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Effect of Genotype and Growth Stage on Distribution of Melilotic Acid, *o*-Coumaric Acid, and Coumarinic Acid in *Melilotus alba* Desr.

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Effect of Genotype and Growth Stage on Distribution of Melilotic Acid, *o*-Coumaric Acid, and Coumarinic Acid in *Melilotus alba* Desr.[1]

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MELILOTIC acid (*o*-hydroxyhydrocinnamic acid), *o*-coumaric acid (*trans-o*-hydroxycinnamic acid), and coumarinic acid (*cis-o*-hydroxycinnamic acid) occur in sweetclover plants predominantly in the form of glucosides (1, 5, 9). Kosuge and Conn (10, 11) suggested that these three compounds may be metabolically related after observing that excised sweetclover shoots rapidly converted labeled coumarin (the lactone form of *cis-o*-hydroxycinnamic acid) to melilotic acid. In a later publication, Kosuge and Conn (12) suggested that coumarin is converted to dihydrocoumarin which is subsequently converted to melilotic acid by the action of the enzyme, dihydrocoumarin hydrolase.

The effects of genetic factors on content of *o*-hydroxycinnamic acid and activity of β-glucosidase have been described in previous publications (2, 4, 5, 14). In brief, the independent allelic pairs, *Cu/cu* and *B/b,* influence the amount of *o*-hydroxycinnamic acid and the presence or absence of β-glucosidase activity, respectively. Plants of the *CuCuBB* and *CuCubb* genotypes are high in content of *o*-hydroxycinnamic acid, but only the former genotype displays β-glucosidase activity, which under suitable conditions effects hydrolysis of the β-glucoside of *cis-o*-hydroxycinnamic acid. Plants of the *cucuBB* and *cucubb* genotypes are low in *o*-hydroxycinnamic acid and only the *cucuBB* genotype exhibits β-glucosidase activity. Akeson et al. (1) reported that melilotic acid content was approximately 3 times as high in leaves of greenhouse-grown *CuCu* plants as in *cucu* leaves, and concluded that the content of melilotic acid also is influenced by the *Cu/cu* alleles.

The effects of certain nongenetic factors on the content of *o*-hydroxycinnamic acid in sweetclover (measured as coumarin in the earlier studies) have been reported by several authors (3, 8, 13, 15, 17, 18). Only preliminary observations on melilotic acid content have been made[3] and recently some attention has been given to the determination of the *cis* and *trans* isomers of *o*-hydroxycinnamic acid in sweetclover (6, 7). However, no coordinated study has been published dealing with the influence of both genotype and growth stage on levels of melilotic acid, *o*-coumaric acid, and coumarinic acid in sweetclover.

In the present report, information is presented on the influence of genotype and maturity on the content and distribution of these three metabolically-related compounds throughout the life cycle of sweetclover.

MATERIALS AND METHODS

Plant Materials

Four closely related, highly inbred lines of biennial sweetclover (*Melilotus alba* Desr.) were used throughout this study as a source of leaf, stem, and root samples. The derivation of these lines, which will be referred to as isogenic lines, has been described previously (5). In the greenhouse, plants of the *CuCuBB* genotype were grown in 14 by 20-inch flats of soil, with rows spaced 3 inches apart in the flat and plants 1½ inches apart in the row. In the field, spring seedings of the *CuCuBB, CuCubb, cucuBB,* and *cucubb* genotypes were made in adjacent plots in 1960 and 1961 so that first- and second-year plants could be sampled under similar conditions during the 1961 growing season. Each genotype was seeded in a single 15-foot row in each of 6 replications for each season of growth. Rows were spaced 3 feet apart.

Because the supply of isogenic seed was limited, other sources were used to furnish material for seed assays. Seed of the varieties Spanish and Evergreen represented the *CuB* phenotype, while the *Cub* phenotype was represented by W-7, an experimental synthetic developed at the University of Wisconsin. The low-coumarin varieties Cumino and Denta were used for the *cu-* phenotype.

