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# Shortening the Biennial Life Span of *Swertia punicea* by Using Tissue Culture to Obtain Season-free Inflorescences

Katsuhiko KONDO,* Minoru FUJISHIMA** and Reiji TAKEDA***

* *Laboratory of Plant Chromosome and Gene Stock, Faculty of Science, Hiroshima University, 1-1-89, Higashi-Senda-Machi, Naka-ku, Hiroshima 730, Japan*

** *Okinawa Section, Department of Plant Biotechnology, Central Research Laboratories, Sekisui Plastics Co., Ltd., Koki 1774, Nago 905, Japan*

*** *Iwakuni Office, Yamaguchi Prefecture Department of Forestry, 3-5-7, Marifu-cho, Iwakuni 740, Japan*

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Tissue segments from stalked second-year-plants of the biennial *Swertia punicea* induced calli when they were cultured on the LS gelrite media containing 1 μM NAA and 1 μM BA, or 1 μM NAA and 10 μM BA, or 1 μM 2, 4-D and 1 μM BA, in combination. Those calli were rapidly propagated on the LS gelrite medium containing 0. 5 μM NAA or 2, 4-D at 25℃ at a relative humidity of 65% under an illumination of 2, 000 lux for 12 hr. After approximately 60 days of the callus culture in the LS gelrite medium containing 0. 5 μM IAA and 0. 5 μM BA, they began to produce numerous adventitious buds which later regenerated plantlets. Those plantlets were chromosomally rather stable clones with the stable karyotype of 26 median-centromeric chromosomes at mitotic metaphase. Large enough plantlets having several leaves of 7-8 cm long and many roots were transplanted in pots *in vivo* and acclimated in environment at above 15℃ and relative humidity above 65% for a week. After about three months at any season if cultivated at above 15℃, they usually bolted stems and produced inflorescences. Thus, this culture system shortened the biennial life-span of *Swertia punicea.*

*Swertia punicea* Hemsl., a rare species of the Gentianaceae native to the southeastern part of the Himalayas, is a biennial herbaceous plant with a rosette form for the first year and an erect form performing pleiochasium inflorescence for the second year. This species, as well as other species of *Swertia*, has been cultivated in Japan these several years since it is known to have a bitter stomachic property for a folklore medicine. A previous chemical study in *S. punicea*[4] resulted in the isolation of oleanolic acid, monohydroxy trimethoxy xanthone, 1-hydroxy 3, 7, 8-trimethoxy xanthone (decussatin), and 1, 7-dihydroxy 3, 8-dimethoxy xanthone (gentiana caulein).

Since those bitter stomachics are concentrated in the inflorescences of *S. punicea* set regularly in every other November to December, they are too long, uneconomically time-consuming. Thus, an exploitation of culture system shortening the biennial life-span, inducing season-free flower and proliferating perennially and continuously plantlets in *S. punicea* is favorable to economical harvest.

## Materials and Methods

Second-year-plants of the biennial *Swertia punicea* Hemsl., which were cultivated in pots in the experimental garden, Hiroshima University, were utilized in this study. Their apical meristems *ca* 0. 2 mm long, leaf pieces *ca* 9 mm area, internodes of stems *ca* 5 mm long, and roots *ca* 5 mm long

**Table 1.** Callus induction from plant segments of *Swertia punicea* in response to various LS media containing auxin and cytokinin for primary culture.

| Auxin | (μM) | Cytokinin BA (μM) 1.0 | 10.0 |
|---|---|---|---|
| 2, 4-D | 0.1 | no response | no response |
| | 1.0 | callus formation | no response |
| NAA | 0.1 | shoot formation | shoot formation |
| | 1.0 | callus formation | callus formation |
| IAA | 0.1 | no response | shoot formation |
| | 1.0 | shoot formation | no response |

**Table 2.** Callus multiplication-rate in *Swertia punicea* after 40 days of subculture in response to various LS media containing auxin and cytokinin.

| Auxin | (μM) | Cytokinin BA (μM) 0 | 0.5 | 5.0 |
|---|---|---|---|---|
| 0 | | 1.51±0.17 | 1.51±0.03 | 1.69±0.28 |
| IAA | 0.5 | 2.44±0.69 | 1.64±0.25 | 1.99±0.41 |
| | 5.0 | 1.92±0.51 | 2.37±0.92 | 2.39±0.67 |
| NAA | 0.5 | 3.48±0.81 | 3.39±0.20 | 3.33±0.36 |
| | 5.0 | 1.76±0.10 | 1.76±0.17 | 1.83±0.04 |

