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Occurrence of *o*-Hydroxycinnamic Acid in Species of *Melilotus* and *Trigonella*

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Occurrence of *o*-Hydroxycinnamic Acid in Species of *Melilotus* and *Trigonella*[1]

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OBERMAYER (9) in 1913 developed the first quantitative method for the determination of coumarin content in sweetclover. Since that time a variety of more refined qualitative and quantitative methods have demonstrated the presence of high levels of coumarin in *Melilotus alba* Desr. and *M. officinalis* (L.) Lam., the two sweetclover species most widely grown for soil improvement, pasture, hay, and silage. In 1933, Suvorov (12) reported that *M. dentata* (Waldst. and Kit.) Pers. contained an insignificant amount of coumarin, and R. A. Brink (1) independently reported the discovery of a low-coumarin strain of *Melilotus* which was later also identified as an annual form of the typically biennial *M. dentata.* Smith (11) was successful in transferring to *M. alba* the low-coumarin character of *M. dentata,* and genetic studies by Goplen et al. (2) demonstrated that this character is governed by a single pair of alleles, designated *Cu/cu.* The *cu* gene is the basis for the low-coumarin varieties, Cumino and Denta. Information concerning the coumarin content of other species of *Melilotus* is extremely limited. V. C. Brink,[3] using a qualitative colorimetric method, reported that only *M. dentata* and *M. segetalis* (Brot.) Seringe were consistently low in coumarin among 13 species of *Melilotus* that were tested. Suvorov (13) reported coumarin percentages from quantitative colorimetric determinations of plants representing 14 species of *Melilotus.* Contents were highest in *M. alba* and *M. officinalis,* lowest in *M. dentata* and *M. sulcata* Desf. and intermediate in the other species assayed. Suvorov also reported that most species of *Trigonella,* a genus closely related taxonomically to *Melilotus,* contain coumarin.

Within recent years, investigators have recognized that free coumarin does not exist in appreciable quantities in normal, intact sweetclover plants (3, 10). The plant constituents previously assayed as coumarin are actually the glucosides of *o*-coumaric acid (*trans-o*-hydroxycinnamic acid) and coumarinic acid (*cis-o*-hydroxycinnamic acid) (3, 7). Kosuge and Conn (8) have desribed a β-glucosidase obtained from *M. alba* that readily hydrolyzes the β-glucoside of coumarinic acid but is virtually inert toward the β-glucoside of *o*-coumaric acid. This fact is utilized by Haskins and Gorz (4) in one method (Method II) of assaying for the *cis* and *trans* isomers of *o*-hydroxycinnamic acid and is also the basis for an improved paper method for the qualitative testing of plant tissues for the presence of coumarinyl glucoside and β-glucosidase activity.[4] With these advances in knowledge and techniques, a study of *o*-hydroxycinnamic acid content and β-glucosidase activity in all available species of *Melilotus* and *Trigonella* seemed in order.

Objectives of the study reported in this paper were to (a) characterize available species of *Melilotus* and *Trigonella* as to content of *o*-hydroxycinnamic acid, (b) evaluate qualitatively β-glucosidase activity in these same species, (c) obtain information concerning the taxonomic relationships of the species under study, and (d) search for a gene for low content of *o*-hydroxycinnamic acid that is different from the *cu* gene obtained from *M. dentata.*

MATERIALS AND METHODS

Sources of seed of the various species of *Melilotus* and *Trigonella* are shown in Table 1. Only 1 accession was grown of each species of *Trigonella* while 2 representative[5] accessions were grown of each *Melilotus* species for which more than 1 lot of seed was available.

For each accession, approximately 10 seeds were planted in each of three 1-pint, plastic-coated milk cartons containing silica sand. Water was used for initial moistening of the sand following

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[2] Research Geneticist, Crops Research Division, ARS, USDA, and Professor of Agronomy, University of Nebraska, respectively. The authors wish to thank George Stevenson and Hugo Gross, Canada Dept. of Agriculture, Brandon, Manitoba, Canada, for identifying and supplying seed of many of the species of *Melilotus* and *Trigonella* used in this study. The technical assistance of Larry Williams and Dean Whited also is gratefully acknowledged.