Sampling and Extraction Procedures

Seed samples—Seed samples representing the various phenotypes were weighed, dropped into boiling water (30 ml. per g. of seed), autoclaved for 15 minutes at 15 psi, cooled, and mixed. The extracts were decanted, centrifuged briefly in a clinical centrifuge, and the supernatant solutions were frozen and stored for subsequent assay. An additional sample of each seed lot was oven-dried for the determination of percentage dry matter.

Greenhouse studies—Approximately 50 plants of the *CuCuBB* genotype were used for the assay of leaves and stems. The upper 10- to 13-cm. portions, removed from plants 15 to 20 cm. in height, were the sources of leaf and stem samples 1 to 8 (Table 1). Samples 9 and 10 were obtained from the remaining parts of 10 of these same plants.

Leaflets to be assayed were removed, counted, bulked, weighed, and immediately dropped into a 25 × 150 mm. tube containing 15 ml. of boiling water per g. of fresh tissue. Petioles, stems, and roots were treated in the same way except that only 5 ml. of boiling water was used per g. of plant material. All samples were autoclaved and treated subsequently as described for seed. Dry matter percentages of the various plant parts were determined by oven-drying a portion of the bulked sample from each plant part.

Field studies—In 1961, samples of the four isogenic lines were collected at two-week intervals throughout the period when suitable plant material was available. First- and second-year plants were sampled on alternate weeks in order to distribute the work load. At each sampling date, four plants were randomly selected from each of the four isogenic lines, were removed from the soil to a depth of approximately 30 cm., and were transported to the laboratory in a box kept moist and cool with cold water and ice cubes.

The youngest fully expanded leaves, the upper 4 cm. of the stems, and the lower 6 to 8 cm. of the exposed roots provided the samples to be assayed. Similar plant parts from the four plants of each genotype were bulked and weighed. A portion of each bulked sample was oven-dried for the determination of percentage dry matter. The remainder was extracted as previously described, and extracts were frozen for later assay.

Assay Methods

The water extracts were assayed for *o*-coumaric and coumarinic acids by the nonenzymatic fluorometric procedure described by Haskins and Gorz (6). This procedure is based upon the fact that in alkaline solution, nonfluorescent coumarinic acid is partially converted to the fluorescent isomer, *o*-coumaric acid, by exposure

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[3] Brink, V. C. The content, distribution, and some metabolic aspects of coumarin in sweetclover (*Melilotus alba* Desr.). Unpublished Ph.D. Thesis, University of Wisconsin. 1941.

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to ultraviolet light. Thus, intensity of fluorescence of a non-irradiated alkaline hydrolysate of sweetclover extract afforded a measure of *o*-coumaric acid content, and fluorescence intensity of a similar hydrolysate after exposure to ultraviolet irradiation provided a measure of the total *o*-hydroxycinnamic acid content. Coumarinic acid level was then calculated by difference. Each plant extract was assayed in duplicate for these compounds.

Melilotic acid was assayed by the method described by Akeson et al. (1). In this procedure the melilotic acid in acid-hydrolyzed plant extracts was first separated from interfering compounds by paper chromatography. Melilotic acid bands from the chromatograms were then eluted, and eluates were reacted with diazotized *p*-nitroaniline to produce a colored solution. Intensity of the color furnished a measure of the melilotic acid content. For seed and greenhouse-grown material, five determinations of melilotic acid were made on each extract, while four determinations were made on each extract prepared from field-grown plants. Values from separate determinations on a single extract were averaged for presentation in this report.

RESULTS

Assay of Seed

The contents of melilotic acid, *o*-coumaric acid, and coumarinic acid in seed of three phenotypes of sweetclover are listed in Table 2. Percentages of each of the three compounds in seed of the *Cu* phenotypes were higher than in seed of *cu* plants. Contents of melilotic acid were intermediate between contents of *o*-coumaric and coumarinic acids.

