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EFFICIENT PRODUCTION OF BIOLOGICALLY ACTIVE DITERPENOIDS BY LEAF ORGAN CULTURE OF *SCOPARIA DULCIS*

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Key Word Index—*Scoparia dulcis*; Scrophulariaceae; leaf organ culture; 4PU; diterpenoid; scopadulcic acid B; scopadulciol.

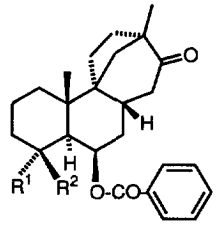
Abstract—Production of scopadulcic acid B and scopadulciol by leaf organ culture of *Scoparia dulcis* was examined by addition of cytokinins to culture media. Of the tested cytokinins, *N*-phenyl-*N'*-(4-pyridyl)urea was the most efficient when supplemented in the mass spectroscopic liquid culture medium at 0.1 μM. © 1997 Elsevier Science Ltd

INTRODUCTION

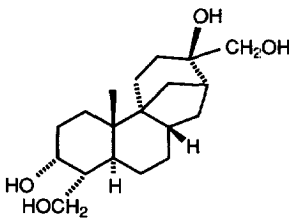
Scoparia dulcis L. (Scrophulariaceae) is a medicinal plant distributed in the torrid zone and produces unique tetracyclic diterpenoids termed scopadulcic acids A (SDA, **1**) and B (SDB, **2**) [1], and scopadulciol (SDC, **3**) [2]. They have a similar carbon skeleton to that of aphidicolin (**4**) isolated from a mould, *Cephalosporium aphidicola*, as an inhibitor of eucaryotic DNA polymerase α. Because of their novel structures as well as interesting biological activities such as inhibitory effects against proton pumps [2, 3], bone resorption [4] and replication of herpes simplex virus type 1 [5, 6], they have received much attention and recently, their racemates have been chemically synthesized [7, 8].

In previous papers, we reported the production of **2** and **3** by cultured tissues, i.e. callus and multiple shoots of *S. dulcis* [9, 10]. The capability of diterpenoid production by these cultured tissues was very poor and differentiation of leaves was thought to be related to diterpenoid production. In addition, diterpenoids were suggested to be produced and accumulated in the leaves. The findings that the ability to biosynthesise secondary metabolites was associated with differentiation of plant organs have been reported in *Papaver somniferum* [11], *Scopolia parviflora* [12], *Hyoscyamus niger* [13] and *Catharanthus roseus* [14, 15].

In this paper, we describe the effect of cytokinins, plant growth substances playing a major role in cell division and differentiation, on production of **2** and **3** in leaf organ cultures of *S. dulcis*.



1: $R^1 = CH_2OH$, $R^2 = COOH$
2: $R^1 = COOH$, $R^2 = CH_3$
3: $R^1 = CH_2OH$, $R^2 = CH_3$



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RESULTS AND DISCUSSION

In order to select a suitable growth stage for leaf organ culture of *S. dulcis*, the time courses of the growth of plants and diterpenoid production by leaves were measured. The diterpenoid content was analysed by HPLC [9]. Germination of seeds was observed at the 3rd day after sowing in the light. As shown in Fig. 1, diterpenoids were detected at the 7th day after germination and their production increased rapidly in parallel with the growth of plants. After 4 weeks of culture following seed germination, the diterpenoid content in leaves reached plateau. Thus, the leaves of 4-week-old plants were used as inocula for leaf organ culture.

2-Isopentenyladenine (2IP), kinetin (K), ^{6}*N*-benzyladenine (BA) and *N*-phenyl-*N'*-(4-pyridyl) urea (4PU) were tested for their effects on the growth of

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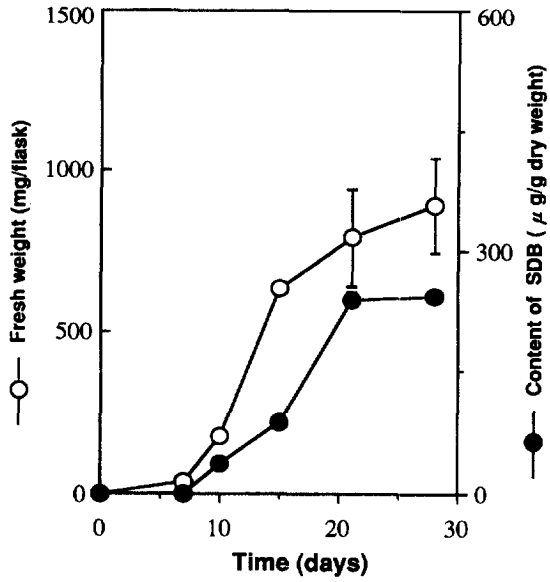