Twenty-four calli were used in each experiment.
Multiplication rate±standard deviation

$$= \frac{\text{fresh weight of callus at 40-day-subculture}}{\text{fresh weight of callus at transplantation}}$$

were aseptically excised for primary culture. The Linsmaier and Skoog medium[2] was supplemented with indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxy acetic acid (2, 4-D) and $N^6$-benzyladenine (BA) at various concentrations in various combinations (**Table 1**). The pH of the media was adjusted to 5.7, and 2 g/*l* gelrite was added to each medium. The cultures were made under a condition of 2000 lux illumination with the day-light of 12 hr at a relative humidity of 65% at a temperature of 25°C. The calli induced were cut into 2×2 cm pieces and subcultured in the above media at intervals of 20 days.

Forty-day-old subcultured calli were transferred onto differentiation media for adventitious budding and plant regeneration (**Table 2**). The plantlets which grew up large enough with several leaves 7–8 cm long and many long roots were transplanted into the mixture of vermiculite and Kiryu-sand (1: 1) in pots *in vivo* and periodically measured in their growth forms.

Growing root-tips of the plants given were harvested and pretreated in an aqueous solution of 0.002 M 8-hydroxyquinoline at 18°C for 3 hr. Then, they were fixed in 45% acetic acid for 5 min at 5°C, before they were macerated in a mixture of two parts of 1-N hydrochloric acid and one part of 45% acetic acid at 60°C for 8 sec, and stained and squashed in 2% aceto-orcein.[5]

## Results and Discussion

Calli of *Swertia punicea* were formed, when the tissue segments were cultured on the LS gelrite media containing 1 μM NAA and 1 μM BA, or 1 μM NAA and 10 μM BA, or 1 μM 2, 4-D and 1 μM BA, in combination (**Fig. 1 A, B**). Thus, the auxin substances effective for callus induction in *S. punicea* were NAA and 2, 4-D at the concentration of 1 μM, respectively (**Table 1**). Frequency of callus induction in an apical meristem culture of *Gentiana*, another member of the Gentianaceae, positively correlated with an increase in NAA and BA concentrations,[3] while that from filament and ovary

cultures positively correlated with an increase in NAA concentration.[3] These phenomena were quite similar to those of the present study with respect to the auxin activity.

LS media containing IAA, NAA and BA at various concentrations in various combinations were

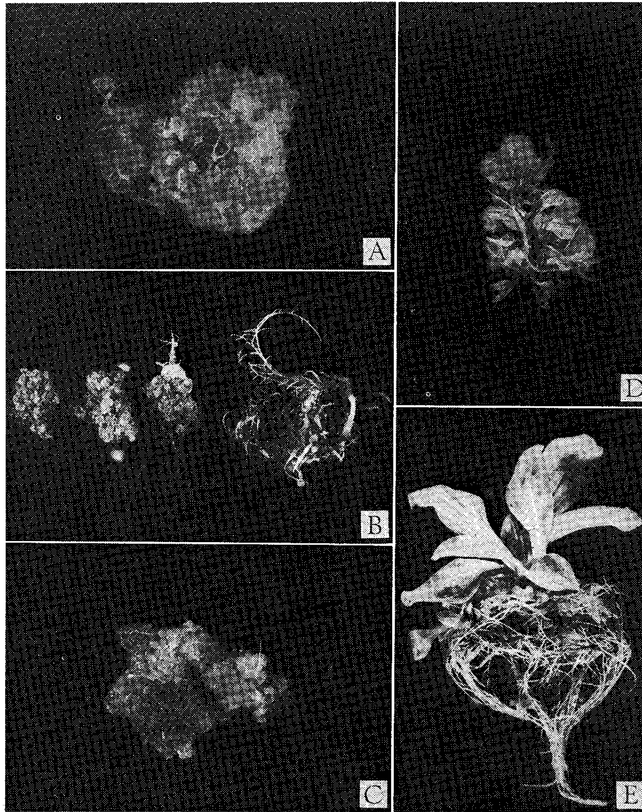

**Fig. 1.** Induction of plantlets from callus. A. 40-day-old stage of callus. B. Initial stage of callus formation from plant segments of: (left to right) apical meristem, leaf piece, stem internode and roots. C. Callus with several adventitious buds and a shoot. D. Numerous shoots and roots to initiate plantlets. E. Plantlet at potting stage.

