I *Actinidia polygama* (Japanese name Matatabi): In Vitro Culture, Micropropagation, and the Production of Monoterpenes and Triterpenoids

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1 Introduction

1.1 Botanical Aspects

The genus *Actinidia* (family Actinidiaceae) consists of 40 species found in tropical and subtropical Asian countries. *Actinidia* species have been utilized for medicinal purposes and as a food source. For instance, the sour fruit of *A. chinensis* is very rich in vitamin C. The roots and racemes of *A. chinensis* are utilized as an astringent, to quench thirst, and as a diuretic. The fruits and leaves of *A. arguta* are considered to be antipyretic, astringent, tonic, thirst-quenching, and insecticidal. The roots and leaves of *A. eriantha* are utilized as an antidote, with antipyretic effect. The fruits of *A. coriacea* are also utilized as an antipyretic.

   Actinidia polygama is indigenous in Japan and Korea (Fig. 1). It is a deciduous shrub which grows on the edge of streams. The stem grows to several meters in length and 5 cm in diameter. The leaf is egg-shaped and alternately arranged. The leaf surface frequently changes to a white color in and around June. The shrub produces male and female white flowers in early summer. The fruits frequently form galls, which are induced by cecidogenous midges, *Asphondylia matatabi*.

1.2 Biologically Active Compounds and Traditional Uses

The fruit galls of *A. polygama* are commonly known as mu tian liao in Chinese traditional medicine. It has also been used as an analgesic, a tonic, an anti-rheumatic and digestive in folk medicine in Japan. However, the active principles have not yet been determined. The volatile components which cause the well-known specific activity of Felidae species were reported (Hazama 1942); cats show an especial liking for this plant, first licking and then eating it. Generally, they salivate, rub their fur onto the

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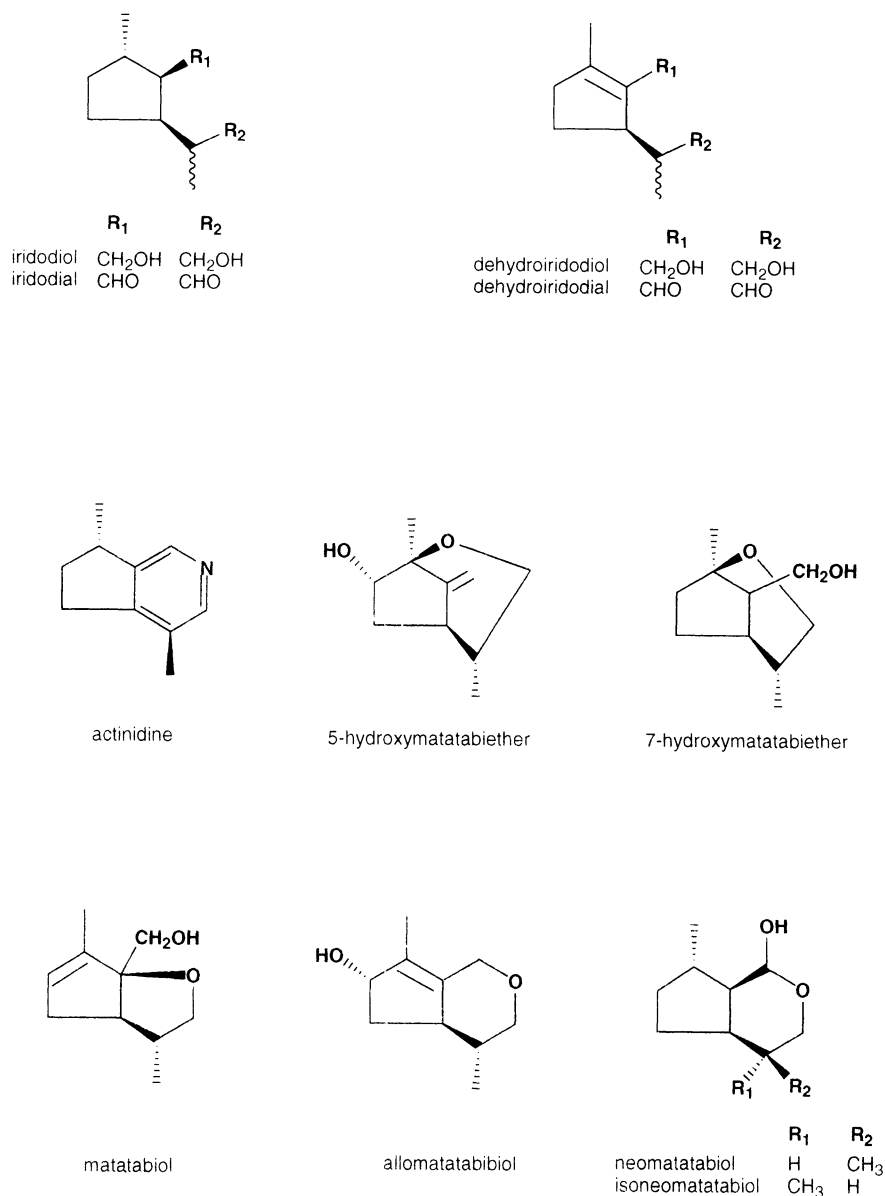[3] Tokyo College of Pharmacy, 1432-1 Horinouchi, Tokyo 192-03, Japan

