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Comparison of Valerenic Acids and Valepotriates Production According to the Culture Conditions for Cultured Roots of *Valeriana fauriei* var. *dasycarpa* Hara

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ABSTRACT : We established a practical method for rapid and large-scale production of *Valeriana fauriei* var. *dasycarpa* Hara roots by bioreactor culture and confirmed valerenic acids and valepotriates production. We also compared valerenic acids and valepotriates production patterns according to various media conditions. Among the media tested, B5 medium gave the maximum biomass production of 101 g fresh weight, which was a 5.03-fold multiplication rate obtained 4 weeks after inoculation of 20 g of fresh weight. The best production of total valerenic acids (7.86 ㎎/ℓ) and valepotriates (8.96 ㎎/ℓ) was B5 medium.

Key words **:** *Valeriana fauriei* var. *dasycarpa* Hara, valerenic acids, valepotriates, bioreactor culture, HPLC-PDA.

INTRODUCTION

Valeriana fauriei var. *dasycarpa* Hara belongs to the family of Valerianaceae is a medicinal herb which grows in Korea, China and Japan. The family Valerianaceae is composed 200-300 species in the world, most of which are distributed over temperate zone (Bos *et al.*, 2002). There are three forma, two varieties and three species of *Valeriana* plant in South Korea. The three species are *V. amurensis, V. dageletiana* and *V. fauriei* (Park, 1972). The roots and rhizomes of *Valeriana* species comprise the drug valerian that has been used as a sedative since it was described by the ancient Greeks and Romans (Houghton, 1988; Gao and Bjrk, 2000). That is widely used for the treatment of tension, irritability, restlessness and insomnia. Valerian is the 8th top-selling herbal supplement in North America (Blumenthal *et al.*, 2001; Letchamo *et al.*, 2004) and in Australia it is in the top 10 selling retail herbs (Wills and Shohet, 2003).

The biological activity of valerian is ascribed to valerenic acids (valerenic acid and their derivatives) and valepotriates (Fig. 1). In addition, the essential oil may contribute to sedative effect (Bos *et al.*, 1998).

The contents of valepotriates and valerenic acids in two Korean valerian roots (*V. officinalis* var. *latifolia* Miq. and *V. fauriei* var. *dasycarpa* Hara) were reported and compared with European valerian roots (*V. officinalis* L.) by HPLC method (Kim *et al.*, 2003).

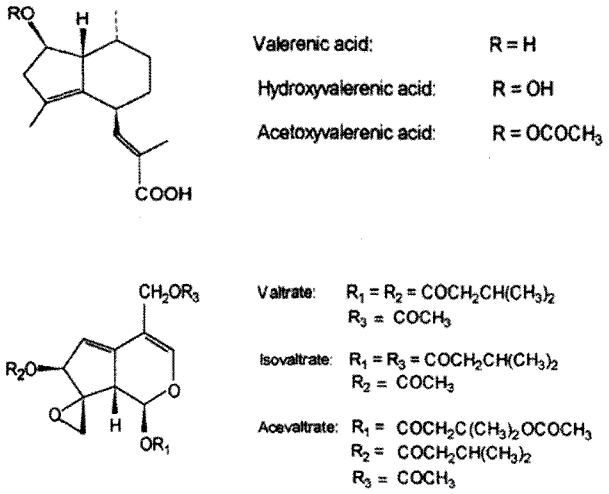

Fig 1. Structures of the valerenic acids and valepotriates.

Due to biotechnological interests, root and rhizome of different *Valeriana* species have been over-collected, usually from naturally growing immature plants that have not yet formed seeds. In addition, collected seeds from naturally population of *V. fauriei* have a short survival period of 2-3 months, even kept at low temperatures.

As an alternative to obtain these active compounds is great pharmaceutical interest, *in vitro* production has been evaluated by callus culture and various tissues from some Valerianaceae such as *V. officinalis, V. wallichii* DC, *V. glechomifolia* , *Centranthus rubber, C. macrosiphon* and *V. edulis ssp.* Procera.

