Original Paper Plant Biotechnology, **21** (4), 275-281 (2004)

Camptothecin Production by *in Vitro* Cultures of *Ophiorrhiza liukiuensis* and *O. kuroiwai*

Takashi ASANO, Ikumi WATASE, Hiroshi SUDO, Mariko KITAJIMA, Hiromitsu TAKAYAMA, Norio AIMI, Mami YAMAZAKI and Kazuki SAITO

Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

**Corresponding author* E-mail address: ksaito@faculty.chiba-u.jp

Received 7 October 2004; accepted 15 November 2004 (Edited by K. Yazaki)

Abstract

Camptothecin-derived compounds are widely used for clinical treatment of human cancer. They are synthesized from natural camptothecin, which is obtained by extraction from intact plants. For the feasible production of camptothecin, tissue cultures of *Ophiorrhiza liukiuensis* and *O. kuroiwai*, an interspecies hybrid of *O. liukiuensis* and *O. pumila*, have been investigated. The aseptic plants and hairy roots of *O. liukiuensis* and *O. kuroiwai* were established in addition to the previously-established *O. pumila*. The camptothecin production by *O. kuroiwai* was better than that by *O. liukiuensis*. 10-Methoxycamptothecin was accumulated in tissue cultures of *O. liukiuensis* and *O. kuroiwai* but not in *O. pumila*. Methyl jasmonic acid slightly enhanced the production of camptothecin in the *O. liukiuensis* hairy roots. These results indicate that the tissue cultures of *O. liukiuensis* and *O. kuroiwai* would be the feasible ways for production of camptothecin and related alkaloids.

Key words: *Ophiorrhiza liukiuensis, Ophiorrhiza kuroiwai, Ophiorrhiza pumila*, Rubiaceae, Indole alkaloids, Camptothecin, 10-Methoxycamptothecin, Hairy root, Aseptic plant, *Agrobacterium rhizogenes*

Abbreviations

MS, Murashige and Skoog; HPLC, high performance liquid chromatography; MJ, methyl jasmonic acid; SA, salicylic acid; YE, yeast extract.

Introduction

Camptothecin (1) (**Fig. 1**) is a water-insoluble monoterpenoid indole alkaloid isolated from extracts of *Camptotheca acuminata*, and it was remarked as a potential drug with anti-tumor activity by the National Cancer Institute (NCI) in the United States (Wall *et al.*, 1966). The interest in camptothecin was stimulated by the characterization of camptothecin as a specific topoisomerase I inhibitor (Hsiang *et al.*, 1985). At present, semi-synthetic water-soluble camptothecin derivatives, topotecan (2) and irinotecan (3), are used throughout the world as clinical anti-tumor agents against cancers of lung, cervix, ovaries (Takeuchi *et al.*, 1991), colon (Giovanella *et al.*, 1989), and other organs (Pizzolato and Saltz, 2003). The worldwide market of irinotecan and topotecan has currently reached one thousand million US dollars per year, which represents approximately one ton of camptothecin in terms of raw material. In addition, water insoluble analogues 9-aminocamptothecin and 9-nitrocamptothecin (rubitecan), both of which were reported to possess activity sufficient to merit clinical evaluation (Thomas *et al.*, 2004). Rubitecan serves as a metabolic precursor to 9-aminocamptothecin and was submitted to the Food and Drug Administration (FDA) in the United States and the European Agency for the Evaluation of Medicinal Products (EMEA) as an orally active camptothecin for the treatment of pancreatic cancer. Consequently the demand of camptothecin will be more expanding in the future. Despite its increasing demand, camptothecin is still supplied exclusively from the intact plants, mainly *C. acuminata* and *Nothapodytes foetida* (Govindachari and Viswanathan, 1972).