[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.

[4] Unpublished data, H. J. Gorz and F. A. Haskins.

[5] Information concerning representative accessions of the various species of *Melilotus* was supplied by George Stevenson, Canada Dept. of Agriculture, Brandon, Manitoba, Canada, in a personal communication.

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planting of the seed, but after seedling emergence, a modified[6] Hoagland and Arnon's (5) nutrient solution No. 2 was added by subirrigation as needed. All plants were grown in growth chambers at a constant temperature of 80° F. and a 50% relative humidity, under continuous exposure to cool white fluorescent lights producing an intensity of approximately 1500 foot-candles at the level of the plants.

Samples for the initial assay were usually harvested when plants were in the late bud or early flower stage. Ten vigorous plants were selected for individual sampling from the 3 cartons of each accession. Depending upon leaf size, the samples consisted of 1 to 3 young leaves that were slightly less than fully expanded. Leaves at this stage of development were chosen to maximize the differences between species. Immediately after removal from the plant, samples were weighed to the nearest 0.1 mg. on a direct-reading balance. Mid-leaflets, used for the determination of dry-matter percentage, were weighed separately from the side leaflets, which were extracted and assayed for content of *o*-hydroxycinnamic acid. Extraction was accomplished by dropping the sample from each plant into 10 ml. of boiling water, and autoclaving for 15 minutes at approximately 15 psi. Autoclaved extracts were cooled, leaflets were removed and discarded, and the extracts were stored in a freezer for later assay.

The extracts were assayed for *cis*- and *trans-o*-hydroxycinnamic acid by the nonenzymatic fluorometric procedure described by Haskins and Gorz (4). A Turner Model 110 Fluorometer was used for the fluorescence readings, and the dilutions used in the published procedure were altered by a factor of about 30 to take advantage of the high sensitivity of this instrument. Readings on nonhydrolyzed extracts were also taken for the determination of free *o*-hydroxycinnamic acid, but in no case was an appreciable quantity of the free acid detected. Thus, all subsequent references to *o*-hydroxycinnamic acid actually refer to the aglycone equivalent of *o*-coumaryl and coumarinyl glucosides.

A qualitative observation of β-glucosidase activity was made for each of the plants used in the *o*-hydroxycinnamic acid assay. One-inch squares were ruled on 6- × 11-inch sheets of Whatman No. 1 filter paper and a portion of a young leaf from each sampled plant was crushed on its appropriate square. When the 10 plants of 1 accession had been sampled, a drop of substrate, consisting of a water extract of sweetclover leaves, was placed on each sample spot. The leaf extract contained approximately 0.12 mg. of glucosidically bound *o*-hydroxycinnamic acid per ml., and about 74% of the compound was present as the *cis* isomer. After the sheets were dried at room temperature, each spot was treated with a drop of 2.5 *N* NaOH and the sheets were placed under an ultraviolet light for approximately 5 minutes. The presence or absence of β-glucosidase activity was denoted by the presence or absence of a bright, yellowish-green fluorescence around the sample spots.

After completion of sampling for *o*-hydroxycinnamic acid content and β-glucosidase activity, height to the apical bud on the main stem of the plant was measured, and all plants were then moved to the greenhouse where natural daylength was supplemented with incandescent light to give a total photoperiod of 18 hours. After seven days, the plants were returned to the laboratory where additional samples for assays of *o*-hydroxycinnamic acid and β-glucosidase were taken. All sampling, extraction, and assay procedures were identical with those described for the first samples.

RESULTS AND DISCUSSION

Data on *o*-hydroxycinnamic acid content, β-glucosidase activity, and height from 19 species of *Melilotus* and 20 species of *Trigonella* are shown in Table 1. Substantial differences in *o*-hydroxycinnamic acid levels were observed among the various species. In the genus *Melilotus,* essentially no *o*-hydroxycinnamic acid was found in *M. dentata, M. messanensis* (L.) All., *M. segetalis,* and *M. sulcata.* The 15 remaining species of *Melilotus* all contained substantial amounts of *o*-hydroxycinnamic acid. Contents were highest in *M. polonica* (L.) Desr., and lowest in *M. infesta* Guss., where the amount observed was less than 50% of the levels found in most of the other 14 species.