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A number of studies have been reported on the production of valepotriates from the transformed roots of the Valerianaceae, including *V. officinalis* L. var. *sambucifolia* Mikan (Grnicher *et al.*, 1992), *Centranthus ruber* DC (Grnicher *et al.*, 1995), *V. wallichii* DC (Banerjee *et al.*, 1998), *Valerianella discoidea* (L.) Loisel (Caetano *et al.*, 1999) and *Valerianella locusta* (Kittipongpatana *et al.*, 2002). The contents of hairy roots culture can enhance valepotriates production. However, in practice genetically-modified organisms or organs are not well accepted as food drugs. Therefore, the present aim of this work was to establish root culture without transformation with *Agrobacterium rhizogenes* and to compare valerenic acids and valepotriate production, valerenic acids and valepotriate pattern in *V. fauriei* root culture using various culture media without plant growth regulators.

MATERIALS AND METHODS

Plant material

Seeds of *V. fauriei* were obtained from the seed bank of the Korea University. The seeds were surface sterilized by rinsing them with 70% ethanol for 1 min, then sterilized with 1% sodium hypochlorite solution containing 0.12% Tween 20 for 5 min, and then rinsed five times with sterile distilled water. The seeds were germinated on hormone-free MS (Murashige and Skoog, 1962) basal salt medium containing 3% sucrose and 0.7% agar and the pH was adjusted to 5.7 ± 0.1 before autoclaving at 121 ℃ for 20 min. The seedlings were grown at 25 ± 1 ℃ under a 16/8-h (light/dark) photoperiod with light provided by cool-white fluorescent lamps.

Root culture

The whole roots were 1-2 ㎝ long cross sliced and cultured in 100 ㎖ Erlenmeyer flask containing 30 ㎖ of liquid B5 (Gamborg *et al.*, 1968) medium without plant growth regulators. The media contained 3% sucrose and the pH was adjusted to 5.7 before autoclaving (20 min. 121 ℃). The flasks were shaken at 100 rpm for aeration. The roots formed were cut from the explants and they were cultured in the same B5 liquid medium and subcultured every 4 weeks. The cultures were maintained for more than 1 year in darkness before use as a source for bioreactor culture.

Bioreactor culture

For determination of optimal medium in root growth and valerenic acids and valepotriate production, various media like B5, MS, WPM (Lloyd and McCown, 1980), RCM (Root Culture Medium) and SH (Schenk and Hildebrandt, 1972) were tested. All media contained 3% sucrose and the pH was adjusted to 5.7 before autoclaving (25 min, 121 ℃). The 20 g of roots were inoculated into a 3 ℓ glass balloon type of bubble bioreactors containing 2 ℓ of liquid culture media and the air volume was adjusted with an air flow meter to give a constant flow rate of 0.2 vvm. The roots were harvested after 4 weeks of culture. The three replicates were frozen, lyophilized, powdered, and pooled prior to the analysis of valerenic acids and valepotriates contents.

The multiplication rate for root cultures was determined as:

$$GI = \frac{\text{Final fresh weight}}{\text{Initial fresh weight}}$$

One-way analysis of variance (ANOVA) was applied with a critical value of $p \leq 0.05$. Significant ANOVAs were followed by the Tukey test.

Sample preparation for valerenic acids and valepotriates quantification

Pure standards of valerenic acid, hydroxyvalerenic acid, acetoxyvalerenic acid, valtrate, isovaltrate and didrovaltrate were kindly supplied by Dr. Bos R. (Groningen University, Netherlands).

Root extracts of *V. fauriei* were prepared by a similar method described by Bos *et al.* (1996) and Bicchi *et al.* (2000). Dry weight of cultured roots were determined after freeze drying 1 g of the finely ground plant material was extracted with 30 ㎖ methanol in an ultrasonic bath for 30 min. The resulting extract was filtered and the residue was re-extracted twice with 15 ㎖ methanol through the same procedure. The three extracts were bulked, evaporated to dryness under vacuum and redissolved in 3 ㎖ methanol and filtered through 0.2 ㎛ pore size a membrane filter (Millipore) and 20 ㎕ of the solution was analyzed by HPLC-PDA. This procedure was carried out in triplicate. One-way analysis ANOVA were carried out for valepotriates and valerenic acids. Significant ANOVAs were followed by the Tukey test. A critical value of $p \leq 0.05$ was used for all statistical tests.