Camptothecin is produced also in some species of the genus *Ophiorrhiza* (Rubiaceae), *e.g.*, *O. mungos* (Tafur *et al.*, 1976), *O. pumila* (Aimi *et al.*, 1989), *O. liukiuensis* (Kitajima *et al.*, unpublished) and *O. kuroiwai* (Kitajima *et al.*, unpublished). 10-Methoxycamptothecin (5) was also isolated in

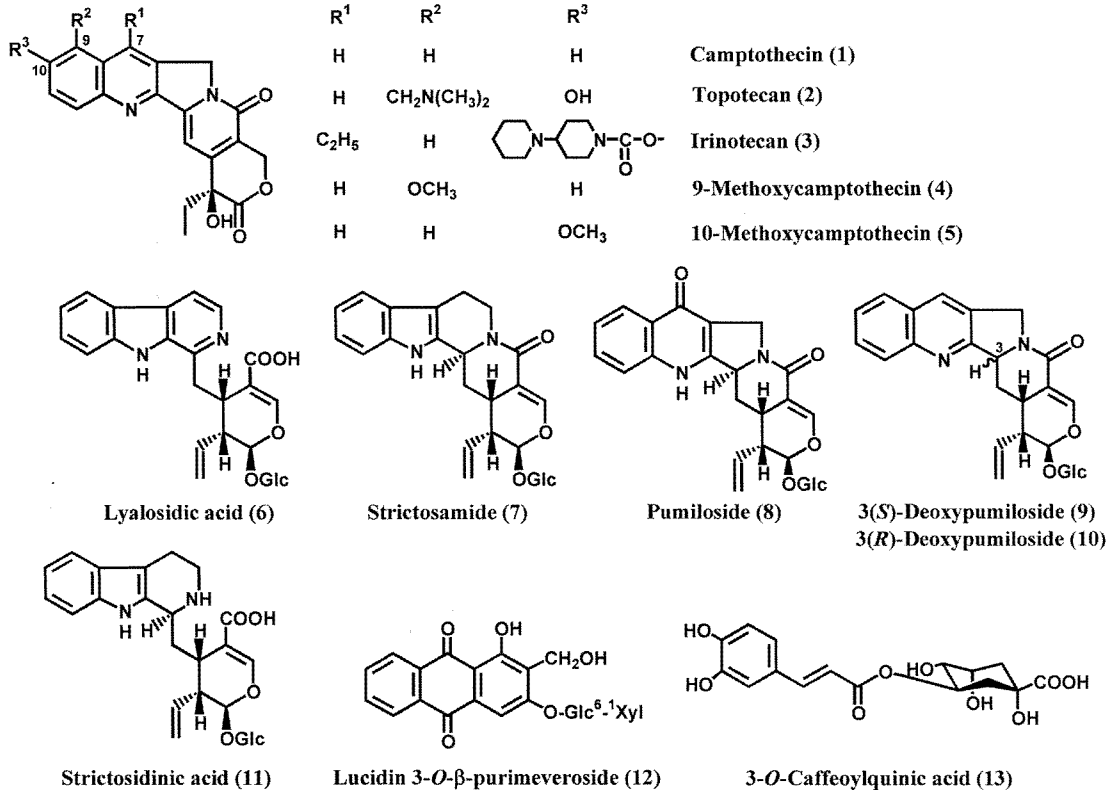

Fig. 1. Chemical structures of clinically used camptothecin derivatives and secondary metabolites detected in tissue cultures of *Ophiorrhiza* species.

O. liukiuensis and *O. kuroiwai*. Camptothecin derivatives with C-10 substitution have been reported to inhibit the growth of cancer cells more effectively than camptothecin (Vladu *et al.*, 2000). Since only few plants accumulate 10-methoxycamptothecin (Tafur *et al.*, 1976; Lorence *et al.*, 2004) and no report is available for production of 10-methoxycamptothecin by tissue cultures, it is intriguing to establish cell cultures that produce not only camptothecin but also 10-methoxycamptothecin. We have investigated the production of camptothecin and chemical constituents in the hairy roots of *O. pumila* (Saito *et al.*, 2001; Sudo *et al.*, 2002; Kitajima *et al.*, 2002; Yamazaki *et al.*, 2003a). The expression study of several biosynthetic genes (Yamazaki *et al.*, 2003b) and the tracer experiments using ^{13}C (Yamazaki *et al.*, 2004) were also conducted by the *O. pumila* hairy roots. Recently, we have shown that *O. kuroiwai* is an interspecies hybrid of *O. pumila* and *O. liukiuensis* (Sudo *et al.*, in preparation).

In this study, the aseptic plants and hairy roots of *O. liukiuensis* and *O. kuroiwai* were established in addition to the previously-established *O. pumila*. We have investigated the accumulation patterns of camptothecin and other secondary metabolites in these tissue cultures, and effects of elicitors on growth and camptothecin production in these tissue cultures.

