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POLYPHENOL PRODUCTION IN HAIRY ROOT CULTURES OF *FRAGARIA* × *ANANASSA*

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Key Word Index—*Fragaria* × *ananassa*; Rosaceae; hairy roots; *Agrobacterium rhizogenes*; tannin; flavonoid; catechin; procyanidin; pedunculagin; taxifolin.

Abstract—Hairy roots of *Fragaria* × *ananassa* cv. Reikou, induced with *Agrobacterium rhizogenes* ATCC 15834, grew well in hormone-free Murashige–Skoog (MS), root culture and Gamborg B5 liquid media. Particularly, in MS medium, hairy roots showed maximum growth (539 mg per flask, dry wt at week 8) producing high contents of polyphenols (especially (+)-catechin (0.59% dry wt at week 8) and procyanidin B-3 (0.80% dry wt at week 7). Polyphenol contents in the intact plant (leaf blade, petiole, calyx, receptacle and root) were also investigated.

INTRODUCTION

Fragaria × *ananassa* cv. Reikou (a hybrid between *Fragaria* × *ananassa* cv. Harunoka and *Fragaria* × *ananassa* cv. Hukuba) is one of the most important cultivars for cross-breeding of strawberries in Japan. In a recent chemical study of this cultivar, six polyphenols, i.e. condensed tannins, (+)-catechin (**1**), (+)-afzelechin-(4α-8)-(+)-catechin (**2**) [1], procyanidin B-3 (**3**) [2], procyanidin B-6 (**4**) [3], a hydrolyzable tannin, pedunculagin (**5**) [4] and a new flavonoid, (+)-taxifolin 3-*O*-α-L-arabinofranoside (**6**) were isolated from the roots [5]. In continuing experiments on tissue cultures of this cultivar, we have succeeded in establishing hairy root cultures (induced by the infection with *Agrobacterium rhizogenes* ATCC 15834). The production of polyphenols (**1**–**6**) and a related flavone, (+)-taxifolin (**7**), in the root cultures was determined. Concomitantly, the polyphenol profile of intact plant tissues was also investigated.

RESULTS AND DISCUSSION

For the induction of hairy roots, leaf segments of *in vitro* plantlets were infected with *A. rhizogenes* ATCC 15834 using the co-culture infection method. Some hairy roots which appeared at infected sites, were transferred to hormone-free Murashige–Skoog (MS) [6] solid medium. One clone which showed the best growth was selected for the present experiments.

The growth of the hairy roots cultured in hormone-free MS, root culture (RC) [7] and Gamborg B5 (B5) [8] liquid media is shown in Fig. 1. Of the three media tested, good proliferation of hairy roots was observed in MS medium. After three weeks of culture in MS medium, the amount of roots rapidly increased until the end of the culture period to reach the maximum (539 mg per flask at week 8); this was over five times greater than RC (96 mg per flask at week 8) and B5 (81 mg per flask at week 8) media.

Polyphenol (**1**–**7**) production in these cultures was also determined (Fig. 2). Hairy roots grown in the three basal liquid media yielded similar polyphenols to those observed in intact plants [5]. In particular, in MS and RC media, the contents of **1** and **3** were relatively high compared to those of the other constituents. In MS medium after four weeks of culture, the content of **3** gradually increased and exceeded that of **1** (maximum; **1**: 0.59% at week 8, **3**: 0.80% at week 7, as dry wt). In contrast, in RC medium, the content of **1** generally exceeded that of **3** during most of the culture period, except at the early stage (1–2 weeks). This observation suggested that polymerization of flavan-3-ols (i.e. formation of **3** from **1**) in hairy roots had occurred more in MS medium than in RC. Polyphenol contents in B5 medium were generally lower (below 0.30% dry wt) than those in MS and RC.

Polyphenol contents in the intact plant (leaf blade, petiole, calyx, receptacle and root) are shown in Fig. 3. In the calyx and root, polyphenol contents were relatively high, in particular **3** (3.26% in calyx and 2.42% in root), **5** (2.75% in calyx) and **6** (3.01% in root). In the receptacle