**Table 3.** Plant regeneration from calli of *Swertia punicea* after 60 days culture in response to various LS media containing auxin and cytokinin.

| Auxin | (μM) | Cytokinin BA (μM) 0 | 0.5 | 5.0 |
|---|---|---|---|---|
| 0 | | regenerated single plantlets, 25.0% ; rooted, 50.0% | regenerated multi-plantlets, 16.7% ; rooted, 50.0% | not regenerated ; rooted, 66.7% |
| IAA | 0.5 | not regenerated ; rooted, 75.0% | regenerated multi-plantlets, 37.5% ; rooted, 41.7% | not regenerated ; rooted, 66.7% |
| | 5.0 | not regenerated ; rooted, 100% | not regenerated ; rooted, 33.3% | regenerated single plantlets, 20.0% ; rooted, 70.0% |
| NAA | 0.5 | not regenerated ; rooted, 66.7% | not regenerated ; rooted, 20.0% | regenerated single plantlets, 11.6%, and multi-plantlets, 4.7% ; rooted, 55.8% |
| | 5.0 | not regenerated ; not rooted | not regenerated ; not rooted | not regenerated ; not rooted |

%=culture vials with regenerated and rooted calli/50 culture vials studied

tested to find the best subculture medium for rapid growth of calli in *Swertia punicea*; fresh weight of the callus at the beginning of transplantation and that at the 40-day-period after the beginning of transplantation were gravimetrically analyzed (**Table 2**). The lowest multiplication rate of about 1. 6 was shown in any auxin-free LS medium and LS medium supplemented with IAA.

The highest callus-multiplication rate of 3. 4 was exhibited in the LS supplemented with 0. 5 $\mu$M NAA (**Table 2**). 0. 5 $\mu$M NAA seemed to be effective for the multiplication rate approximately 20 days after transplantation. However, NAA at higher concentrations more than 5 $\mu$M prohibited callus growth