[6] The amounts of potassium nitrate and calcium nitrate used were, respectively, 1.5 and 2.0 times the published amounts.

The intermediate level in *M. infesta* is of interest since similar percentages were found in several *Trigonella* species but not in other species of *Melilotus.* These levels may indicate that *M. infesta* is more closely related to some of the *Trigonella* species than are the remaining species of *Melilotus.* Evidence supporting this view is found in the recent discovery[7] that plants of *M. infesta* and several species of *Trigonella* were virtually immune to the sweetclover weevil (*Sitona cylindricollis* Fahr.) while plants of all other *Melilotus* species were heavily fed upon.

Seven species of *Trigonella* contained significant amounts of *o*-hydroxycinnamic acid, but only *T. calliceras* Fisch., *T. cretica* (L.) Boiss., and an unnamed species contained the high levels characteristic of most *Melilotus* species. *T. anguina* Delile, *T. arabica* Delile, *T. balansae* Boiss. and Reut., and *T. uncinata* Soland. contained intermediate levels that were somewhat similar to the level observed in *M. infesta.* Essentially no *o*-hydroxycinnamic acid was found in the remaining 13 species of *Trigonella,* despite Suvorov's (13) report that "most" *Trigonella* species contain coumarin.

Extracts from plants of each of the species of *Melilotus* (except *M. polonica*) and *Trigonella* which contained appreciable amounts of *o*-hydroxycinnamic acid were chromatographed on 9- × 11-inch sheets of Whatman No. 1 filter paper using the 4 solvents described by Kosuge (7). Authentic samples of the β-glucosides of *cis*- and *trans-o*-hydroxycinnamic acid, supplied by Drs. Kosuge and Conn, University of California at Davis, were included on each sheet. Convincing evidence was obtained that all extracts contained glucosidically bound *o*-hydroxycinnamic acid.

In all species sampled, extracts prepared from plants grown in growth chambers contained more than 90% of the *o*-hydroxycinnamic acid as the *trans* isomer. Extracts from the same plants grown in a greenhouse for 1 additional week averaged only 35% as the *trans* isomer, but varied widely in percentage, presumably due to variations in light intensity from day to day in the greenhouse. These findings support previous suggestions (4, 6) that the *trans* to *cis* conversion in the plant is a nonenzymatic, photochemical reaction and indicate that the radiant energy supplied by cool white fluorescent lamps is relatively inefficient in effecting the conversion. The lack of appreciable quantities of the *cis* isomer in leaf extracts from the growth chambers further indicates that no effective isomerization of the *trans* isomer occurred in leaves of any of the species assayed.

As indicated in the introductory section of this paper, the *cu* allele apparently is responsible for the low *o*-hydroxycinnamic acid content of *M. dentata.* The possibility that this same gene is responsible for the low content in other species of *Melilotus* and *Trigonella* was not investigated in the present study. Attempts are in progress to make interspecific and intergeneric crosses which, if successful, will permit the investigation of this possibility and which may lead to the incorporation of new, useful germ plasm into the agronomic species, *M. alba* and *M. officinalis.*

The activity of β-glucosidase was readily detected in leaves of all species of *Melilotus* and *Trigonella* that contained appreciable quantities of *o*-hydroxycinnamic acid. No activity was found in the species that were essentially free of *o*-hydroxycinnamic acid. While genetic stocks are available in *M. alba* which contain all possible combina-

[7] Unpublished data, G. R. Manglitz and H. J. Gorz.