High performance Liquid chromatographic photodiode array detector (HPLC-PDA) separations were performed using a Waters 600 controller system equipped with a Waters 996 photodiode array detector and waters 600 pump and Waters 717 plus auto-sampler. The data acquisition was achieved using the Millennium^{32} PDA program. The mobile phase consisted of a linear gradient from 100% of solvent A (water: acetonitrile: phosphoric acid, 80: 20: 0.05) to 100% of solvent B (water: acetonitrile: phosphoric acid, 20: 80: 0.05) in 40 min, applied at a flow-rate of 1 ㎖/min. (Table 1). The injection volume was 20 ㎕, and the detection wavelength was 220 ㎚ for valerenic acids and 256 ㎚ for valepotriates . The μBondapak^{TM} C_{18} (3.9×300 ㎜) reverse phase column was used.

Table 1. The operation conditions of HPLC-PDA for analysis of valerenic acids and valepotriates compounds.

Column	μBonda pak™ C_{18}(3.9×300 ㎜)		
Mobile phase	Gradiant		
	Solvent A		Solvent B
	80% H2O: 20% acetonitrile 0.05% phosphoric acid		20% H2O: 80% acetonitrile 0.05% phosphoric acid
Flow rate	1.0 ㎖/min.		
Detector (UV)	Waters 996 photodiode array detector at 220 ㎚ and 256 ㎚		
Injection volume	20 ㎕		
Time program	Time (min.)	Solvent A (%)	Solvent B (%)
	0.0	100	0
	40.0	0	100

Valerenic acids and valepotriates were identified by comparing their retention times and UV spectra recorded by PDA with those of authentic samples. Valerenic acids and valepotriates content expressed as a percentage of dry weight.

RESULTS AND DISCUSSION

Effect of culture media on roots growth

The seed (Fig. 2a), seedling (Fig. 2b) and plantlet (Fig. 2c) of *V. fauriei* were cultured on MS basal medium without growth regulators. Each sliced root was developed 3-4 of new 3-4cm long lateral roots (Fig. 2d) in B5 medium without growth regulators after 4 weeks. The roots formed were subcultured on the same liquid medium at 4 weeks of intervals (Fig. 2e).

For determination of optimal medium for the *V. fauriei* roots growth and valerenic acids and valepotriates productions, 2 ℓ balloon type of glass bubble bioreactors (Fig. 2f) were used.