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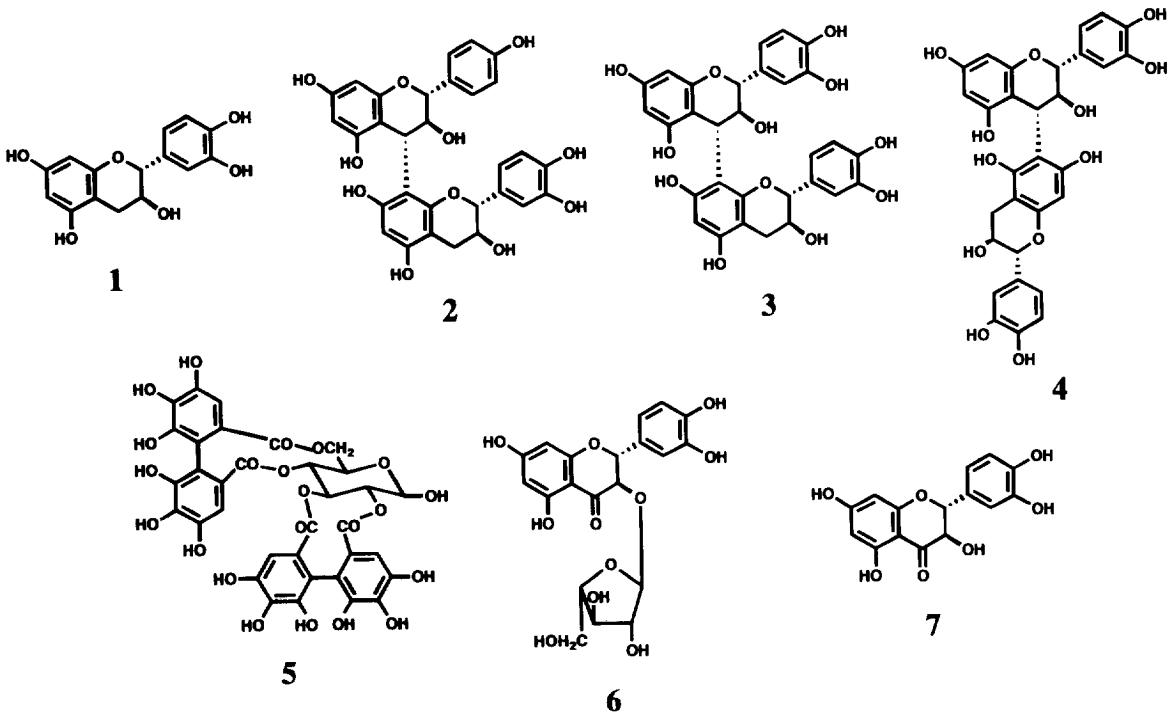

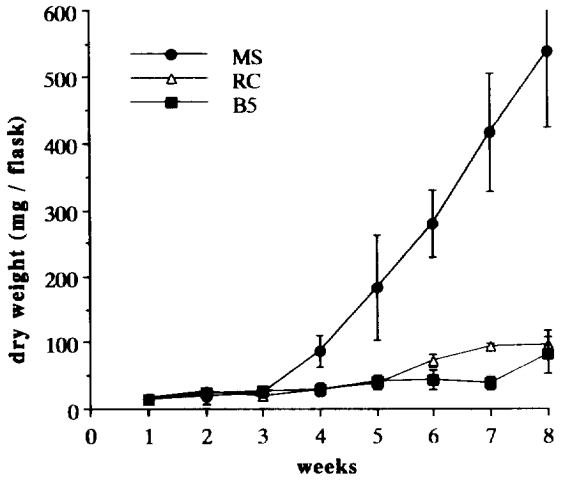

Fig. 1. Growth of hairy roots of *Fragaria* × *ananassa* cultured in MS, RC and B5 liquid media (bars represent standard errors).

(flesh and achene), only **1** was detected in small amounts (0.09%).

With the determination of the effects of culture conditions, hairy root cultures of this species could be a very useful system for the study of polyphenol (tannins and flavones) biosynthesis, although the polyphenol content is lower than that of the parent plant (in MS medium, the content was almost one-third of the root of the intact plants). Although some experiments on the transformation of *Fragaria* plants (strawberry) using *A. tumefaciens* [9–12] or *A. rhizogenes* [13] have been reported, this is the first study on the secondary metabolites produced in transformed cells of strawberry.

EXPERIMENTAL

All culture media, MS (containing 30 $g l^{-1}$ sucrose), RC (containing 15 $g l^{-1}$ sucrose) and B5 (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 and induction of hairy roots. Apices (0.7–0.8 mm, in length) of *Fragaria* × *ananassa* Duch. cv. Reikou, cut from sterilized shoots, were aseptically placed on MS solid medium containing 0.1 $mg l^{-1}$ indoleacetic acid and 0.1 $mg l^{-1}$ kinetin, and cultured under light (16 hr photoperiod per a day, 3000 lx). Axenic plantlets thus obtained were used for *Agrobacterium* infection. *Agrobacterium rhizogenes* ATCC 15834 subcultured on YEB agar medium [14] was 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 solution of this bacterium (200 μl) and leaf segments of plantlets *in vitro* were inoculated to 1/2 MS liquid medium (20 ml in 100 ml flask) and co-cultured for 4 days in the dark at 100 rpm. Infected segments, after being rinsed with sterile H_2O, were transferred to 1/2 MS solid medium containing antibiotic (claforan 0.5 $mg ml^{-1}$) to eliminate the bacteria. Axenic hairy roots were transferred to hormone-free MS solid medium and subcultured in the dark. One clone which showed the best growth was selected and used for expts. Transformation of hairy roots was proved by the detection of opines, agropine and mannopine [15] using paper electrophoresis. Intact plants of *F.* × *ananassa* cv. Reikou (for determination of polyphenol content by HPLC analysis), were cultivated in a field at Tsukuba and collected in June,