Published in Crop Science (March-April 1964) v. 4, no. 2

Table 1. Mean levels (dry weight basis) of total *o*-hydroxycinnamic acid (*o*-HCA) and presence or absence of β-glucosidase activity in 19 species of ***Melilotus*** and 20 species of ***Trigonella.***

Genus and species	Source of seed	Data from growth chambers				Data from greenhouse			β-glucosidase activity
		Age, days	Height, cm.	Total o-HCA*, % ± S. E.	% trans†	Height, cm.	Total o-HCA*, % ± S. E.	% trans†	
Melilotus-(Eumelilotus)‡									
M. alba Desr.	Hubam (Annual)	36	39	6.67 ± .498	96	51	4.07 ± .761	32	+
M. alba Desr.	Spanish	29	34	5.32 ± .338	96	47	4.45 ± .767	28	+
M. altissima Thuill.	Bdn. 58-242	36	34	6.28 ± .367	93	42	4.07 ± .785	29	+
M. altissima Thuill.	Bdn. 963	34	33	5.69 ± .919	93	45	3.45 ± .965	34	+
M. dentata (Waldst. & Kit.) Pers.	Nebr. A205-2(x)	38	41	0		48	0		-
M. dentata (Waldst. & Kit.) Pers.	Wis. P784-3 (Ann.)	48	53	0		62	0		-
M. hirsuta Lipsky	Bdn. 58-44	42	30	6.04 ± .751	94	35	5.53 ± .361	40	+
M. hirsuta Lipsky	Nebr. E6-6(x)	38	23	6.20 ± .555	94	30	6.59 ± .351	47	+
M. officinalis (L.) Lam.	Goldtop	31	33	5.38 ± .521	97	45	5.01 ± .164	33	+
M. officinalis (L.) Lam.	Madrid	34	33	5.30 ± .397	98	40	2.59 ± .183	18	+
M. polonica (L.) Desr.	Nebr. C31-2(x)	48	42	7.30 ± .489	97	52	7.94 ± .904	53	+
M. polonica (L.) Desr.	Nebr. D123-2(x)	48	44	8.31 ± .502	97	51	6.71 ± .865	58	+
M. suaveolens Ledeb.	Golden Annual	42	44	4.71 ± .512	95	48	3.55 ± .790	34	+
M. suaveolens Ledeb.	Bdn. 59-52	36	27	3.99 ± .716	97	33	2.40 ± .453	28	+
M. taurica (Bieb.) Seringe	Bdn. 546	62§	33	7.54 ± .505	95	36	6.30 ± .831	39	+
M. taurica (Bieb.) Seringe	F. C. 23,281	62	39	5.74 ± .789	95	41	4.01 ± .612	47	+
M. wolgica Poir.	Bdn. 1022	41	55	7.45 ± .386	96	63	3.53 ± .489	29	+
M. wolgica Poir.	Bdn. (Brno)	41	68	7.76 ± .774	95	76	4.06 ± .365	30	+
Melilotus-(Micromelilotus)‡									
M. elegans Salzm.	Bdn. 61-134	52	79	6.02 ± .244	100	105	6.37 ± .129	31	+
M. indica (L.) All.	Bdn. 58-5	38	42	4.32 ± .319	97	62	4.55 ± .179	37	+
M. indica (L.) All.	Bdn. 525	45	52	6.29 ± .285	93	72	4.27 ± .492	22	+
M. infesta Guss.	Bdn. 61-98	43	36	2.35 ± .107	100	49	1.79 ± .114	30	+
M. italica (L.) Lam.	Bdn. 58-256	42	39	6.00 ± .157	98	49	4.20 ± .175	43	+
M. italica (L.) Lam.	Bdn. 523	42	39	5.98 ± .138	100	48	3.58 ± .163	39	+
M. macrocarpa Coss. & Durieu	Bdn. 61-97	57	26	6.17 ± .412	100	37	4.57 ± .424	56	+
M. messanensis (L.) All.	Bdn. 524	48	30	0		50	0		-
M. messanensis (L.) All.	Bdn. 859	48	37	0		58	0		-
M. neapolitana Ten.	Bdn. 58-245	43	40	6.99 ± .322	100	55	4.88 ± .461	28	+
M. segetalis (Brot.) Seringe	Bdn. 535	60	28	0		38	0		-
M. segetalis (Brot.) Seringe	Bdn. 863	66	21	0		31	0		-
M. speciosa Durieu	Bdn. 59-51	38	51	3.90 ± .170	100	62	6.50 ± .380	37	+
M. speciosa Durieu	Bdn. 536	31	39	5.71 ± .348	95	45	7.08 ± .486	36	+
M. sulcata Desf.	Bdn. 58-263	31	29	0		39	0		-
M. sulcata Desf.	Bdn. 1019	43	38	0		47	0		-
Trigonella									
T. anguina Delile	P.I. 227394	28	6	1.17 ± .103	99	15	1.46 ± .066	42	+
T. arabica Delile	Bdn. 60-80	28	23	2.36 ± .121	97	34	1.58 ± .094	29	+
T. arcuata Meyer	P.I. 222273	54	11	0		14	0		-
T. balansae Boiss. and Reut.	P.I. 222211	25	14	0.68 ± .068	100	26	0.77 ± .056	32	+
T. brachycarpa (Fisch.) Moris	P.I. 244326	49§	5	0		8	0		-
T. calliceras Fisch.	Bdn. 60-158	49	29	5.45 ± .216	99	35	6.42 ± .424	24	+
T. caerulea (L.) Seringe	P.I. 244288	33	26	0		37	0		-
T. corniculata L.	P.I. 244289	35	18	0		31	0		-
T. cretica (L.) Boiss.	Bdn. 60-159	35	10	4.63 ± .189	91	23	3.96 ± .343	16	+
T. foenum-graecum L.	Bdn. 60-81	40	20	0		34	0		-
T. gladiata Stev.	Bdn. 60-156	63§	3	0		6	0		-
T. kotschyi Boiss.	P.I. 206775	45§	22	0		32	0		-
T. monantha C.A. Mey.	P.I. 227677	45	14	0		21	0		-
T. monspeliaca L.	P.I. 227051	45	7	0		11	0		-
T. noeana Boiss.	P.I. 251412	45	15	0		24	0		-
T. polycerata L.	Bdn. 60-160	54	12	0		17	0		-
T. radiata (L.) Boiss.	Bdn. 60-188	49§	4	0		7	0		-
T. spicata Sibth. and Sm.	P.I. 206284	54	19	0		25	0		-
T. uncinata Soland.	P.I. 226533	25	12	0.95 ± .127	97	24	0.72 ± .068	28	+
T. sp.	Bdn. 61-48	63§	38	6.07 ± .510	98	42	6.14 ± .312	32	+

