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ROSMARINIC ACID AND RELATED PHENOLICS IN HAIRY ROOT CULTURES OF *OCIMUM BASILICUM*

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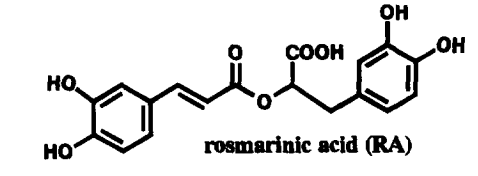
Key Word Index—*Ocimum basilicum*; Lamiaceae; hairy root; *Agrobacterium rhizogenes*; rosmarinic acid; lithospermic acid; lithospermic acid B.

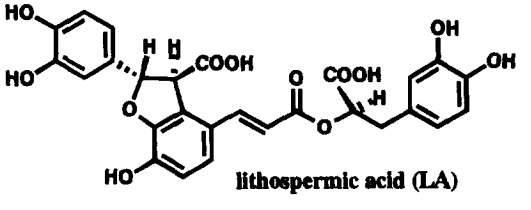
Abstract—Five clones of *Ocimum basilicum* hairy roots, A-1 and A-2 (included by *Agrobacterium rhizogenes* ATCC 15834), and J-1, J-2 and J-3 (induced by *A. rhizogenes* MAFF 03-01724), grew well in hormone-free Murashige–Skoog, Gamborg B5 and Woody Plant liquid media. In these cultures, a large amount of rosmarinic acid was produced (maximum: 14.1% dry wt, by J-1 in MS medium) together with small amounts of the related phenolics, lithospermic acid (*ca* 1.70% dry wt) and lithospermic acid B (*ca* 0.17% dry wt).

INTRODUCTION

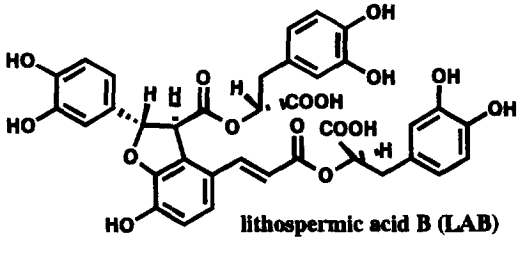
Ocimum basilicum L., a popular lamiaceous plant known as sweet basil, is used as a kitchen herb and as an ornamental in house gardens. It is known to contain the antioxidant phenolic compound rosmarinic acid (RA) [1], one of the most common caffeic acid esters occurring in Lamiaceae (*Coleus* [2], *Salvia* [3], *Rosmarinus* [4], etc.) and Boraginaceae (*Lithospermum* [5], *Anchusa* [6], etc.). In the present study, hairy root cultures of this plant, induced by two types of *Agrobacteria* (*A. rhizogenes* ATCC 15834 and *A. rhizogenes* MAFF 03-01724 strains), were established and the chemical constituents (RA and the related phenolic compounds lithosphermic acid [7] (LA) and lithospermic acid B [8] (LAB)) in the cultures were determined.



rosmarinic acid (RA)



lithospermic acid (LA)



lithospermic acid B (LAB)

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RESULTS AND DISCUSSION

Five clones of *O. basilicum* hairy roots, A-1 and A-2 (induced by *A. rhizogenes* ATCC 15834) and J-1, J-2 and J-3 (induced by *A. rhizogenes* MAFF 03-01724) were cultured in three hormone-free liquid media—Murashige–Skoog (MS) [9], Gamborg B5 (B5) [10] and Woody Plant (WP) [11]. After inoculation into these media, all clones showed rapid proliferation at the early stage of cultures (Fig. 1). particularly in WP medium, the biomass of the roots increased without an obvious lag time, with the maximum biomass being observed at week 3 (except for one clone, A-2). The highest amount of biomass recorded for any culture was 702.8 mg dry wt per flask. This was for A-2 grown in MS medium for six weeks.

