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Culture and *o*-Hydroxycinnamic Acid Content of Excised *Melilotus* Roots

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Culture and *o*-Hydroxycinnamic Acid Content of Excised *Melilotus* Roots[1]

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COUMARINIC and *o*-coumaric acids (respectively the *cis* and *trans* isomers of *o*-hydroxycinnamic acid) occur almost entirely as glucosides in intact sweetclover plants (5, 6). These compounds are most abundant in leaves, intermediate in level in stems, and least abundant in roots (1). Even in roots, however, total *o*-hydroxycinnamic acid contents of 0.5% (dry weight basis) are not uncommon. Sweetclover tissues also possess a β-glucosidase which removes glucose from coumarinyl glucoside when the plant tissue is disrupted (5, 8, 9). The resulting coumarinic acid spontaneously lactonizes yielding coumarin. Other studies have shown that both *o*-hydroxycinnamic acid content and β-glucosidase activity are influenced by genetic factors (2, 9).

Investigations employing C^{14}-labeled shikimic acid, phenylalanine, and *trans*-cinnamic acid have shown that in excised shoots of white-flowered sweetclover (*Melilotus alba* Desr.) the biosynthesis of *o*-coumaryl glucoside and coumarin involves the shikimic acid pathway (7). A similar biosynthetic pathway in root cultures of yellow-flowered sweetclover (*M. officinalis* (L.) Lam.) is suggested by the observation that in such cultures C^{14} from C^{14}-labeled phenylalanine was incorporated into coumarin (11).

In the first successful attempts to culture excised sweetclover roots, the roots grew slowly but could be cultured indefinitely (12). The present report deals with investigations of some of the factors influencing growth of excised sweetclover roots and with the production of *o*-hydroxycinnamic acid in isolated root cultures.

MATERIALS AND METHODS

Two biennial sweetclover varieties, 'Spanish' (white-flowered) and 'Goldtop' (yellow-flowered) were used in these studies. Both varieties are characterized by high levels of glucosidically bound *o*-hydroxycinnamic acid and β-glucosidase activity. Roots of these varieties were cultured on a modified White's medium (10) of the following composition (numbers indicate mg. of compound per liter of distilled water): NH_4NO_3, 400; $Ca(NO_3)_2 \cdot 4H_2O$, 144; KNO_3, 80; $MgSO_4 \cdot 7H_2O$, 72; $MnSO_4 \cdot H_2O$, 3.9; $ZnSO_4 \cdot 7H_2O$, 2.7; KI, 0.75; H_3BO_3, 1.6; KH_2PO_4, 38; KCl, 65; thiamine hydrochloride, 0.1; pyridoxine hydrochloride, 0.5; nicotinic acid, 0.5; glycine, 2.0; and Sequestrene NaFe (an iron chelate), 25. The carbon source was sucrose (20 g./l.). When solid medium was needed, Difco Bacto agar was added at a concentration of 15 g./l.

Roots for culturing were obtained initially from newly germinated seeds. The seeds were first submerged in 95% ethanol for 2 minutes, then in a 1.25% sodium hypochlorite solution (diluted Clorox) for 5 minutes, and finally they were rinsed 4 times with sterile distilled water. The seeds were then allowed to germinate on sterile moist paper towels in Petri dishes. After a 48-hour germination period the terminal 2-cm. portion was removed from the root of each seedling. These root portions were transferred immediately either to agar medium in 10-cm. Petri dishes or to liquid medium in 250-ml. Erlenmeyer flasks. Transfers could then be made from the new root growth on the agar to fresh agar plates or to liquid medium. One root clone was maintained on agar for over 18 months and through 40 passages without apparent change

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in rate or habit of growth. The transfers were most successful if the terminal 4- to 5-cm. portion of a rapidly growing root branch was used as the transfer segment. Such portions were characterized by a lustrous whiteness and by the presence of small branch roots 3 to 5 cm. from the tip. All transfers were made in a covered chamber that was moistened with dilute Clorox immediately before use.

Unless otherwise indicated, the following procedure for culturing the excised roots was used in the experiments reported here. Segments of Spanish sweetclover roots several passages removed from the original seedling root were transferred to 250-ml. Erlenmeyer flasks, each of which contained 100 ml. of liquid medium. Several segments having a total dry weight equivalent of 1.5 to 2.0 mg. were placed in each flask. Flasks were then incubated on a rotary shaker (New Brunswick Gyrotory)[3] operated at a speed of approximately 200 rpm for 3 weeks. The flasks were exposed to normal laboratory temperature (23 to 30° C.) and light during incubation.

After the incubation period, roots were extracted by immersion in boiling water followed immediately by autoclaving at 15 psi for 25 minutes. Water extracts were assayed for *o*-coumaric and coumarininc acids by the nonenzymatic fluorometric procedure described in an earlier communication (4). Fluorescence readings were made with the Turner model 110 fluorometer equipped with primary filter No. 7–60 and secondary filter No. 8.