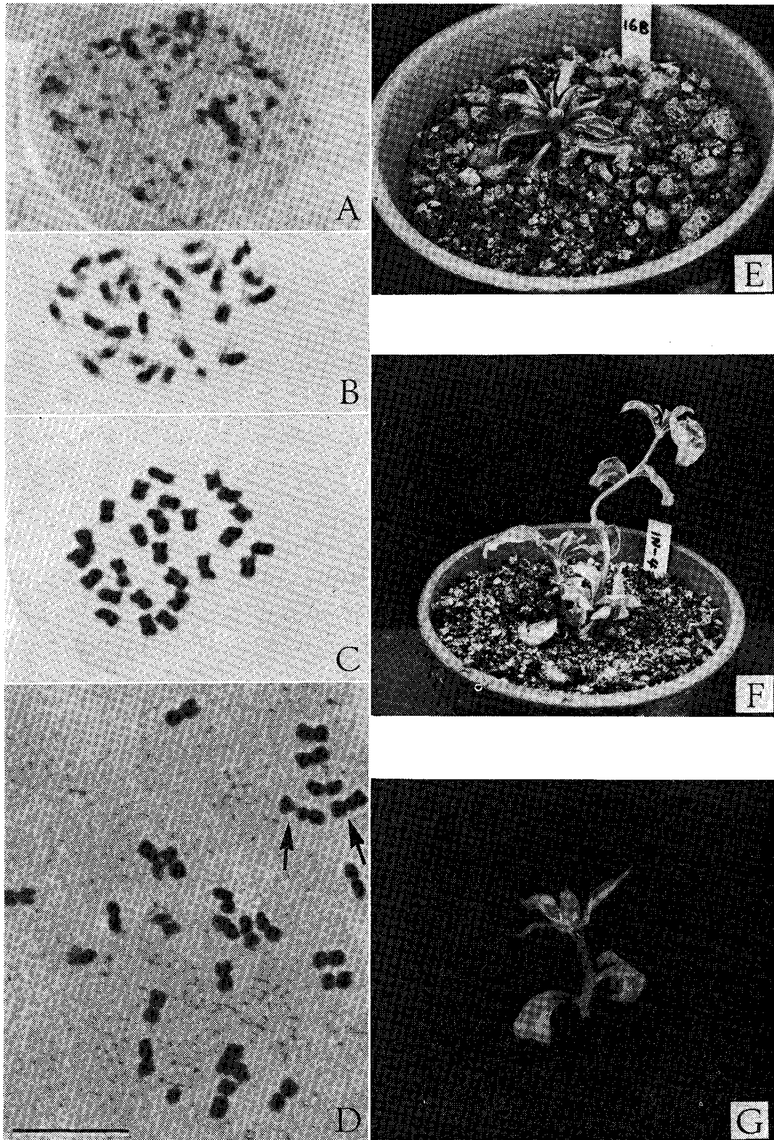


**Fig. 2.** Somatic chromosomes (A–D; bar = 10 $\mu$m) and potted plants (E–G). A. Resting chromosomes of the simple chromocenter type. B. Prophase chromosomes of the proximal type. C. $2n=26$ median-centromeric chromosomes at metaphase. D. Metaphase chromosome complement with two secondary constrictions (arrows). E. Plant at the beginning of acclimatization. F. Plant with erect stem. G. Single flower.

and plant regeneration. These proliferated calli were soft and pale yellow in color.

The highest regeneration efficiency of 38% in the calli of *Swertia punicea* was displayed on the LS supplemented with 0.5 μM IAA and 0.5 μM BA (**Table 3**). Those calli first generated numerous green-colored adventitious buds and roots (**Fig. 1 C**) and eventually plantlets (**Fig. 1 D**). The calli placed on the LS containing 5 μM IAA and 5 μM BA produced only single plantlets with regeneration efficiency of 20%, while those with 0.5 μM NAA and 5 μM BA showed 16% regeneration efficiency and multi-plantlets with regeneration rate of 4.7% (**Table 3**). Furthermore, the calli on the hormone-free LS medium performed single plantlets with regeneration efficiency of 25% and those supplemented with 0.5 μM BA performed a mass of plantlets with regeneration efficiency of 17%. In contrast, an apical meristem culture of *Gentiana* resulted that the best shoot regeneration was exhibited on the LS medium containing 0.089 or 0.89 μM BA.

The parental plants of *Swertia punicea* raised from seeds had the karyomorphological characteristics[5] of the simple chromocenter type of the resting nucleus, the proximal type of the mitotic-prophase chromosome, the somatic chromosome number of $2n=26$, and the median centromeric type of the mitotic-metaphase chromosome. Two chromosomes of the mitotic-metaphase complement had secondary constrictions. From the longest (3.0 μm) to the shortest (1.8 μm) chromosomes a gradual decrease in length was observed. Similarly, the regenerated plantlets of *Swertia punicea* from calli showed here regularly the same karyomorphological characteristics not only to each other but also to the parental plants (**Figs. 2 A–D**). Thus, those plantlets were of karyomorphologically rather stable clones.

After well-grown, small plants of *Swertia punicea* with several leaves and roots (**Fig. 1 E**) were transplanted to a mixed soil of vermiculite and Kiryu-sand (1 : 1) (**Fig. 2 E**) and gradually acclimatized for potting, most of them began to grow shoots (**Fig. 2 F**). Then, after approximately three months at any season if cultivated at above 15℃, they faced completely adult phase to bolt pleiochasium inflorescences with numerous flowers (**Fig. 2 G**).

The TLC and HPLC analyses of secondary metabolite productions revealed that the flowering adult plants of the cultured *Swertia punicea* had all of the major components of the species.[1,4]

Thus, this tissue culture method seems promising for season-free, short-term harvest of *Swertia punicea* inflorescences.

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**≪和文要約≫**

組織培養利用による二年生稀少種 *Swertia punicea* の大量増殖と
生活環の短縮化による花茎の短期収穫

近藤勝彦*，藤島　稔**，竹田礼次***

* 広島大学理学部附属植物遺伝子保管実験施設，** 積水化成品工業株式会社中央研究所バイオ研究室沖縄分室，*** 山口県岩国林業事務所

二年生 *Swertia punicea* を培養して，花茎を短期間に誘導できる植物体を大量に再分化させうるカルスを得た．カルスは，LS 培地で NAA または 2,4-D を 0.5 μM 添加した時，最もよく増殖した．また再分化には LS 培地で IAA 0.5 μM と BA 0.5 μM を添加したものが最適であった．