis or isomerization of this compound, even after 48 hours, it appeared to be a 6,8-di-*C*-glycosyl apigenin in which the 2 sugars were identical, in which case the 2 molecular configurations resulting from acid interconversion would be identical [4]. In fact, its spectral behaviour was identical to that of vicenin (6,8-di-*C*-glucosylapigenin) [5]. However, attempts to establish its identity by chromatographic comparison with known di-*C*-glycosylapigenin derivatives were not successful (see Experimental). A preliminary mass spectrum of a permethylated derivative indicated that the compound was most likely a 6,8-di-*C*-pentosylapigenin. Attempts to complete the identification are continuing. The chemical difference between red seedlings (genotype *CC*) and green seedlings (genotype *cc*) appeared to be due to a single anthocyanin pigment present only in the former genotype. Efforts to isolate and purify the anthocyanin, however, were largely unsuccessful, so that it was not possible to characterize it completely. Hydrolytic and spectral studies indicated that it was a cyanidin glycoside. This conclusion is consistent with the recent report [6] that the principal anthocyanin in seedlings of several *Melilotus* species is a cyanidin glycoside.

DISCUSSION

The absence of certain flavonoids in the seed coats and seedlings of the *cc* genotype, in contrast to their presence in the *CC* genotype, indicates that the *C/c* alleles affect some aspect of flavonoid biosynthesis. Flavonoid biosynthesis is not completely blocked in the *cc*

* Contribution from the Agricultural Research Service, USDA, and the Nebraska Agricultural Experiment Station, Lincoln. Published as Paper No. 3930, Journal Series, Nebraska Agr. Exp. Sta. The work reported was conducted under project 12-88, Nebraska Agr. Exp. Sta.

genotype, however, since embryos of both genotypes contained a flavonoid. Thus the *C/c* alleles appear to control the 3'-hydroxylation of flavonoids; whereas a 4'-hydroxylated flavonoid occurs in both genotypes, 3',4'-dihydroxylated flavonoids occur only in the dominant *CC* genotype. Genes with similar effects have been reported in other plant species [3, 7]. Since caffeic acid esters occur in both *Melilotus* genotypes, it is clear that the phenolase-type enzyme [8] controlling 3'-hydroxylation of flavonoids in this plant must be specific at the C_{15}-level. Unfortunately, efforts to detect phenolase activity in sweetclover tissues of both genotypes were unsuccessful, despite the use of a number of different enzyme extraction techniques.

EXPERIMENTAL

Plant material. Closely related lines were derived from selfed seeds produced on plants grown from 2 selected F_4 progenies in a previous inheritance study [1] and had been selfed for 4 subsequent generations. The flavonoid and anthocyanin compounds present in seeds and seedlings of the *CC* genotype and in a commercial variety, Emerald, were found to be identical based on chromatographic comparisons in 6 solvent systems. Therefore, seeds and seedlings of the more abundant Emerald were used to obtain sizeable amounts of these compounds for identification purposes.

Identification of flavonoids. Flavonoids were extracted from seed tissue with a biphasic solvent extraction procedure [9] using $CHCl_3$–MeOH–H_2O (1:1:1). They were purified using ascending PC in *n*-BAW (*n*-BuOH–HOAc–H_2O; 4:1:5, upper layer), *t*-BAW (*t*-BuOH–HOAc–H_2O; 3:1:1), *n*-BEW (*n*-BuOH absolute EtOH–H_2O; 4:1:2.2), 15HOAc (HOAc–H_2O, 15:85), 3NaCl (3% aq NaCl), and H_2O (dist. H_2O). Comparative R_f (×100) data for the apigenin *c*-glycoside of seed embryos and for violanthin and vicenin were respectively: 54, 66 and 43, in *n*-BAW; 37, 60 and 37 in *t*-BAW; 60, 72 and 51 in *n*-BEW; 68, 85 and 79 in 15HOAc; 32, 56 and 40 in 3NaCl; 43, 78 and 65 in H_2O. The identity of the purified constituents was established by spectrophotometric and chromatographic comparisons with authentic standards [5]. An enzymatic hydrolysis mixture of 1·0 mg of emulsin (β-glucosidase) and 1·0 mg of unknown flavonoid in 2·0 ml of 0·5 M NaOAc buffer (pH 5·0), incubated at 37° for 24 hr, was used to check for the presence of *O*-glycosidic linkages. To check for acid hydrolysis of sugars from the unknown flavonoids, 3·0 mg of unknown was dissolved in 3·0 ml of 2N HCl and 3·0 ml of MeOH, and the mixture was heated for various time intervals at 100°. The flavonoids present in the hydrolytic mixtures were extracted with *n*-BuOH and chromatographed with unhydrolyzed unknown to determine the effect of hydrolysis.

Isolation of anthocyanin. The anthocyanin present in seedlings of the *CC* genotype was extracted by homogenizing defoliated stems of 2- to 3-week-old seedlings in a medium of MeOH–12 N HCl (97:3). Chromatographic and spectrophotometric procedures used in attempts to isolate, purify, and identify the anthocyanin were similar to published procedures [3].

Extraction and chromatography of phenolic acids present in seedling genotypes. Phenolic acid extracts of 2- to 3-week-old seedlings of both genotypes were obtained by a procedure reported previously [10]. The phenolic acid extracts were then chromatographed alongside cinnamic acid standards (including caffeic acid). Solvents used included *n*-BAW, *n*-BEW, *n*-BPW (*n*-BuOH–C_5H_5N–H_2O; 14:3:3), and BzAW (C_6H_6–HOAc–H_2O; 125:72:3).

Extraction and assay of sweetclover tissue for phenolase. The monophenolase activity (3'-hydroxylation) was assayed by following the appearance of caffeic acid (at 325 nm) produced from the hydroxylation of *p*-coumaric acid. The standard reaction mixture contained in a 3·0 ml vol: 0·1 M KPi buffer (pH 7·0), 2×10^{-4} M *p*-coumaric acid, 1×10^{-3} M ascorbic acid, and an aliquot of an enzyme preparation. *o*-Diphenolase activity (dehydrogenation of caffeic acid to its *o*-quinone) was assayed by following the disappearance of ascorbate at 265 nm (11, 12). The standard reaction mixture contained the following components in a 3·0 ml vol: 0·1 M KPi buffer, 1×10^{-4} M caffeic acid, 1×10^{-4} M ascorbic acid, and an aliquot of an enzyme preparation. Crude enzyme preparations were ordinarily prepared by grinding freshly excised leaves of 3-week-old seedlings in 50 mM KPi buffer (pH 6) in a vol equal to 2 × their fr. wt in the presence of PVP (0·2 of the tissue fr. wt) and sand. Homogenates were filtered through cheesecloth and centrifuged for 1 min at 500 *g* to remove cellular debris, PVP, and sand. The supernatant was assayed directly or frozen for later assay. Other published extraction techniques [11–15] also were attempted.

Acknowledgments—We acknowledge the kindness of Drs. T. J. Mabry and H. Wagner in furnishing samples of *C*-glycosylflavonoids for comparison with our unknowns and thank Prof. J. Chopin (Lyon, France) for his assistance in the preliminary MS identification of the pentosyl nature of the sugar residues in the apigenin derivative.

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