* Sensitivity of the method was such that o-hydroxycinnamic acid values recorded as zero represent levels not exceeding 0.03%. † (Mean content of trans-o-HCA ÷ by mean content of total o-HCA) × 100. ‡ Schulz, O. E. Monographie der Gattung Melilotus. Bot. Jahrb. 29:660-735. 1901. § Sampling in these species was done before any of the plants were in the bud stage, either because of unthrifty appearance of the plants or extreme lateness in flowering.

tions of high and low levels of *o*-hydroxycinnamic acid and β-glucosidase activity, it is of interest that none of the naturally occurring species varies from the high-high or low-low status. Perhaps plants containing the glucosides of *o*-hydroxycinnamic acid have no selective advantage without the concurrent presence of β-glucosidase activity.

SUMMARY

Leaf samples from available species of *Melilotus* and the closely related genus, *Trigonella,* were assayed for free as well as glucosidically bound *cis-* and *trans-o*-hydroxycinnamic acids. A qualitative evaluation of β-glucosidase activity also was made. In none of the species were appreciable quantities of the free acids detected. Of the 19 species of *Melilotus* sampled, 14 contained high levels of the *o*-hydroxycinnamic acid glucosides, one *(M. infesta)* was intermediate, and four were essentially free of these glucosides. The intermediate level in *M. infesta* is unlike that in any other species of *Melilotus,* but is similar to the contents observed in four species of *Trigonella.* Essentially no *o*-hydroxycinnamic acid was found in 13 species of *Trigonella,* but contents in three species were comparable with the high levels found in *Melilotus.* β-Glucosidase activity was observed in only those species of *Melilotus* and *Trigonella* that contained appreciable quantities of glucosidically bound *o*-hydroxycinnamic acid.

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