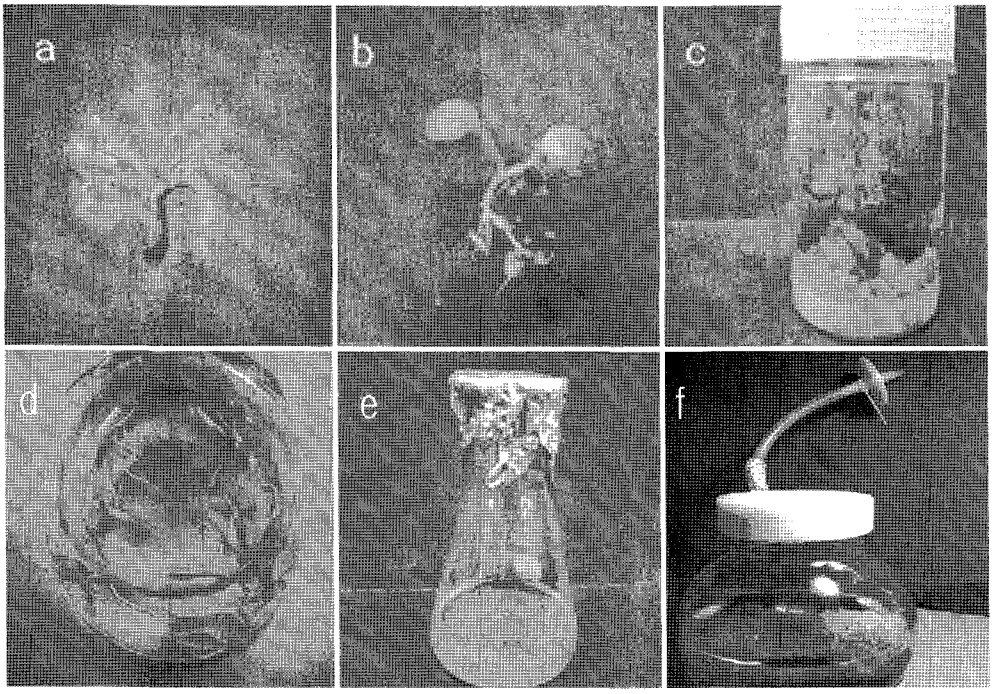

Fig. 2. *In vitro* cultured *V. fauriei*.
The seeds (a) were surface sterilized and germinated seedling (b) and plantlet (c) cultured on MS basal solid medium containing 3% sucrose without growth regulators. Lateral roots (d) formed 3-4 ㎝ long after 4 weeks of cultured in 100 ㎖ Erlenmeyer flask containing 30 ㎖ of liquid B5 medium and 3% sucrose without growth regulators. Roots were subcultured on the same liquid medium at 4 week intervals (e). Roots cultured in 3 ℓ of balloon type of bubble bioreactors containing 2 ℓ of liquid medium (f) inoculation with 20 g of fresh weight and 3% sucrose without growth regulators after 4 weeks culture.

Table 2. Effect of culture media on multiplication rate (expressed by fresh weight) of roots of *V. fauriei*. Each value is the mean of three replicates.

Media	**Multiplication rate** (Fresh weight)
B5	5.03 ± 0.11a
MS	2.41 ± 0.43c
SH	3.67 ± 0.12b
WPM	2.40 ± 0.52c
RCM	0.82 ± 0.08d

Means followed by different letters are at 5% level by LSD and Tukey HSD.

The multiplication rate for adventitious root cultures was determined as:

$$GI = \frac{\text{Final fresh weight}}{\text{Initial fresh weight}}$$

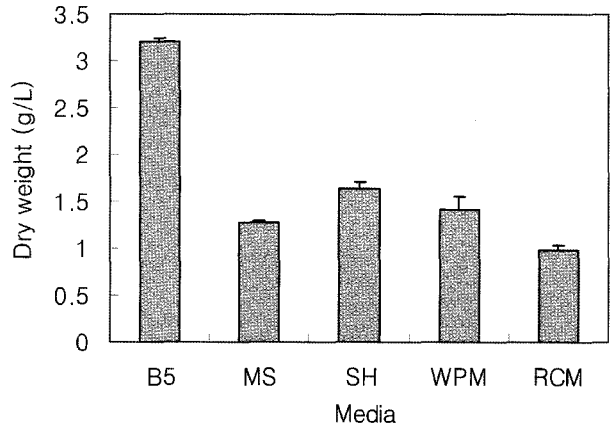

Fig. 3. Effect of culture media on root growth (expressed by dry weight) of *V. fauriei*.
Each medium contained 3% sucrose. The 20 g of roots were cultured for 4 weeks in 3 ℓ balloon type of glass bubble bioreactor containing 2 ℓ of various culture media in darkness. The air volume was adjusted with an air flow meter to give a constant flow rate of 0.2 vvm. Each value is the mean of three replicates. The dry weight was determined for each sample after freeze drying.

Among the media tested, the best root growth was Ochieved in B5 medium and followed by the other of SH, MS and WPM medium. However, the roots were deteriorated in RCM medium. B5 medium gave the maximum biomass production of 101 g fresh weight and a 5.03-fold multiplication rate. The 3.67, 2.41 and 2.40-fold (Table 2) multiplication rate ware obtained in SH, MS and WPM media, respectively, when 20 g of fresh weight was inoculated. The statistical analyses showed that the growth of roots was strongly affected by the kind of basal salts tested (Table 2).