Fig. 1. Time courses of growth and diterpenoid contents of seedlings of *S. dulcis* (SDB type) cultured in MS medium at 26° under continuous light irradiation (6000 lux). Mean ± s.e. ($n = 3$). –○–: fresh weight (mg flask^{-1}); –●–: content of SDB (μg g^{-1} dry weight).

tissues and diterpenoid production in leaves. As a result, only 4PU was found to be efficient at 0.1 μM for leaf organ culture of *S. dulcis* (Fig. 2). At concentrations more than 0.5 μM, 4PU induced callus and caused much decrease in diterpenoid production. Similar results were obtained in both plant chemotypes (SDB and SDX types). In the cases of other cytokinins, the diterpenoid production was also better than controls when each cytokinin was added at 0.1 μM in the culture medium. However, remarkable rooting was observed in all these cultures, which was distinct from the cultures treated with 4PU. In addition, etiolation of leaf organs was induced when kinetin was added in the culture medium at more than 0.5 μM. The etiolated tissues were found to contain significantly less diterpenoid in comparison with green leaf organs (data not shown).

The time courses of growth and diterpenoid production of tissues were then examined by using the leaf organ culture medium containing 4PU at 0.1 μM. As illustrated in Fig. 3, the growth of tissues increased rapidly from the 6th day, while the diterpenoid content per tissue weight increased markedly up to the 3rd day and then increased slowly. On the other hand, the total diterpenoid per flask increased in parallel with the tissue growth until the 10th day. On the 12th day, remarkable rooting was observed and resulted in dispersion of diterpenoid content. From these results, the most efficient diterpenoid production by leaf organ culture was found to be attained when subcultures were performed every 10 days.

4PU was originally synthesised by Bruce *et al.* [16] and reported by Okamoto as a highly active cytokinin [17]. It also showed a marked effect on shoot formation in tobacco pith disc and callus with a single application [18]. So far, there have been no reports in the literature concerning the effects of 4PU on production of secondary metabolites of higher plants. In this study, 4PU was found to increase number and size of leaves as well as their content of diterpenoids at 0.1 μM but induce de-differentiation of tissues and cause a remarkable decrease in diterpenoid production at more than 0.5 μM. The poor capability of the de-differentiated tissues for production of diterpenoids indicates that the biosynthesis of SDB and SDC is strongly related to differentiation of leaves. On the other hand, etiolation of leaf organs induced by treatment with higher concentration of kinetin (more than 0.5 μM) caused a marked decrease in diterpenoid production. These findings suggest that the biosyn-

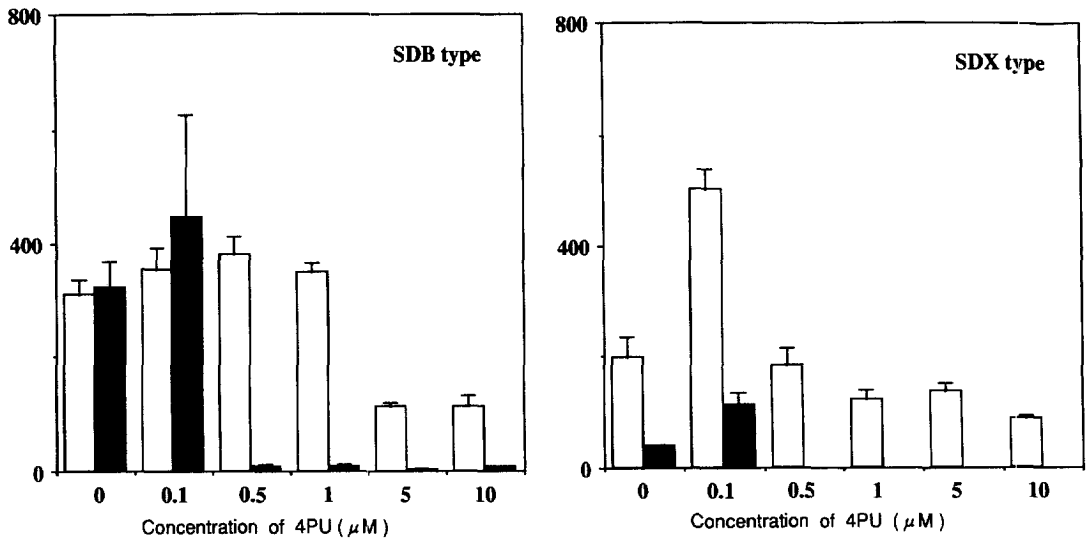

Fig. 2. Effect of concentration of 4PU in culture medium on the growth and contents of diterpenoids in leaf organ cultured tissues of *S. dulcis*. The tissues were cultured in MS liquid medium at 26° under continuous light irradiation (6000 lux). Mean ± s.e. ($n = 3$). □: dry weight (mg flask^{-1}); ■: total content of diterpenes (μg flask^{-1}).

