A Modified Procedure for Assay of Melilotic Acid in Sweetclover

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Kouge and Conn (5, 6) observed that excised sweetclover shoots effect a rapid conversion of administered coumarin to melilotic acid (o-hydroxyhydrocinnamic acid). Although doubt has been expressed as to the existence of molecular coumarin in intact sweetclover tissues (3, 8), the closely related compounds, o-coumaric acid and coumarinic acid (the trans and cis isomers, respectively, of o-hydroxycinnamic acid) are clearly present in glucosidic form (2, 4) and these compounds may possibly serve as normal precursors of melilotic acid. In previous studies on sweetclover (1) the glucosides of o-coumaric and coumarinic acids have been assayed together as bound coumarin, and the genetic control of bound coumarin level has been demonstrated. Because of the possible metabolic relationship between o-hydroxycinnamic and melilotic acids, genes influencing the derivation of these lines was described earlier (3). Briefly, the genotypes used in this study were Cu/Cu---high in o-hydroxycinnamic acid, high in o-glucosidase activity; Ca/Cu---high in o-hydroxycinnamic acid, high in β-glucosidase activity; cu/Cu---low in o-hydroxycinnamic acid, high in β-glucosidase activity; and cu/cu---low in o-hydroxycinnamic acid, lacking in β-glucosidase activity. Each genotype was represented by approximately 50 plants grown in flats of soil in the greenhouse.

PLANT MATERIAL AND PROCEDURE

Four lines of sweetclover (Melilotus albus Desr.) homozygous with respect to the Cu/cu and B/b alleles were used in this study. The derivation of these lines is described earlier (3). Briefly, the characters of the lines with respect to glucosidically-bound o-hydroxycinnamic acid and the activity of β-glucosidase are as follows: Cu/Cu---high in o-hydroxycinnamic acid, high in o-glucosidase activity; Ca/Cu---high in o-hydroxycinnamic acid, lacking in β-glucosidase activity; cu/Cu---low in o-hydroxycinnamic acid, high in β-glucosidase activity; and cu/cu---low in o-hydroxycinnamic acid, lacking in β-glucosidase activity. Each genotype was represented by approximately 50 plants grown in flats of soil in the greenhouse.

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The terminal 3- to 4-inch portions of several branches from each plant were removed and the cut ends of the stems were immediately immersed in water. These stems were then taken to the laboratory where the youngest fully-expanded leaf was harvested from each stem. The total sample consisted of approximately 2 g. (fresh weight) of young leaves for each genotype. One side-leaflet of each leaf was used in a composite sample for dry-weight determination, and the other side-leaflet and the mid-leaflet were used in a composite sample for extraction and assay.

Melilotic acid and melilotyl glucoside were extracted by dropping the leaflets into boiling water (15 ml/g. fresh tissue) to inactivate β-glucosidase and autoclaving immediately at 15 psi for 15 minutes. The resulting extract was filtered from the leaflets and centrifuged approximately 5 min. in a clinical centrifuge. The supernatant was used in subsequent steps of the procedure.

To 2 ml. of the supernatant, 1 ml. of 3.0 N HCl was added, and the solution was autoclaved for 30 min. at 15 psi to hydrolyze melilotyl glucoside. The hydrolyzed solution was partially neutralized by the addition of 1 ml. of 2.5 N NaOH, centrifuged to eliminate a precipitate which had formed, and chromatographed. Both hydrolyzed and nonhydrolyzed samples of extract were chromatographed on a 9 × 11 inch sheet of Whatman No. 1 filter paper. Aliquots chromatographed usually contained between 1.5 and 5% of melilotic acid, but these quantities do not represent the limits of the method. The aliquots were applied in a band along a line near the lower edge of the paper. In addition, a spot of the sample was made near one end of this same line. This spot was oversptotted with approximately 2% of authentic melilotic acid (obtained from the K and K Laboratories), to assist in later location of the melilotic acid band. Chromatograms were developed with an ascending solvent consisting of 3 volumes of n-propanol and 1 volume of 5% aqueous ammonia.

After ascent of the solvent, air-dried chromatograms were examined under ultraviolet light, and the yellowish-green fluorescent o-coumaric acid band was marked. Because this band was slightly below and clearly separated from the melilotic acid band, its position helped to locate the melilotic acid band. The edge of the sheet on which authentic melilotic acid had been chromatographed was sprayed with diazotized sulfanilic acid (10) and 5% NaHCO3. Color intensity was determined with a Bausch and Lomb Spectronic 20 colorimeter set for a wavelength of 490 mμ.

Because the chromatograph paper contains some materials which react with the color reagents, care was taken to use chromatogram sections approximately equal in size for elution. In addition, an eluate from a developed chromatogram to which no plant extract had been applied was used in preparing the colorimeter blank.

Melilotic acid equivalence values were read from a standard curve, and melilotic acid contents of the tissues were calculated on a dry-weight basis.

RESULTS

Adequacy of procedure—Efficiency of hydrolysis of melilotyl glucoside was tested by autoclaving portions of a plant extract representing the Cu/Cu genotype in NaOH and HCl under various conditions. For NaOH, concentrations of 0.8 N, 1.7 N, and 3.3 N were used, and the solutions were autoclaved for 30 min. For HCl, a concentration of 1.0 N and autoclaving times of 7, 15, 30, and 60 min. were employed. Melilotic acid levels observed following the 30-min. HCl treatment were higher than those resulting from the NaOH treatments and the HCl treatments of shorter duration, and approximately equal to those obtained from the longer HCl treatment. Thus, the 30-min. autoclav-