Assay of Various Tissues from Greenhouse-grown Plants

Expressed as percentages of dry weight, levels of melilotic acid, *o*-coumaric acid, and coumarinic acid were highest in leaf tissue, intermediate in stem tissue, and lowest in the roots (Table 3). Percentages of each of the compounds were higher in young leaves and stems than in older tissues of the same type. Age-related differences in *o*-coumaric acid percentage were particularly striking. In general, coumarinic acid percentage was highest, *o*-coumaric acid was intermediate, and melilotic acid was lowest within each of the various tissues tested.

Table 1—Description of samples used in assays of melilotic acid, *o*-coumaric acid, and coumarinic acid in various parts of greenhouse-grown sweetclover plants.

Sample No.	Description	Dry wt., mg. /leaflet	Sample No.	Description
1	Leaflets tightly pinched	0.27	6	Petioles from leaves sampled
2	Leaflets partly open	0.76	7	Stems - upper portion, 4 cm. long
3	Leaflets fully open	1.92	8	Stems - middle portion, 5 to 10 cm. long
4	Leaflets fully expanded	2.92	9	Stems - basal portion, 6 to 7 cm. long
5	Leaflets - older	3.50	10	Roots

Table 2—Levels of melilotic acid, *o*-coumaric acid, and coumarinic acid in seed representing 3 phenotypes of sweetclover.

Variety or strain	Phenotype	Content (percentage of dry wt.)		
		Melilotic acid	*o*-Coumaric acid	Coumarinic acid
Evergreen	CuB	0.120	0.048	1.49
Spanish	CuB	0.100	0.048	1.32
W-7	Cub	0.071	0.048	1.36
Denta	cu-	0.048	0.007	0.27
Cumino	cu-	0.030	0.009	0.22

Table 3—Contents of melilotic acid, *o*-coumaric acid, and coumarinic acid in leaves, stems and roots of greenhouse-grown sweetclover plants of genotype ***CuCuBB***.

Sample No.	Melilotic acid		*o*-Coumaric acid		Coumarinic acid	
	%*	γ/leaflet	%	γ/leaflet	%	γ/leaflet
1	0.171	0.47	3.03	8.4	3.17	8.8
2	0.128	0.97	2.28	17.4	3.88	29.4
3	0.089	1.71	1.43	27.5	3.45	66.0
4	0.062	1.82	0.63	18.3	3.11	90.9
5	0.055	1.92	0.36	12.7	2.49	87.7
6	0.077		0.21		1.54	
7	0.070		0.24		1.31	
8	0.035		0.05		0.52	
9	0.026		0.03		0.34	
10	0.032		0.03		0.19	

* All percentages are calculated on the basis of dry weight.