The RA content (percentage dry wt) of these clones cultured in three media (MS, B5 and WP) is shown in Fig. 2. The RA content observed in the five hairy root clones with time showed a different pattern from that of

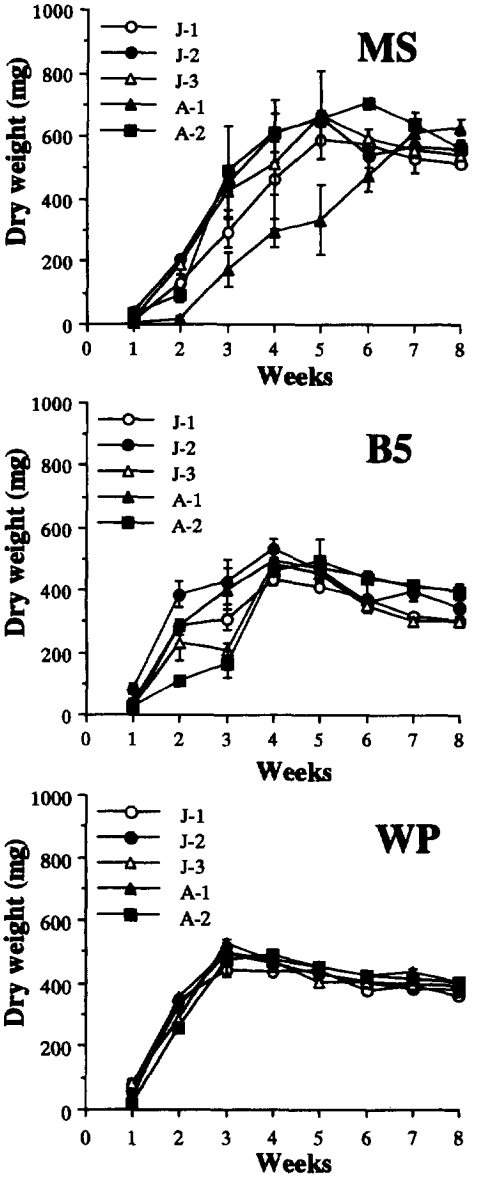

Fig. 1. Growth of *Ocimum basilicum* hairy roots cultured in MS, B5 and WP liquid media in the dark.

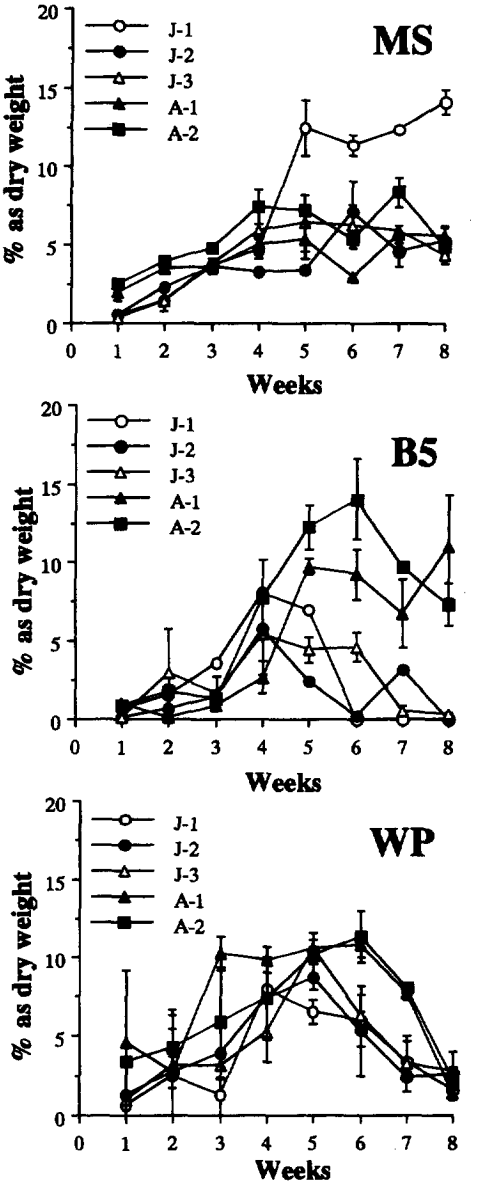

Fig. 2. Rosmarinic acid (RA) content in *Ocimum basilicum* hairy roots cultured in MS, B5 and WP liquid media in the dark.