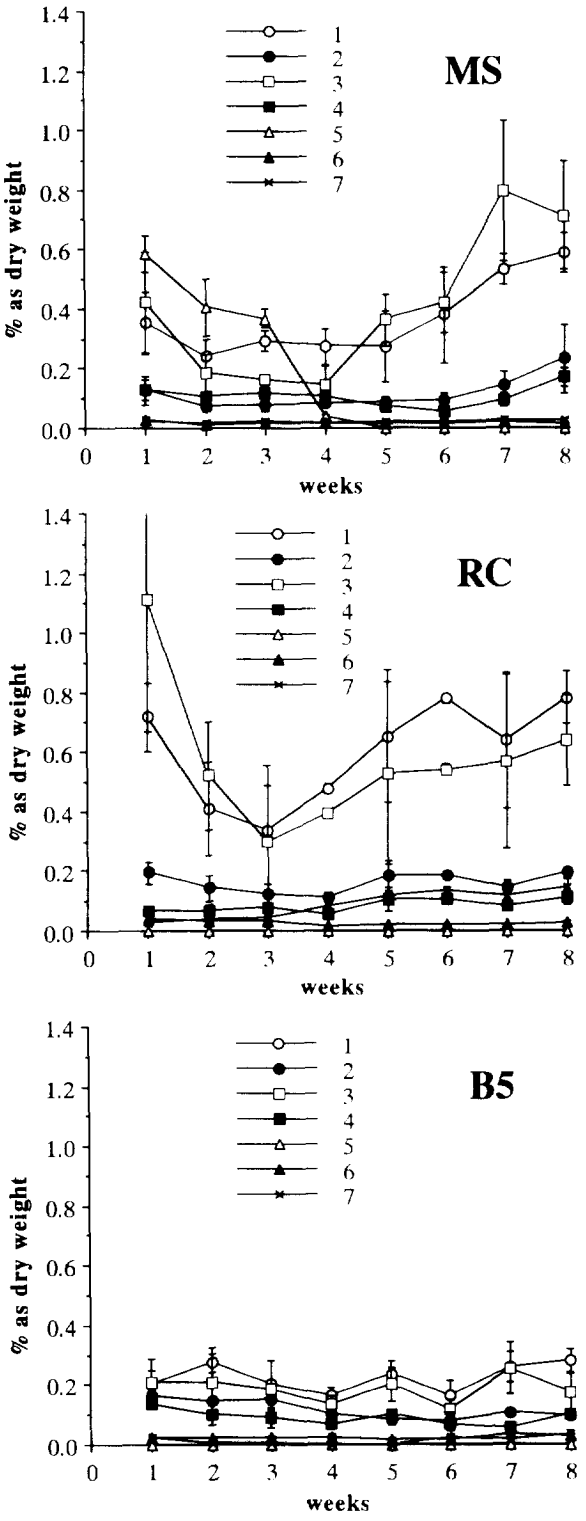

Fig. 2. Polyphenol contents in hairy roots of *Fragaria* × *ananassa* cultured in MS, RC and B5 liquid media (bars represent standard errors).

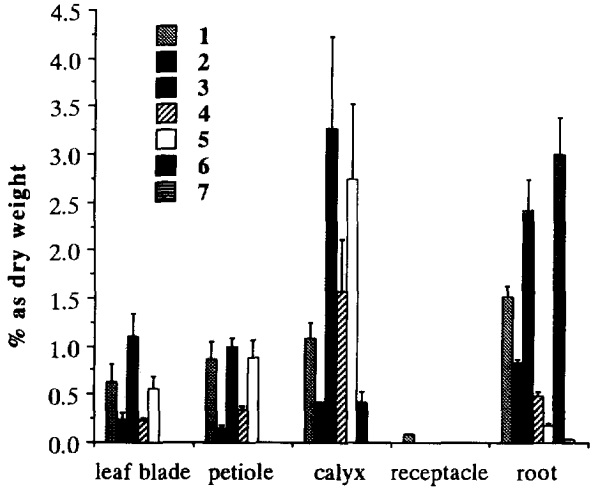

Fig. 3. Polyphenol contents in *Fragaria* × *ananassa* (bars represent standard errors).

1993. Voucher specimens are deposited at the Faculty of Agriculture, Saga University.

Hairy root cultures in basal liquid medium. Fr. hairy roots (*ca* 50 mg) were inoculated into hormone-free MS, RC and B5 liquid media (50 ml in 100 ml flask) and cultured (100 rpm on a rotary shaker) in the dark. Growth (root wt) and polyphenol production were determined once a week for 8 weeks.

HPLC analysis of polyphenols. Lyophilized samples (5–20 mg) were mashed and extracted with MeOH (1–2 ml) for 15 hr at room temp. Each extract, after filtration through a Millipore filter (0.45 μm), was analyzed (5–10 μl) by HPLC. HPLC conditions were as follows: column TSK-gel ODS 80Ts (4.6 mm i.d. × 250 mm); mobile phase MeCN–1 mM tetrabutylammonium (pH 2.9 with HOAc) (1:9–4:1, in 30 min), flow rate 0.7 ml min^{-1}; column temp. 40°; detection 280 nm (UV). R_t values (min): **5** (12.8), **3** (14.5), **1** (15.5), **2** (17.6), 4 (18.5), **6** (23.1), **7** (23.7).

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