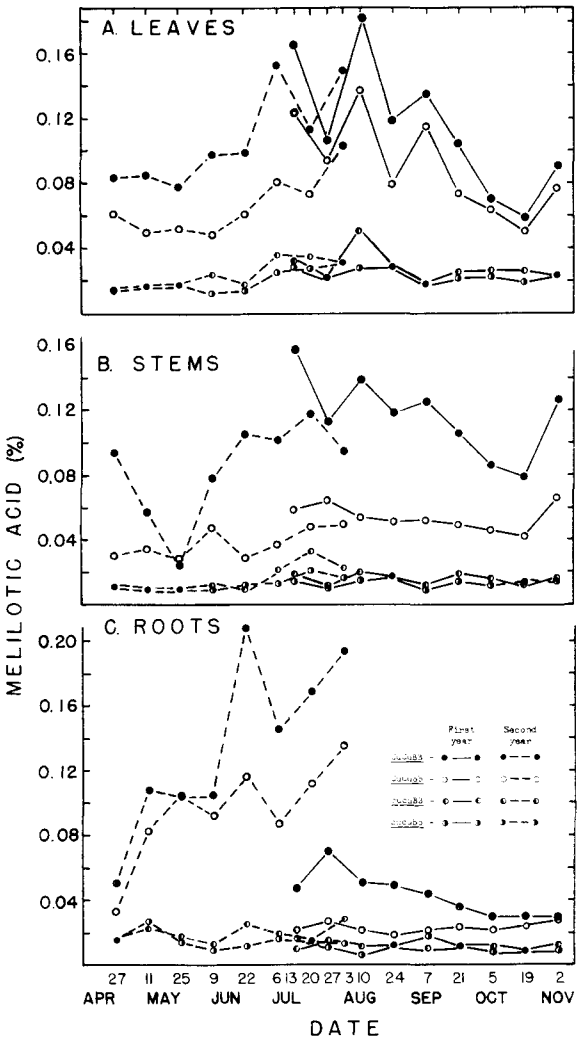

Figure 1—Levels of melilotic acid in tissues from 4 genotypes of sweetclover sampled periodically throughout the life cycle.

On the basis of quantity of compound per leaflet, contents of melilotic acid and coumarinic acid increased with age of the young leaves and appeared to level off as the leaves attained full expansion. Content of *o*-coumaric acid per leaflet, on the other hand, increased with age of the leaves to the fully opened stage and then decreased with further aging.

Seasonal Variation in Field-grown Plants

Melilotic acid—As shown in Figure 1, melilotic acid percentages were higher in all tissues of *CuCu* plants than in those of *cucu* plants. Furthermore, leaves, stems, and roots of *CuCuBB* plants were generally higher in content of melilotic acid than the corresponding parts of *CuCubb* plants.

No consistent seasonal changes were observed in the melilotic acid contents of *cucu* plants. In *CuCu* plants, on the other hand, reasonably consistent trends were observed. Thus, in leaves, stems, and roots of first-year plants of the *CuCuBB* genotype, melilotic acid percentage was relatively

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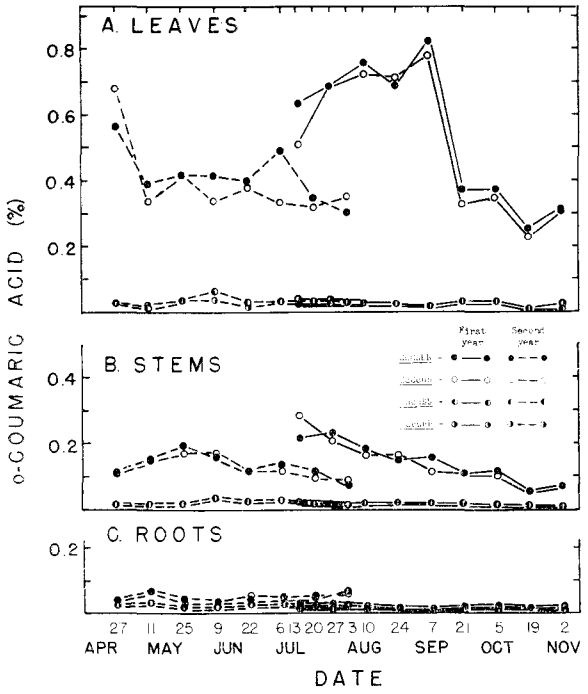

Figure 2—Levels of o-coumaric acid in tissues from 4 genotypes of sweetclover sampled periodically throughout the life cycle.

high in the samples harvested during July and August, and samples harvested between early September and mid-October were progressively lower in content. A similar pattern was observed in leaves of *CuCubb* plants, but little variation was noted in stems and roots. In leaves and stems of *CuCu* plants, a sharp increase in melilotic acid content was observed in late fall. This increase was not found in roots. Several nights of below-freezing temperatures during this late-fall period may have had some influence on melilotic acid levels. In the second year of growth, melilotic acid percentage was low in leaves and roots of *CuCu* plants in early spring and increased as the plants reached maturity. The relative increase in roots was much greater than that in leaves. In stems of *CuCubb* plants, little variation was noted while in stems of *CuCuBB* plants, melilotic acid percentage was high in early spring, decreased to a minimum on May 25, and then increased until the plants were mature.