biomass production. Although in all media, the hairy roots produced substantial amounts of RA, particularly high levels (over 14% dry wt) were observed in MS (J-1: 14.1%, at week 8) and B5 (A-2: 14.0%, at week 6) media. These levels were almost 3.5-fold higher than that (3.98% dry wt, in leaf portion) of the intact plant and were also almost identical to those obtained in suspension cultures of *C. blumei* [2] and *S. officinalis* [3], among others, cultured under highly optimized conditions for RA production. It was noteworthy that only the clones transformed with *A. rhizogenes* MAFF 03-01724 (J-1, J-2, J-3) showed a marked decrease of RA content after reaching the maximum levels at weeks 4 in B5 medium, although in WP medium the RA content of all clones successively decreased in the latter part of the culture period. The maximum yield (mg per flask) of RA in all cultures examined was 73.5 mg/flask^{-1} produced by J-1 in MS medium at week 5.

LA was also produced in all clones in the three media tested (Fig. 3). High LA contents were observed in MS medium. Maximum LA production was obtained by J-clones in the latter period of the cultures in MS medium (J-1, 1.57% at week 8; J-2, 1.19% at week 6; J-3, 1.70% at week 8).

Although the content was fairly low (below 0.17%, dry wt), LAB was certainly produced in some clones when cultured in B5 (A-2, J-1 and J-3) and WP (A-1 and A-2 and J-1) media (Fig. 4). However, in MS medium, no LAB was detected.

Thus, hairy root cultures of *O. basilicum* are able to polymerize caffeic acid to a trimer (LA) and a tetramer

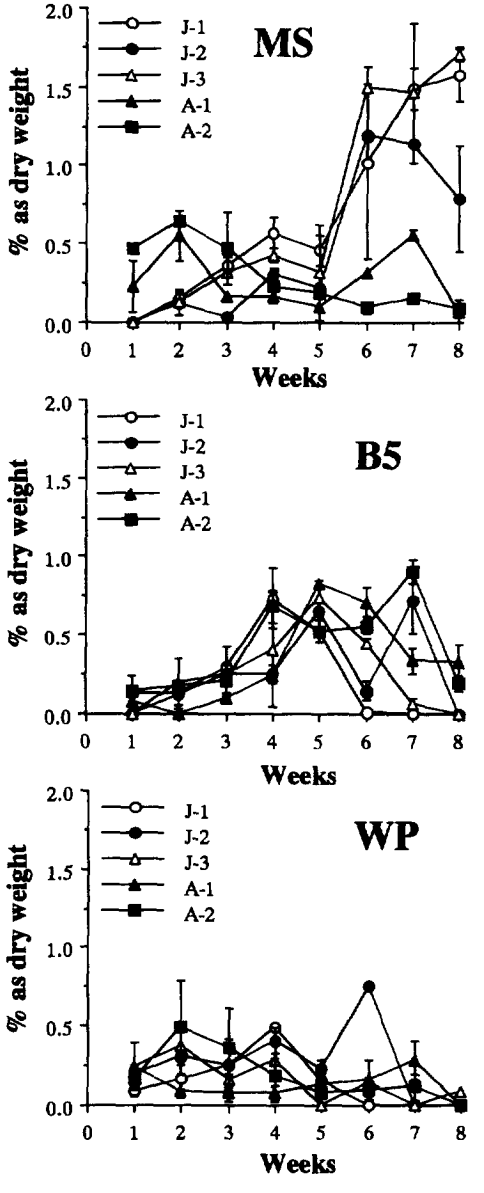

Fig. 3. Lithospermic acid (LA) content in *Ocimum basilicum* hairy roots cultured in MS, B5 and WP liquid media in the dark.