Materials and Methods

Plant materials

The seeds of *O. liukiuensis* (Hayata, 1912) were collected in Ishigaki-Island, Okinawa, Japan. The plants of *O. kuroiwai* (Makino, 1906) were collected in Iriomote-Island, Okinawa, Japan and grown in a green house.

Culture of aseptic plants

The seeds of *O. liukiuensis* were sterilized and germinated on half-strength Murashige and Skoog (1962) (MS) medium containing 1% sucrose and 0.2% gellan gum in a test tube. The young leaves of *O. kuroiwai* were sterilized by 1% sodium hypochlorite solution and cultured on 1/2 MS medium containing 1% sucrose, 0.2% gellan gum, 0.5 μM 1-naphthaleneacetic acid and 5 μM kinetin. After 40 days, regenerated shoots were excised and subcultured on 1/2 MS medium containing 1% sucrose and 0.2% gellan gum in a test tube. The aseptic plant of *O. pumila* was cultured as described previously (Kitajima *et al.*, 1997). All aseptic plants were maintained at 25℃ with a photoperiod of 18 h light (2000 lux.) / 6 h dark.

Induction and culture of hairy roots

Agrobacterium rhizogenes strain 15834 was in-

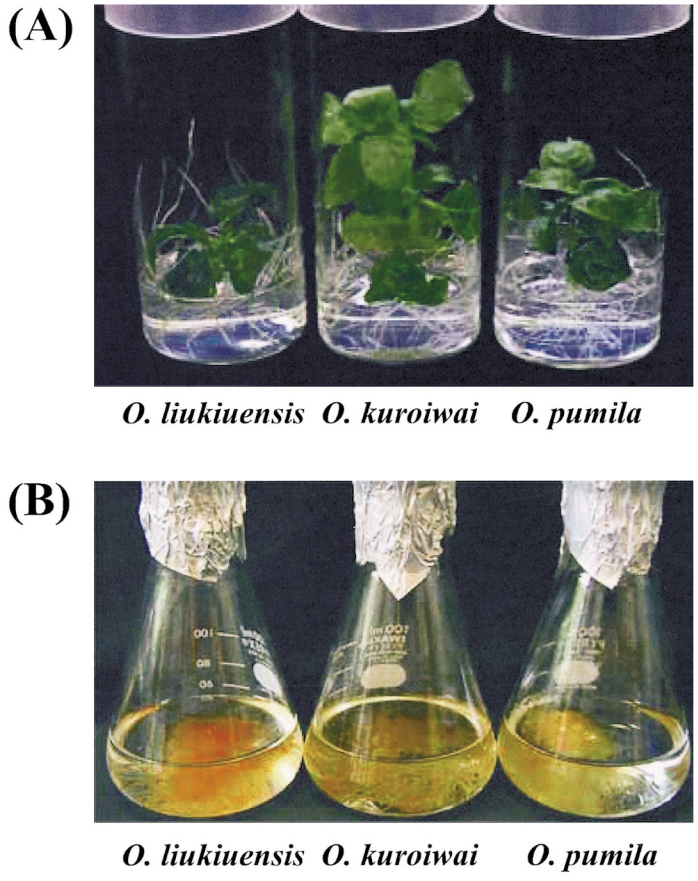

Fig. 2 Established tissue cultures of *Ophiorrhiza liukiuensis*, *O. kuroiwai* and *O. pumila*. (A) Aseptic plants cultured for 5 weeks on 1/2 MS medium containing 1% sucrose and 0.2% gellan gum in test tubes. (B) Hairy roots cultured for 4 weeks in B5 liquid medium containing 2% sucrose.

oculated onto the sections of the stems excised from the aseptic plants of *O. liukiuensis* and *O. kuroiwai* according to the procedure as described previously (Saito *et al.,* 2001). After several weeks, the hairy roots which emerged from stem fragment were excised and cultured on B5 medium (Gamborg *et al.,* 1968) containing 2% sucrose and 0.2% gellan gum supplemented with 200 mg l^{-1} cefotaxime (Claforan®) at 25℃ under dark condition. Transgenic states of hairy roots were checked by the detection of the *rolB* gene in genomic DNA by polymerase chain reaction analysis as described previously (Watase *et al.,* in press). The established hairy root cultures were subcultured every 3-4 weeks in B5 liquid medium containing 2% sucrose at 25℃ on rotary shaker (80 rpm) under dark condition. For time course experiments, the culture was prolonged up to 7 weeks.