o-Coumaric acid—With respect to content of *o*-coumaric acid (Figure 2), all samples from *cucu* plants were extremely low and little seasonal variation was noted. Appreciable quantities of *o*-coumaric acid were found in leaves and stems of *CuCu* plants, but very little was observed in root samples. *CuCuBB* plants were very similar to *CuCubb* plants in content of *o*-coumaric acid at most sampling dates. In the first year of growth, leaves from *CuCu* plants increased slightly in percentage of *o*-coumaric acid to a maximum in late August or early September and then decreased sharply during September and October. In the first-year stems, highest values were observed at the first sampling dates in July, and contents decreased gradually until late October and November. In the second year of growth, percentages of *o*-coumaric acid in leaves were highest at the first sampling date in spring, decreased sharply to the second sampling date, and then remained relatively constant to maturity. Contents of stems increased slightly in early

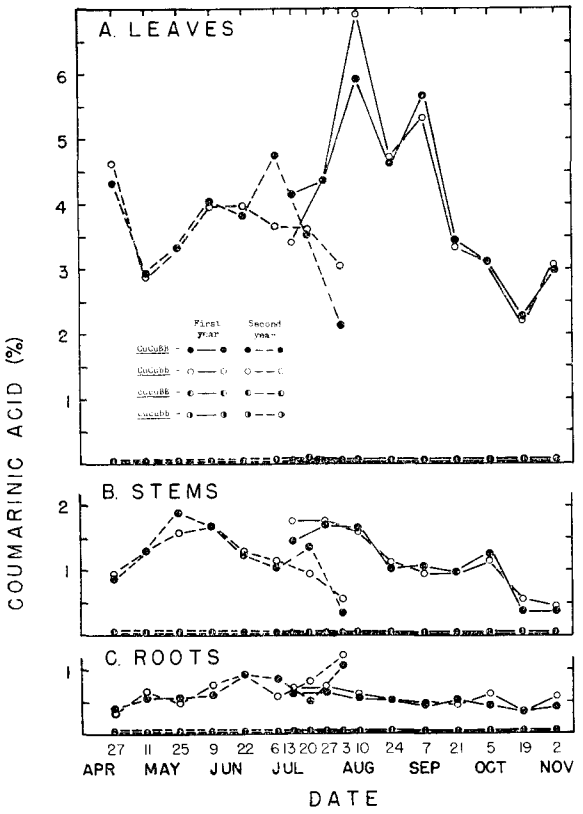

Figure 3—Levels of coumarinic acid in tissues from 4 genotypes of sweetclover sampled periodically throughout the life cycle.

spring to a maximum on May 25 and then slowly decreased.

Coumarinic acid—As indicated by the data in Figure 3, coumarinic acid values in all parts of *CuCu* plants at all sampling dates were much higher than those in *cucu* plants, and the *CuCuBB* and *CuCubb* genotypes were similar to each other with respect to content of this compound. In the first season of growth, percentages in leaves were high at the earliest sampling date in July, increased somewhat during August and early September, and decreased rapidly during the remainder of the growing season. Levels in first-year stems were highest at the first sampling dates in July and gradually decreased until late fall. Contents in first-year roots remained relatively uniform. In the second year of growth, the most consistent changes in content of coumarinic acid in leaves and stems of the *CuCu* genotypes were the decreases associated with advancing age of the plant. These decreases were preceded by an earlier rise in content in the stem samples, and by some early-season fluctuation in the samples of leaf tissue. The content of roots was low early in the season and increased gradually as the plants matured.