Chromatographic investigations utilized Whatman No. 1 filter paper and an ascending solvent consisting of 3 volumes of n-propyl alcohol and 1 volume of 1% aqueous ammonia.

RESULTS AND DISCUSSION

Factors Affecting Growth of Excised Roots in Liquid Culture

Initial pH. By additions of 0.1 *N* NaOH and 0.1 *N* HCl to liquid medium, the pH series shown in Figure 1 was prepared. Three flasks were prepared for each of the 11 pH points shown in the figure. Three root segments with a total dry weight equivalent of approximately 1 mg. were added to each of the 33 flasks, and the flasks were incubated in the usual manner. Within the range from pH 4.5 to pH 7.0, no appreciable differences in type or amount of growth were observed. In those flasks where the initial pH was 8.0 or higher, roots developed a brown color, increased abnormally in diameter, and failed to form normal branch roots. In all flasks where the dry weight increase amounted to more than 10 mg., the pH of the medium after incubation was between 4.2 and 4.6 regardless of the initial pH.

Light exposure. Excised root tips were added to 12 flasks of medium. Six of the flasks were covered with aluminum foil to exclude light, and the remaining six flasks were left uncovered. The flasks were then incubated on the shaker under a bank of cool white fluorescent lamps which produced a light intensity of approximately 1,400 ft.-c. at the level of the flasks. The timer-controlled lights were turned on at 6:00 a.m. and off at 8:00 p.m. each day. Temperature measurements disclosed no difference between the medium in uncovered flasks and that in covered flasks. After the usual incubation period dry weights of the roots (mean ± SE) in the lighted and darkened flasks were 28.6 ± 4.4 mg. and 59.7 ± 8.6 mg., respectively. Although fluorescent light at an intensity of 1,400 ft.-c. apparently prevented optimal growth of the cultured roots, no adverse effects on growth were observed when roots were cultured in the ordinary light of the laboratory.

Duration of incubation. Excised roots were added to 10 flasks of medium and the flasks were incubated on the shaker. Two flasks were removed at the end of each week of incubation until all 10 flasks had been removed, and roots were dried and weighed. As shown in Figure 2, an initial lag period of about 1week was followed by a period of 4 weeks during which growth was approximately linear with time. Discoloration of roots and medium, observed after five weeks of incubation, indicated that some degradation of tissue had occurred and suggested that the period of linear growth rate had ended.

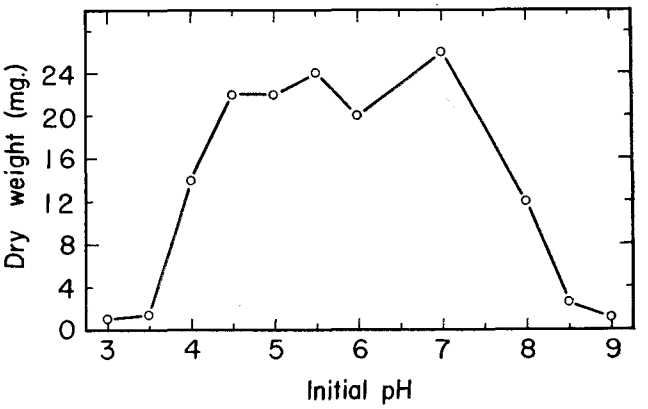

Figure 1. Influence of initial pH of the culture medium on growth of Spanish sweetclover roots in liquid culture. Each point represents the mean of 3 determinations.

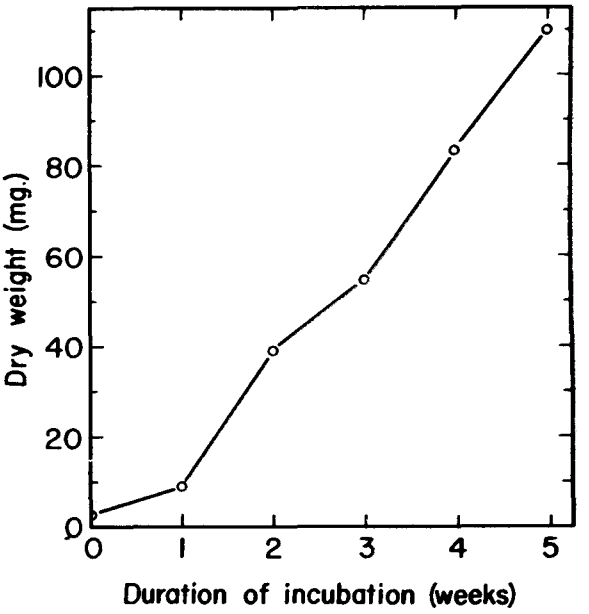

Figure 2. Influence of duration of incubation on growth of Spanish sweetclover roots in liquid culture. Each point represents the mean of 2 determinations.

o-Hydroxycinnamic Acid Content

As determined fluorometrically, the total *o*-hydroxycinnamic acid content of the roots in 6 culture flasks after 3 weeks of incubation was 0.024 ± 0.004% (mean ± SE, dry weight basis). The content in roots from chamber grown plants 4 weeks old, on the other hand, was 0.173 ± 0.015%. Several observations suggested that a major part of the small amount of fluorescence displayed by extracts of cultured roots was due to substances other than *o*-hydroxycinnamic acid. In ultraviolet light the fluorescence of an alkaline hydrolysate of cultured root extract

[3] Mention of specific instruments and companies is for identification only and does not imply endorsement by the U. S. Department of Agriculture.