Elicitor treatments

The hairy roots were transferred into 50 ml B5 liquid medium containing 2% sucrose in 100-ml Erlenmeyer flask. Methyl jasmonic acid (MJ) (Sigma-Aldrich, St. Louis, MO, U. S. A.) and salicylic acid (SA) (Kanto, Tokyo, Japan) were dissolved in dimethylsulfoxide to the concentration of 0.5 M and added to be a final concentration of 100 μM. Yeast extract (YE) (Difco, Becton Dickinson, Franklin Lakes, NJ, U. S. A.) was dissolved in water, autoclaved and added to culture medium at a final concentration of 500 μg ml^{-1}. One week after the addition of elicitors, the hairy roots and the media were collected and extracted for alkaloid analysis.

Analysis of camptothecin-related alkaloids

For alkaloid extraction, the plant tissues were homogenized in a mortar with pestle, and 1 ml methanol per 100 mg tissue was added and mixed. After sonication for 15 min, the homogenates were centrifuged at 10,000*g* for 10 min. The supernatants were applied on reverse-phase HPLC. For the separation in HPLC, a TSK gel ODS-80TM (4.6 mm x 150 mm) column with a solvent system of methanol : H_2O (1 : 1) were used. Camptothecin and related alkaloids exhibiting fluorescence were monitored by their characteristic fluorescence (excitation at 365 nm and emission at 428 nm). As standards for quantification, camptothecin (1) was purchased from Sigma-Aldrich (St. Louis, MO, U. S. A.) and 10-methoxycamptothecin (5) was purified from intact plants of *O. liukiuensis* in our group (Kitajima *et al.,* unpublished).

Non-targeted metabolite profiling

The methanol extracts were analyzed by HPLC-photodiode array detection-electrospray ionization mass spectrometer (Agilent 1100 series, Palo Alto, CA; Finnigan LC-DECA, Thermo Quest, San Jose, CA). The peaks were identified by the combination of their retention times, UV spectra and mass spectra comparing with those of the standard compounds as described previously (Yamazaki *et al.,* 2003a).

Results

Establishment of tissue cultures

The aseptic plants of *O. liukiuensis* were initially obtained from plants germinated from sterilized seeds. The aseptic plants of *O. kuroiwai* were obtained from the excised tissues from the leaves of plants grown in pots. The shoots were regenerated from the excised leave tissues and subcultured. The established aseptic plants (**Fig. 2A**) were subcultured by transferring the shoots on the solid medium under a photoperiod of 18 h light / 6h dark.

For induction of hairy roots, the aseptic plants of *O. liukiuensis* and *O. kuroiwai* were infected with *A. rhizogenes* 15834 by scratching the stems. After 70 days for *O. liukiuensis* and 15 days for *O. kuroiwai,* rapidly growing roots were emerged and subcultured. After the several times of subcultures, 11 lines for *O. liukiuensis* and 7 lines for *O. kuroiwai* were survived and grew rapidly. The transgenic states of these rapidly-growing hairy root lines were confirmed by the detection of the *rol B* gene in the genomic DNA by polymerase-chain-reaction analysis (data not shown). The established hairy roots (**Fig. 2B**) were cultured in the liquid medium under the dark.

Camptothecin production in aseptic plants

The camptothecin contents in shoots and roots of 5-week old aseptic plants of *O. liukiuensis, O. kuroiwai* and *O. pumila* were analyzed by HPLC. As shown in Fig. 3, the highest level of camptothecin production per tissue weight was observed in roots of *O. pumila*. However, the production per tube was the highest by *O. kuroiwai* because of the highest growth rate over other two species. The concentration and total amount of camptothecin in *O. liukiuensis* were lower than those of *O. kuroiwai* and *O. pumila*.