Seasonal averages—Average percentages of melilotic acid, *o*-coumaric acid, and coumarinic acid in leaves, stems, and roots from first- and second-year plants of the four genotypes are presented in Table 4. The data on which the means in this table were based were subjected to Duncan's new multiple-range test (16). Results of this test indicated that, with respect to contents of each of the three compounds, all parts of both first- and second-year *CuCu* plants were significantly higher than corresponding parts of *cucu*

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Table 4—Average percentages of melilotic acid, *o*-coumaric acid, and coumarinic acid in leaves, stems, and roots of first- and second-year sweetclover plants representing 4 genotypes.

Type of tissue and genotype	Mean percentage ± S. E. (dry-weight basis)		
	Melilotic acid	o-Coumaric acid	Coumarinic acid
First-year growth			
Leaves			
CuCuBB	0.114 ± 0.010	0.544 ± 0.071	4.056 ± 0.413
CuCubb	0.090 ± 0.010	0.511 ± 0.072	4.047 ± 0.481
cucuBB	0.028 ± 0.003	0.022 ± 0.003	0.037 ± 0.006
cucubb	0.023 ± 0.001	0.027 ± 0.003	0.035 ± 0.007
Stems			
CuCuBB	0.116 ± 0.008	0.144 ± 0.020	1.102 ± 0.162
CuCubb	0.054 ± 0.003	0.142 ± 0.023	1.151 ± 0.162
cucuBB	0.016 ± 0.001	0.019 ± 0.001	0.020 ± 0.003
cucubb	0.013 ± 0.001	0.018 ± 0.002	0.022 ± 0.003
Roots			
CuCuBB	0.043 ± 0.005	0.023 ± 0.002	0.521 ± 0.034
CuCubb	0.024 ± 0.002	0.022 ± 0.002	0.570 ± 0.042
cucuBB	0.011 ± 0.001	0.015 ± 0.001	0.012 ± 0.002
cucubb	0.011 ± 0.001	0.015 ± 0.001	0.012 ± 0.001
Second-year growth			
Leaves			
CuCuBB	0.107 ± 0.010	0.413 ± 0.029	3.611 ± 0.260
CuCubb	0.066 ± 0.007	0.393 ± 0.040	3.644 ± 0.198
cucuBB	0.024 ± 0.003	0.032 ± 0.005	0.054 ± 0.013
cucubb	0.020 ± 0.002	0.028 ± 0.008	0.047 ± 0.009
Stems			
CuCuBB	0.084 ± 0.011	0.134 ± 0.013	1.215 ± 0.164
CuCubb	0.038 ± 0.003	0.127 ± 0.011	1.175 ± 0.126
cucuBB	0.016 ± 0.003	0.022 ± 0.003	0.037 ± 0.009
cucubb	0.012 ± 0.002	0.021 ± 0.022	0.037 ± 0.009
Roots			
CuCuBB	0.136 ± 0.019	0.052 ± 0.005	0.703 ± 0.081
CuCubb	0.096 ± 0.011	0.049 ± 0.005	0.733 ± 0.098
cucuBB	0.020 ± 0.002	0.031 ± 0.002	0.008 ± 0.002
cucubb	0.015 ± 0.002	0.028 ± 0.001	0.003 ± 0.001

plants. Within the *cucu* genotype, *BB* plants did not differ significantly from *bb* plants in level of the compounds. Similarly, *CuCuBB* plants did not differ significantly from *CuCubb* plants in contents of *o*-coumaric and coumarinic acids. However, leaves, stems, and roots of first- and second-year *CuCuBB* plants contained significantly more melilotic acid than comparable parts of *CuCubb* plants.

DISCUSSION

The study of greenhouse-grown *CuCuBB* plants provided evidence that the plant part sampled and the relative age of the tissue assayed had a strong influence on levels of melilotic acid, *o*-coumaric acid, and coumarinic acid. In leaves, the richest source of the three compounds, the apparent association between leaflet size and contents of the compounds indicated that all three compounds were synthesized most rapidly during leaf expansion. This observation is in agreement with a previous report concerning *o*-coumaric and coumarinic acids in sweetclover (6).