Dry weight was measured after freeze drying for quantification of valepotriates and valerenic acids contents. Dry weight

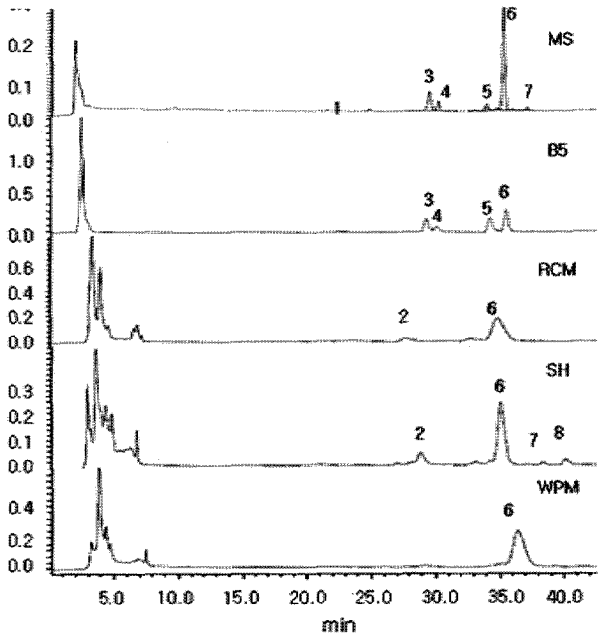

Fig. 4. Comparison of valerenic acids with valepotriates HPLC-PDA profiles patterns between five *in vitro* cultured roots. Present are 1: hydroxyvalerenic acid, 2: acetoxyvalerenic acid, 5: valerenic acid, 6: isovaltrate, 7: valtrate. MS: root cultured in MS medium, B5: root cultured in B5 medium, RCM: root cultured in RCM medium, SH: root cultured in SH medium, WPM: root cultured in WPM medium.

was the highest when the *V. fauriei* root was grown in B5 medium. The dry weight of *V. fauriei* root grown in B5, SH, WPM, MS and RCM media were 3.21, 1.63, 1.42, 1.27 and 0.99 g/ℓ (Fig. 3), respectively.

Effects of culture conditions on valerenic acids and valepotriates production patterns

The identities of the main peaks were established by comparison of retention time (t_R) values and UV spectra with those of standards. The presence of hydroxyvalerenic acid (t_R between 26 and 28 min), acetoxyvalerenic acid (t_R between 31 and 32 min), valerenic acid (t_R between 35 and 36 min), isovaltrate (t_R between 37 and 38 min) and valtrate (t_R between 39 and 40 min) was confirmed.

The HPLC-PDA pattern of valerenic acids and valepotriates in the various culture media were compared (Fig. 4). The contents were significantly affected by the culture conditions. The highest contents of total valerenic acids were shown in MS medium cultured roots (3.74 mg/g). In other media of WPM, B5, SH, and RCM media total yield valerenic acids were 0.20 mg/g, 2.45 mg/g, 1.74 mg/g and 0.46 mg/g, respectively (Table 3). The highest contents of total valepotriates were shown in MS medium cultured roots (4.81 mg/g). In other media of WPM, B5, SH, and RCM media total yield valepot-

Table 3. Comparison of retention time with content or peak area and yield valerenic acids and valepotriates from in various media-cultured roots of *Valeriana fauriei*. (The compounds A, B, C, D, and E were not identified. The compound 1, 2, 5, 6 and 7 were matched with Fig. 4. The is not detected. The peak area is arbitrary units) (1 g of Dry weight based).