Metabolite profiles of aseptic plants

The accumulation patterns of secondary products in the aseptic plants of *Ophiorrhiza* species were profiled by HPLC/DAD/ESI/MS (**Fig. 4 A-F**). The metabolite patterns of *O. liukiuensis* and

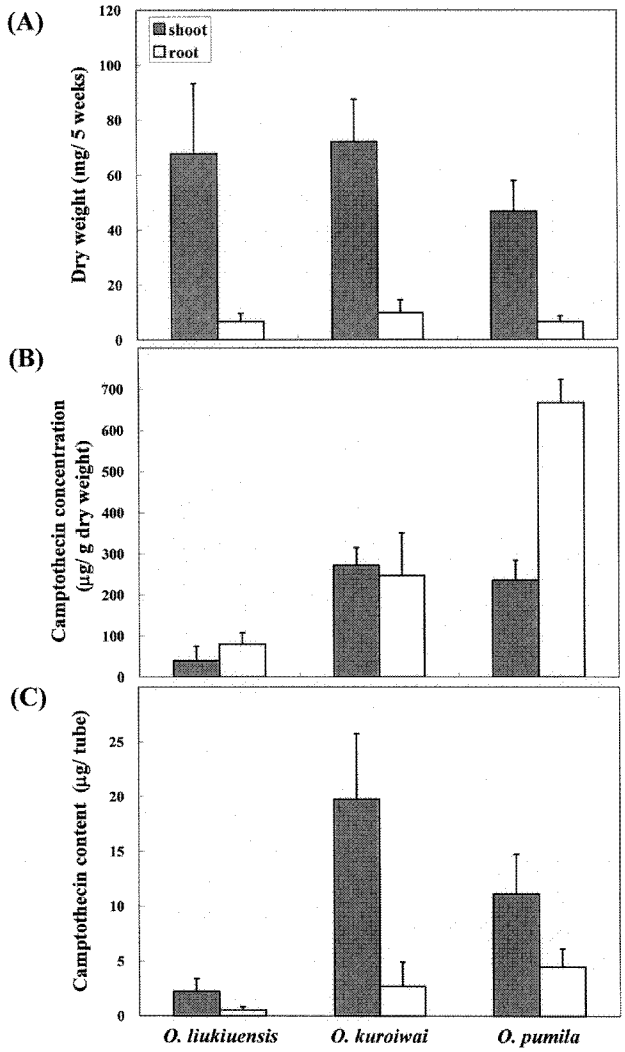

Fig. 3. Growth of aseptic *Ophiorrhiza* plants and camptothecin production for 5 weeks. (A) Dry weight of shoot and root of a plant. (B) Camptothecin concentration per dry weight. (C) Camptothecin content in a whole plant.

O. kuroiwai were similar both in shoot and root, being distinct from that of *O. pumila* regarding a few compounds. 10-Methoxycamptothecin (5) was detected in the roots of *O. liukiuensis* and *O. kuroiwai*, but not in *O. pumila*. Lyalosidic acid (6) was accumulated in the shoots of *O. liukiuensis* and *O. kuroiwai*, but not in *O. pumila*. 3(*S*)- and 3(*R*)-Deoxypumilosides (9, 10) were only detected in *O. pumila*. In contrast, camptothecin (1), 9-methoxycamptothecin (4), strictosamide (7), pumiloside (8), strictosidinic acid (11) and 3-*O*-caffeoylquinic acid (13) were detected in all three species.

Camptothecin production and metabolite profiles of hairy roots

Higher amount of camptothecin was accumulated in the different hairy root lines of *O. kuroiwai* than in those of *O. liukiuensis* (**Table 1**). For further analysis, the best lines of *O. liukiuensis* and *O. kuroiwai* were selected from a viewpoint of growth and camptothecin productivity, and subcultured in the liquid medium (**Fig. 2B**). The metabolite patterns in the hairy roots (**Fig. 4 G-I**) of *O. liukiuensis* and *O. kuroiwai* were not identical to those of aseptic plants. In addition to camptothecin-related compounds, several unidentified anthraquinones deduced from their UV spectra were present in the hairy roots of *O. liukiuensis* and *O. kuroiwai* as reported for *O. pumila* (Yamazaki *et al.*, 2003a).

Time-course study of hairy root cultures

The hairy roots were cultured in 50 ml liquid medium in a 100 ml flask up to 7 weeks. As shown in Fig. 5, the fresh weight of hairy roots of *O. liukiuensis* and *O. kuroiwai* increased to 7.1 g and 8.5 g respectively per flask after 6 weeks. After 7 weeks, the hairy roots and medium color turned brown, and the roots were moribund. The quantitative analysis of alkaloids in hairy roots was carried out about camptothecin and 10-methoxycamptothecin, which is detected in *O. liukiuensis* and *O. kuroiwai* but not in *O. pumila*. The maximum accumulation of camptothecin per flask reached 230 μg in 6-week-old hairy roots of *O. liukiuensis* and 471 μg in 7-week-old hairy roots of *O. kuroiwai*, respectively. This camptothecin production by *O. kuroiwai* was almost the same as that by *O. pumila* (Saito *et al.*, 2001). The level of 10-methoxycamptothecin per flask reached 25 μg in 6-week-old hairy roots of *O. liukiuensis* and 15 μg in 5-week-old hairy roots of *O. kuroiwai*, respectively. Thus, *O. kuroiwai* was better than *O. liukiuensis* for camptothecin production; however, regarding 10-methoxycamptothecin production, oppositely *O. liukiuensis* was a better producer than *O. kuroiwai*.