The influence of the *Cu/cu* alleles on levels of the three compounds was clearly demonstrated in the study of field-grown plants. For example, as indicated by the mean values in Table 4, leaves of the *CuCu* genotype were higher than *cucu* leaves in contents of melilotic acid, *o*-coumaric acid, and coumarinic acid by factors of 3 to 5, 13 to 25, and 67 to 116, respectively. Despite the differences in magnitude of effect, the evidence supports the suggestion that the three compounds are metabolically related and that the *cu*-controlled step in the biosynthetic pathway precedes the formation of all three compounds.

The apparent influence of the *B/b* alleles on melilotic acid content is particularly interesting, since no effect of this pair of alleles on levels of *o*-coumaric acid and coumarinic acid was detected. The work of Kosuge and Conn (10, 12) demonstrating that sweetclover shoots could convert administered coumarin to melilotic acid and the observation of Schaeffer et al. (14) that preparations of *BB* leaves were highly active with respect to β-glucosidase whereas *bb* leaf preparations were virtually inactive, together suggest the basis for a possible explanation of the observed effect of the *B/b* alleles on melilotic acid synthesis. Thus, in *CuCuBB* plants, one might postulate that β-glucosidase hydrolyzed a small portion of the coumarinyl glucoside to produce coumarinic acid which, under the conditions existing in the tissues, would be expected to lactonize to form coumarin. This coumarin could be hydrogenated to form dihydrocoumarin, and dihydrocoumarin could be hydrolyzed to yield melilotic acid as described by Kosuge and Conn (12). A glucose residue could then be added to the melilotic acid molecule to produce melilotyl glucoside, the compound normally found in sweetclover tissues. In *CuCubb* plants, on the other hand, reduced β-glucosidase activity would be expected to result in reduced formation of coumarin, and thus in lowered levels of melilotic acid. A disparity exists, however, between the extents to which activity of β-glucosidase and content of melilotic acid are reduced by the action of the *b*-allele. Preparations of *BB* leaves were several hundred times as active with respect to β-glucosidase as preparations of *bb* leaves (14), but leaves of the 2 genotypes differed by less than a factor of 2 in melilotic acid content (Table 4). If melilotic acid can be synthesized only by a pathway involving β-glucosidase-mediated hydrolysis of coumarinyl glucoside, the relatively high levels of melilotic acid observed in *CuCubb* plants are surprising. In view of the high melilotic acid contents of *CuCubb* plants, the existence of an alternate biosynthetic pathway is suggested. This alternate pathway might reasonably be expected to involve the hydrogenation of *o*-coumaryl glucoside or coumarinyl glucoside to produce melilotyl glucoside.

SUMMARY

Percentages of melilotic acid, *o*-coumaric acid, and coumarinic acid were highest in leaves, intermediate in stems, and lowest in roots of greenhouse-grown sweetclover plants of the *CuCuBB* genotype. In leaves, percentages of each of the three compounds decreased as the leaves grew older, but amounts per leaflet increased with age except for content of *o*-coumaric acid, in which a brief increase was followed by a sharp decrease. Seed from plants of the *Cu* phenotype was higher in content of each of the three compounds than seed from *cu* plants. Levels of *o*-coumaric and coumarinic acids were much lower in seed than in young leaves.

First- and second-year field-grown plants of the *CuCuBB*, *CuCubb*, *cucuBB*, and *cucubb* genotypes were sampled at intervals throughout the 1961 growing season. Leaves, stems, and roots were assayed for melilotic acid, *o*-coumaric acid, and coumarinic acid. Melilotic acid content was lowest in each of the tissues assayed, and percentages of each of the three compounds were considerably higher in *CuCu* plants than in plants of the *cucu* genotype. The *B/b* alleles appeared to influence melilotic acid synthesis since *CuCuBB* plants contained significantly more melilotic acid than *CuCubb* plants. Levels of *o*-coumaric acid and coumarinic acid were not affected by the *B/b* alleles. Contents of the three compounds were found to vary considerably during the growing season.

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