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was grayish blue in contrast to the bright yellowish green fluorescence of *o*-coumaric acid in base. The fluorescence of an alkaline hydrolysate of cultured root extract was reduced by a 15-minute exposure of the extract to ultraviolet light, whereas the fluorescence of a hydrolyzed extract of roots taken from intact plants was increased by such exposure. Furthermore, chromatography of an extract of cultured roots failed to disclose any *o*-hydroxycinnamic acid glucoside. On chromatograms of extracts of roots from intact plants, this substance was readily detected as an ultraviolet (260 mμ)-absorbing band at Rf 0.3 to 0.4.

The cultured roots referred to in the preceding paragraph came from a well-established clone that had been carried through numerous transfers on artificial medium. To investigate the possible accumulation of *o*-hydroxycinnamic acid in roots taken directly from germinating seeds, the following experiment was done. Seeds of the Goldtop variety were germinated and terminal portions averaging 2 cm. in length were excised from the seedling roots. Thirty flasks of liquid medium were prepared, 5 root segments were introduced into each flask, and the flasks were incubated with shaking. At each of the times indicated in Figure 3, six flasks were removed from the shaker and roots were weighed and extracted. As shown in the figure, *o*-hydroxycinnamic acid content (% of dry weight) decreased rapidly during incubation of the roots. This decrease resulted from a rapid increase in root weight while the quantity of *o*-hydroxycinnamic acid remained unchanged. Thus the mean values (± SE) for fresh weight and *o*-hydroxycinnamic acid contents of the root segments initially introduced into each flask were 42 ± 8 mg. and 0.23 ± 0.02 micromoles, respectively, and corresponding values per flask after 2 weeks of growth were 300 ± 80 mg. and 0.24 ± 0.02 micromoles. No net change in quantity of *o*-hydroxycinnamic acid was observed during the first 3 weeks of incubation, and data based on roots cultured more than 3 weeks were considered unreliable because of the interference of extraneous fluorescent substances.

In another experiment excised roots from germinated seeds were incubated with the *o*-hydroxycinnamic acid precursors, shikimic acid, phenylalanine, and *trans*-cinnamic acid. No net increase in quantity of *o*-hydroxycinnamic acid was observed in any of the treatments, despite the previously cited report (11) that excised sweetclover roots incorporated C^{14} from C^{14}-labeled phenylalanine into coumarin.

The obvious conclusion to be drawn from the experiments reported here is that although excised sweetclover roots grow very well under the conditions used, they do not effect a net synthesis of *o*-hydroxycinnamic acid under these conditions. Unfortunately, the artificial establishment of conditions equivalent to those existing in and around the root of an intact plant is impossible, and therefore the behavior of cultured roots cannot furnish conclusive proof regarding the behavior of roots in their normal habitat. As previously noted (1) roots of intact sweetclover plants contain appreciable quantities of *o*-hydroxycinnamic acid. The present work, like previous studies employing grafted plants (3), does not permit an unequivocal answer to the question as to whether the compound is normally produced *in situ* in the roots or is translocated to the roots from the upper parts of the plant.

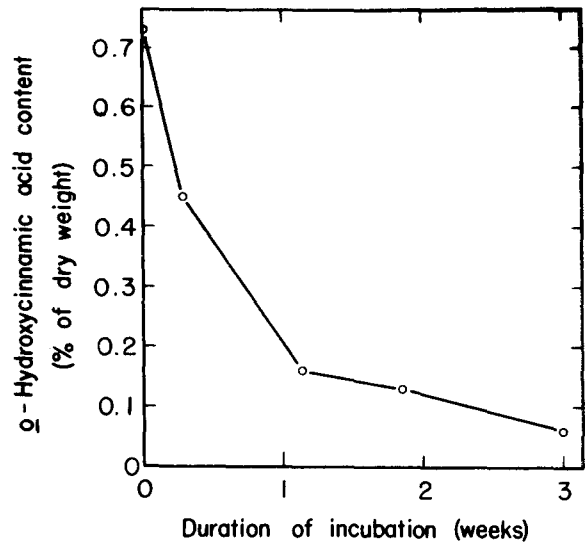

Figure 3. Influence of duration of incubation on *o*-hydroxycinnamic acid content in excised Goldtop sweetclover roots. Each point represents the mean of 6 determinations.

SUMMARY

Excised roots of biennial sweetclover were successfully cultured through repeated transfers on a modified White's medium solidified with agar. Roots also grew well in shaker-agitated liquid cultures over the pH range 4.5 to 7.0. Exposure of the cultures to bright illumination resulted in submaximal growth. No net synthesis of *o*-hydroxycinnamic acid was observed in the cultured roots.

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