As reported for *O. pumila* hairy roots (Saito *et al.*, 2001), the hairy roots of *O. liukiuensis* and *O. kuroiwai* excreted camptothecin and 10-methoxycamptothecin to the culture medium. The excreted rates to the medium by *O. liukiuensis* and *O. kuroiwai*, respectively, were 32% and 23% for camptothecin and 14% and 13% for 10-methoxy camptothecin after 7 weeks.

Effects of elicitors for the hairy roots

The effects of MJ, SA and YE on growth and accumulation of two alkaloids, camptothecin (1) and 10-methoxycamptothecin (5), were investigated (**Fig. 6**). In *O. liukiuensis* hairy roots, MJ enhanced the alkaloid production about 1.3-fold; however SA and YE slightly decreased the produc-

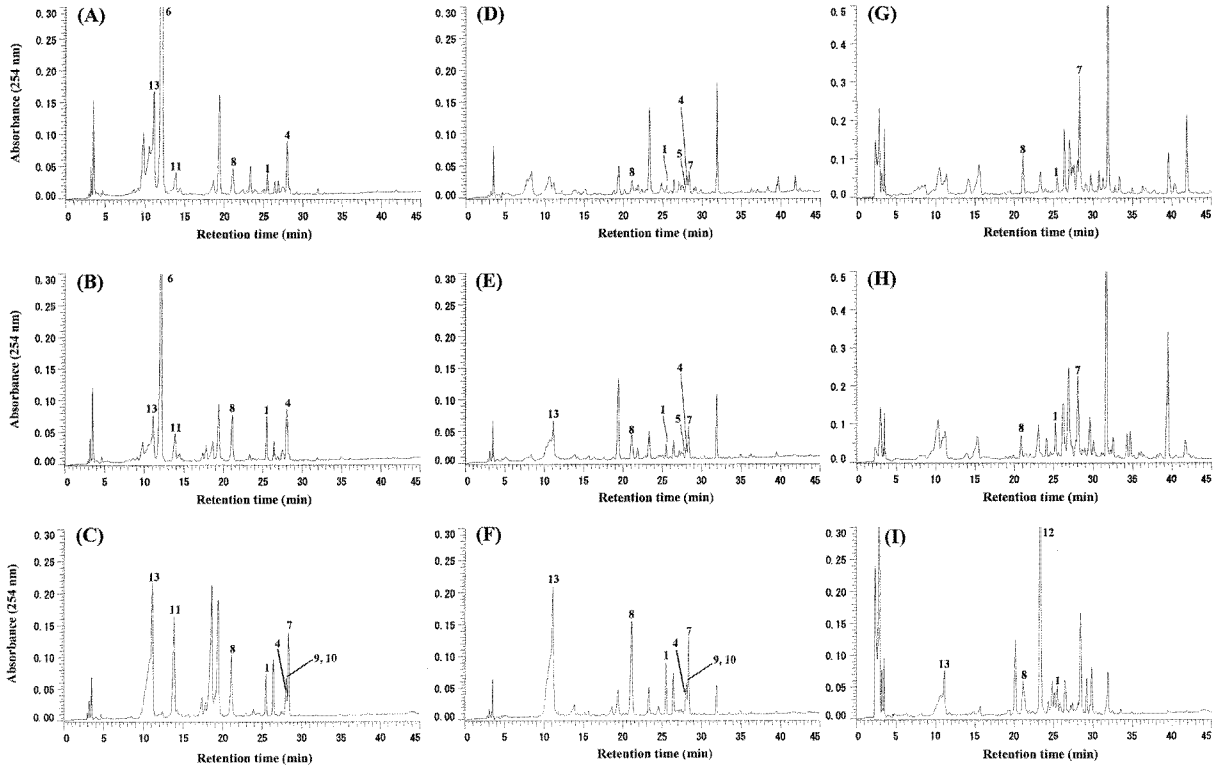

Fig. 4. HPLC chromatograms of the extracts of plant cultures. (A) *O. liukiuensis* sterile plant shoots, (B) *O. kuroiwai* sterile plant shoots, (C) *O. pumila* sterile plant shoots, (D) *O. liukiuensis* sterile plant roots, (E) *O. kuroiwai* sterile plant roots, (F) *O. pumila* sterile plant roots, (G) *O. liukiuensis* hairy roots, (H) *O. kuroiwai* hairy roots, (I) *O. pumila* hairy roots. The peak numbers correspond to the compound numbers in **Fig. 1.** The conditions of HPLC are as followings: column, Mightysil RP-18 (4.6 x 250 mm); gradient, linear gradient from solvent A to solvent B (0-35 min), isocratic at 100% of solvent B (35-40 min), solvent A (20% methanol, 0.2% acetic acid in H_2O), solvent B (90% methanol, 0.025% acetic acid in H_2O); flow rate, 0.8 ml min^{-1}; column temperature, 37℃.

Table 1 Hairy roots induced from *Ophiorrhiza* species

Plant species	Number of established lines	Camptothecin content ($\mu g\, g^{-1}$ D. W. ± S. D. [1])