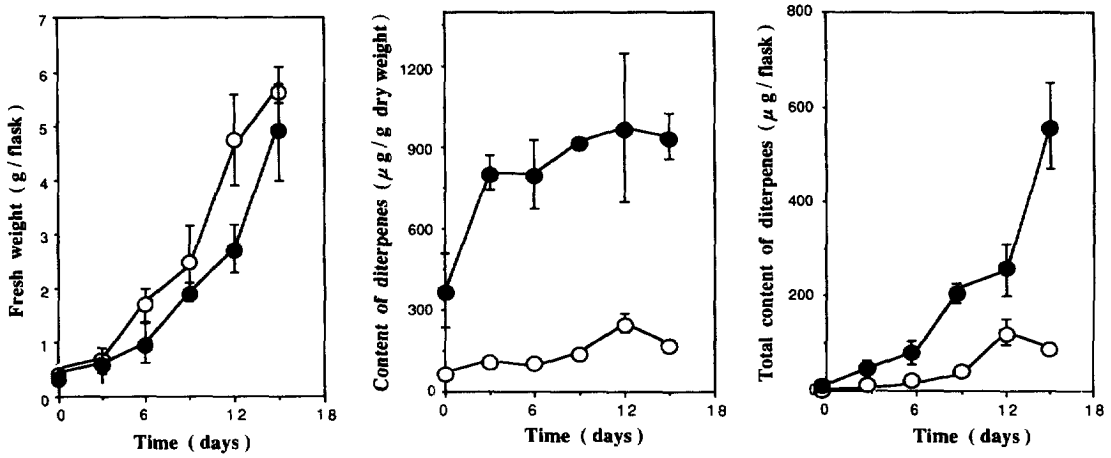

Fig. 3. Time courses of cell growth and content of diterpenes in leaf organ cultured tissues of *S. dulcis*. MS liquid medium containing 0.1 μM of 4PU was used. Mean ± s.e. (n = 3). –○–: SDB type; –●–: SDX type. One result of duplicates is shown.

thesis of the diterpenoids in *S. dulcis* might be catalysed with enzymes distributing in chloroplasts.

Recently, Rohmer *et al.* proved the involvement of a non-mevalonate pathway in terpenoid biosynthesis [19]. In higher plants, mono- and diterpenoids were proposed to be biosynthesized in chloroplasts through a non-mevalonate pathway from glucose [20]. Eisenreich *et al.* confirmed that the mevalonate pathway was not involved in the biosynthesis of the taxoid on the basis of the ^{13}C labelling patterns of taxuyunnanine C [21]. Therefore, there is a possibility that SDB and SDC might be biosynthesised from glucose through the non-mevalonate pathway although we previously proposed that mevalonic acid is a biosynthetic precursor of these diterpenoids [22]. Since aphidicolin was proved to be biosynthesised through the mevalonate pathway [23], it is very interesting to examine the involvement of non-mevalonate pathway in the biosynthetic routes of SDB and SDC.

EXPERIMENTAL

Plant materials. Seeds of *Scoparia dulcis* were obtained from plants (SDB type and SDX type) grown in the Herbal Garden of Toyama Medical and Pharmaceutical University. The seeds were surface sterilized by immersion in 70% EtOH for 1 min and then in 2% NaOCl for 15 min. They were rinsed × 3 with sterile H_2O and sown on 1/4 MS agar medium at 26° under continuous irradiation (6000 lux). The leaves (*ca* 5 mm length) obtained from 4-week-old plants were cut out and used for leaf organ culture.

Leaf organ culture. The leaf segments (15–20) of *S. dulcis* obtained as described above were transferred into MS liquid medium containing 2IP, K, BA or 4PU (0.1, 0.5, 1.0, 5.0 and 10.0 μM). Each flask (100 ml) was agitated on a reciprocal shaker at a speed of 140 strokes min^{-1} at 26° under illumination. The resulting leaf organ cultures were maintained at 26° under continuous illumination and ten leaf segments in each flask were subcultured into fresh media every 2 weeks. For the study of time courses of cell growth and diterpenoid production, the third generation of leaf organ cultures was used.

Sample preparation for diterpene analysis. Harvested tissues were freeze-dried and pulverized. The powder was extracted with $CHCl_3$ and the samples for diterpene analysis was prepd as reported elsewhere [22].

HPLC analysis of diterpene content. The content of diterpenes in cultured tissues was determined by HPLC method as reported elsewhere [22].

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