Retention time (min) / (content mg/g)	In MS medium	In WPM medium	In B5 medium	In SH medium	In RCM medium
26.66 ± 0.70 (1) (Hydroxyvalerenic acid)	1.46 a	–	0.15 b	–	
27.96 ± 0.40 (A) (Peak area)	–	–	830,373	684,019	2,118,246
29.27 ± 0.37 (B) (Peak area)	4,911,865	385,272	1,245,368	–	1,812,049
31.88 ± 0.89 (2) (Acetoxyvalerenic acid)	0.03 b	–	–	0.14 a	0.03 b
34.02 ± 0.40 (C) (Peak area)	5,279,689	458,985	1,064,486	–	2,168,774
35.50 ± 0.19 (D) (Peak area)	195,207	–	666,220	–	–
36.89 ± 0.56 (5) (valerenic acid)	2.25 a	0.20 b	2.3a	1.60 a	0.43 b
37.95 ± 0.36(6) (isovaltrate)	3.24 a	1.39 bc	1.93 b	0.80 c	1.57 bc
39.20 ± 0.10 (7) **(valtrate)**	1.57 a	0.52 ab	0.86 ab	0.12 b	0.09 ab
41.31 ± 0.13 (E) (Peak area)	167,103	55,737	708,264	251,154	159,530
Total valepotriates Content (mg/g)	4.81	1.91	2.79	0.92	2.47
Total valerenic acids content (mg/g)	3.74	0.20	2.45	1.74	0.46

riates were 1.91 mg/g, 2.79 mg/g, 0.92 mg/g, 2.47 mg/g, respectively. The reported values are the means of the results of triplicated analyses. The statistical analyses showed culture media and condition significantly affect on the valerenic acids and valepotriates production based on dry weight.

An additional unidentified peak with t_R of 27.96 ± 0.40 min (A), 29.27 ± 0.37 min (B), 34.02 ± 0.40 min (C), 35.50 ± 0.19 min (D) and 41.31 ± 0.13 min (E) are in the chromatogram *of V. fauriei.* These compounds will be identified continuously in the future.

Effects of culture media on valerenic acids and valepotriates production

Because secondary product formation and culture growth often exclude each other, a decrease in the accumulative capacity during culture growth is inevitable.

The total production contents of valerenic acids and valepotriates were determined as:

Total production (mg/ℓ) = Dry weight production (g/ℓ) × content (mg/g)

Comparison of total production of valerenic acids and vale-

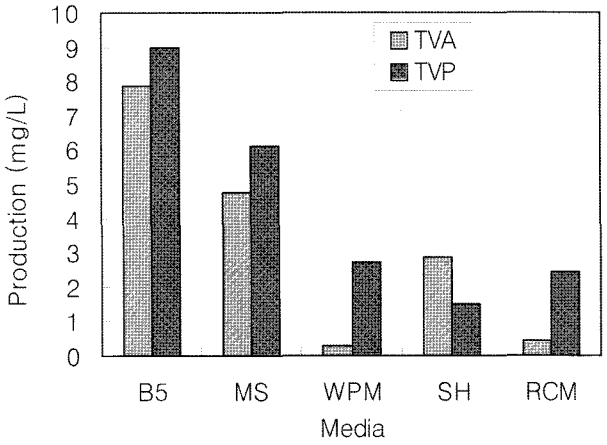

Fig. 5. Effect of culture media on valerenic acids and valepotriates production of *V. fauriei*. Each medium contained 3% sucrose. The 20 g of roots were cultured for 4 weeks in 3 ℓ balloon type of glass bubble bioreactor containing 2 ℓ of various culture media in darkness. The air volume was adjusted with an air flow meter to give a constant flow rate of 0.2 vvm. Each value is the mean of three replicates. The dry weight was determined for each sample after freeze drying. TVA: total valerenic acids production, TVP: total valepotriates production. The total production contents of valerenic acids and valepotriates were determined as: Total production (mg/ℓ) = Dry weight production (g/ℓ) × content (mg/g).

potriates in different media-cultured roots were shown in Fig. 5. The highest total valerenic acids and valepotriates production were in B5 medium-cultured root, and the production were 7.86 ㎎/ℓ and 8.96 ㎎/ℓ, respectively.

These results indicated that mineral nutrients and vitamins were strongly effected the valerenic acids and valepotriates patterns and production. Hormone-free B5 medium could be useful for production of valerenic acids and valepotriates. So we have been studying the optimal conditions for enhancing roots growth and valerenic acids and valepotriate production by using various culture conditions.

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