O. liukiuensis	11	83.0 ± 27.4
O. kuroiwai	7	219.3 ± 31.4

[1] dry weight ± standard deviation

tion. This decreasing effect of SA and YE was also observed in *O. kuroiwai* hairy roots, but MJ had no effects.

Discussion

In the present study, we have established *in vitro* cultures of aseptic plants and hairy roots of *O. liukiuensis* and *O. kuroiwai* in addition to the previously-reported *O. pumila* (Saito *et al.*, 2001). Aseptic plants and hairy roots of all these three species accumulated camptothecin (1) and its plausible biosynthetic intermediate, pumiloside (8) (Aimi *et al.*, 1989; Carte *et al.*, 1990). The aseptic plants of *O. kuroiwai* that is an interspecies hybrid of *O. liukiuensis* and *O. pumila* showed the best growth rate and subsequently the highest production of camptothecin in the shoots. This is presumably due to a heterosis effect of a hybrid species, that has been described as increased size and yield in crossbred as compared to the corresponding inbred lines (Shull, 1948).

Metabolite patterns of *O. kuroiwai* cultures were generally more similar to those of *O. liukiuensis* than those of *O. pumila*, accumulating 10-methoxycamptothecin (5) and lyalosidic acid (6). 10-Methoxycamptothecin may be a desirable compound as an efficient synthetic precursor of topotecan and irinotecan, and also for development of new anti-tumor compounds with C-10 substitution (Vladu *et al.*, 2000). Thus, *O. kuroiwai* and *O. liukiuensis* would be useful plant resources for production of 10-methoxycamptothecin for further development of camptothecin-related medicines.

MJ induced alkaloids production, particularly camptothecin, in the *O. liukiuensis* hairy roots, not