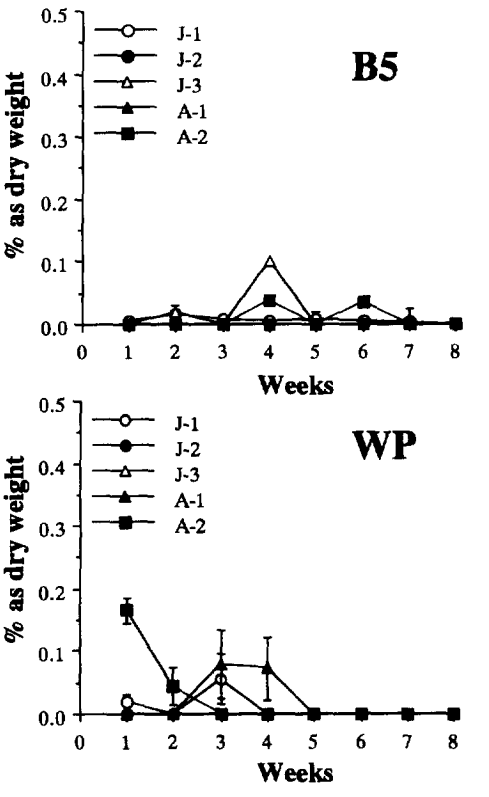

Fig. 4. Lithospermic acid B (LAB) content in *Ocimum basilicum* hairy roots cultured in B5 and WP liquid media in the dark.

(LAB). Our results also indicated the importance of the selection of the hairy root clones (together with the *A. rhizogenes* strains used for hairy root induction) and culture conditions (medium, culture period etc) for the production and the biosynthetic study of useful secondary metabolites (RA, LA and LAB) in *O. basilicum*.

EXPERIMENTAL

General. All culture media, MS (containing 30 g l^{-1} sucrose), B5 (containing 20 g l^{-1} sucrose) and WP (containing 20 g l^{-1} sucrose) were adjusted to pH 5.7 before autoclaving at 121° for 15 min. All cultures were grown at 25°. Data shown are the mean of three experiments.

Plant material. Seeds of *O. basilicum* L., purchased at a local market, were sterilized with 2% NaOCl (8 min) and germinated aseptically on hormone-free MS solid medium (solidified with 2.5 g l^{-1} gelrite). The axenic plantlets *in vitro* were used for infection by *Agrobacterium*.

Hairy root cultures. Agrobacterium rhizogenes ATCC 15834 and *A. rhizogenes* MAFF 03-01724 subcultured on YEB agar medium [12] were transferred to YEB liquid medium (20 ml in 100 ml Erlenmeyer flask) and precultured for 1 day in the dark on a rotary shaker (100 rpm). The soln of the bacterium (200 μl) and leaf disks (5 × 5 mm) of the plantlets were inoculated into 1/2 strength MS liquid medium (20 ml in 100 ml flask) and co-cultured for 2 days in the dark at 100 rpm. The infected leaf segments, after being rinsed with sterile H_2O, were transferred to 1/2 strength MS solid medium containing antibiotic (Claforan 0.5 mg ml^{-1}). After about 3 weeks in culture, several hairy roots appeared at the infected sites. The hairy roots were cut off and were placed on 1/2 strength MS solid medium in the dark. Five clones (A-1 and A-2 by ATCC 15834, and J-1, J-2 and J-3 by MAFF 03-01724), which showed good growth, were selected and used for this study. The transformation of the hairy roots was proved by the detection of opines (agropine

and mannopine [13] in A-1 and A-2 and mikimopine [14] in J-1, J-2 and J-3) using paper electrophoresis. Voucher specimens are deposited at Faculty of Agriculture, Saga University.

Hairy root cultures in three basal liquid media. Five clones (*ca* 0.2 g, fresh wt) were each inoculated into three flasks containing hormone-free liquid media (MS, B5 and WP, 50 ml per 100 ml flask) and cultured (100 rpm, on a rotary shaker) in the dark. The growth (root wt) and phenolic production (RA, LA and LAB) were determined weekly (for 8 weeks).

HPLC analysis of phenolic compounds. Lyophilized samples (*ca* 20 mg) were mashed and extracted with MeOH (2 ml) for 16 hr at room temp. Each extract, after filtration through a millipore filter (0.5 μm), was subjected to HPLC analysis; column: CAPCELL PAK C18 AG 120 (4.6 mm × 250 mm), mobile phase; 1 mM tetra-butylammonium (adjusted to pH 2.8 with AcOH)–MeCN (4:1 → 1:4, in 25 min); flow rate: 0.65 ml min^{-1}; column temp. 40°, detected.: 330 nm; R_t (min): RA (15.5), LA (20.7) and LAB (22.5). RA was purchased from Extrasynthese (FRANCE) and LA and LAB, extracted from *S. miltiorrhiza* [15], were a gift from Dr G. Nonaka, Kyushu University.

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