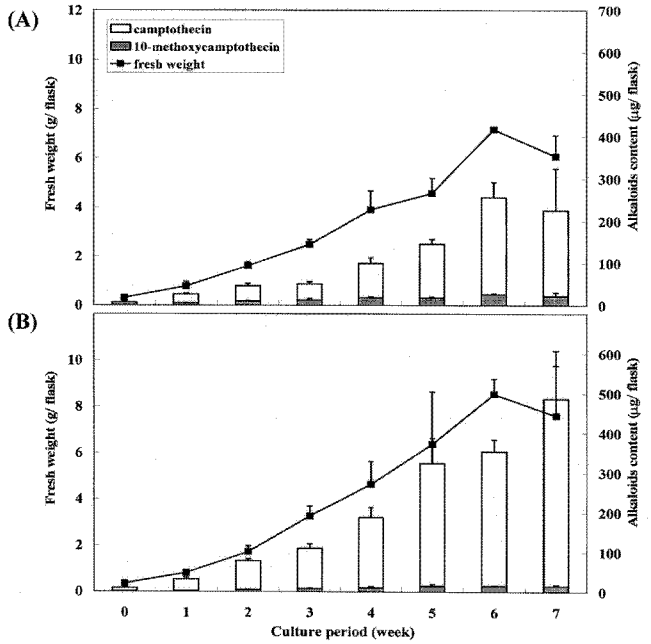

Fig. 5. Time course of growth and alkaloid production of hairy roots. The hairy roots were inoculated in 50 ml of B5 liquid medium containing 2% sucrose in a 100 ml Erlenmeyer flask. These hairy roots were cultured at 25℃ on a rotary shaker (80 rpm). The contents of camptothecin and 10-methoxycamptothecin were determined by HPLC as described in Materials and Methods. (A) *O. liukiuensis* hairy roots, (B) *O. kuroiwai* hairy roots. *Bars*: Standard deviation (*n* = 3 flasks).

remarkably but significantly. This effect was not observed in *O. kuroiwai* and *O. pumila*. The production of other camptothecin derivatives was not affected by the addition of MJ. Thus *O. liukiuensis* hairy root system can be suitable for investigation of inducible production of camptothecin by elicitors.

In conclusion, the tissue cultures of *O. liukiuensis* and *O. kuroiwai* would be alternative experimental systems for study of camptothecin production and possible resources for developments of anti-cancer drugs originated from plants.

Acknowledgements

This study was supported in part by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by CREST of Japan Science and Technology Agency, and by Research for the Future Program (grant no. 00L01605; "Molecular Mechanisms on Regulation of Morphogenesis and Metabolism Leading to Increased Plant Productivity").

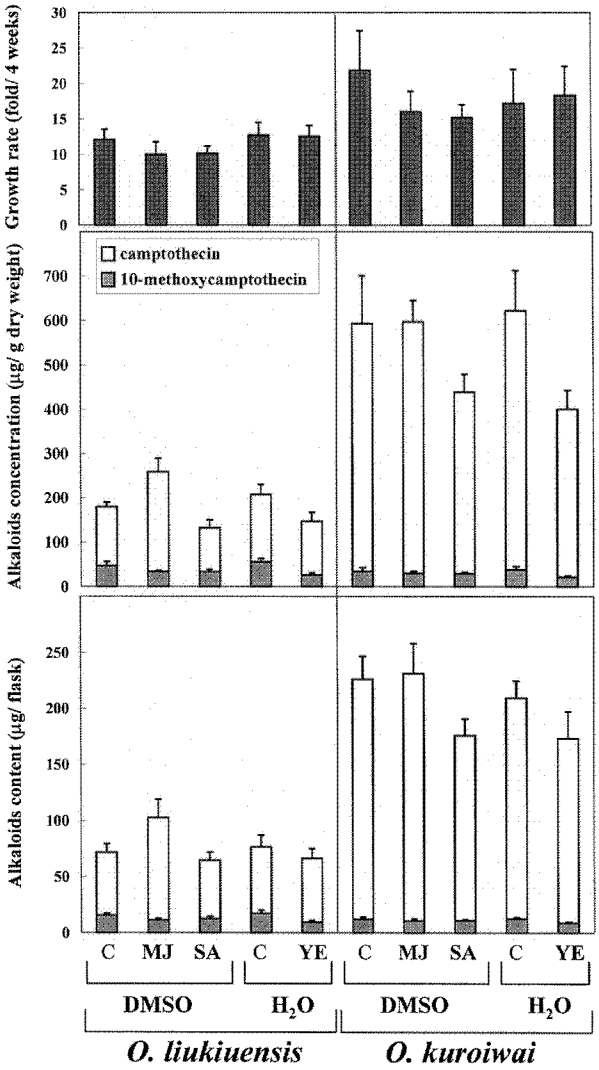

Fig. 6. Effects of elicitors on growth and alkaloid production of hairy roots of *O. liukiuensis* and *O. kuroiwai*. Elicitors were added 3-week-old hairy root cultures in 50 ml of B5 liquid medium containing 2% sucrose in a 100 ml Erlenmeyer flask. The final concentrations of elicitors were: 100 μM methyl jasmonic acid (MJ), 100 μM salicylic acid (SA), 500 μg ml^{-1} yeast extract (YE). For control experiments (C), dimethylsulfoxide (DMSO) for MJ and SA or water for YE were added, respectively. One week after the addition of elicitors, the hairy roots and the liquid media were harvested and extracted for the quantification of camptothecin and 10-methoxycamptothecin. *Bars*: Standard deviation (*n* = 5 flasks).

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