Fig. 1. *Actinida polygama* plant.

fruit gall, and finally sleep. The fruit galls contain cyclopentanoid monoterpenes like iridomyrmecin, which is the most active stimulant for cats (Sakan et al. 1960). In 1949, Pavan reported that iridomyrmecin isolated from the anal glands of the Argentine ant, *Iridomymex humilis*, possessed insecticidal activity. The workers of *I. humilis* use this secretion for attack and defense against their insect enemies, and presumably this ability to wage chemical warfare is one of the factors responsible for the expansion of the species. The occurrence of similar defensive substances which may protect the plant against phytophagous insects has been noted. Sakan et al. studied the plant and isolated a number of other monoterpenoids. Figure 2 shows the biologically active monoterpenoids of *A. polygama*. They consist of four groups, aldehydes (iridodial, dehydroiridodial), alcohols (iridodiol, dehydroiridodiol, and 5-hydroxyiridodiol: strongly attractive for *Chyrysopa septempunctata* and *C. japana*), lactones (iridomyrmecine, dihydronepetalactone, nepetalactone, neonep etalactone: attractive to cats), and an artificial compound (actinidine: attractive to cats). Alcohols are specifically attractive to Felidae species. Aldehydes were reported to be a bitter principle. It is most remarkable that cyclopentanoid monoterpenes were found to strongly attract only the male adults of *Chrysopa septempunctata* (lacewing, stink flies) and *C. japana* in the amount of $10^{-6}\mu$g of neomatatabiol and isoneomatatabiol and $10^{-3}\mu$g of dehydroiridodiol, as indicated in Table 1.

In addition, the fruit galls of *A. polygama* contain several triterpenoids (Sashida et al. 1992). Some types of terpenoids have long been recognized as carriers of important physiological functions and they are also substances of vital importance for living organisms, i.e., some steroids, carotenoids,

ecdysone, and, in plants, phytol, which is an essential component of the chlorophyll molecule. Furthermore, triterpenoids constitute one of the largest groups with highly diversified biological activities including antiinflammatory, antineoplast, antibacterial, antifungal activities, diuretic, antidiabetic, and
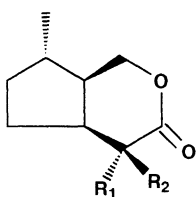
	R$_1$	R$_2$
iridodiol	CH$_2$OH	CH$_2$OH
iridodial	CHO	CHO

	R$_1$	R$_2$
dehydroiridodiol	CH$_2$OH	CH$_2$OH
dehydroiridodial	CHO	CHO

actinidine   5-hydroxymatatabiether   7-hydroxymatatabiether

matatabiol   allomatatabibiol

	R$_1$	R$_2$
neomatatabiol	H	CH$_3$
isoneomatatabiol	CH$_3$	H

A

Fig. 2A,B. Structures of biologically active monoterpenoids from *Actinidia polygama*. (Sakan et al. 1964)
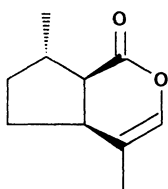
nepetalactone
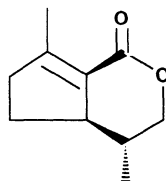
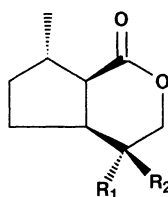neonepetalactone

	R₁	R₂
	R_1	R_2
iridomyrmecin	H	CH3
isoiridomyrmecin	CH3	H

	R_1	R_2
dihydronepetalactone	H	CH3
isodihydronepetalactone	CH3	H

B

Fig. 2A,B. *Continued*

Table 1. Potent cyclopentanoid monoterpenes isolated from *Actinidia polygama* attractive to male adults of *Chrysopa septempunctata* and *C. japana*. (Sakan et al. 1964)

Compound	Active amount (μg)
Iridodiol	1
5-Hydroxymatatabiether	1
7-Hydroxymatatabiether	1
Allomatatabiol	1
Matatabiol	10^{-3}
Dehydroiridodiol	10^{-3}
Neomatatabiol	10^{-6}
Isoneomatatabiol	10^{-6}

metabolite-displacing activity (Kapoor and Chawla 1986; Padmaja et al. 1993). Inhibitory effects of triterpenoids and their saponins on skin tumor promotion and Epstein-Barr virus activation have been studied by Konoshima et al. (1987, 1992, 1994). In view of these facts, the triterpenoids of *A. polygama* were studied.

2 In Vitro Culture Studies

Although numerous studies have been conducted on various in vitro aspects (Huang et al. 1988) of *Actinidia chinensis* and *A. deliciosa*, i.e., micropropagation (Monette 1986), protoplast culture (Cai et al. 1993), genetic transformation (Oliveira et al. 1994), conservation of germplasm (Monette 1995), etc., no studies exist on *Actinidia polygama* except our work (Shoyama et al. 1989; Sashida et al. 1992, 1994), which is summarized in this chapter.
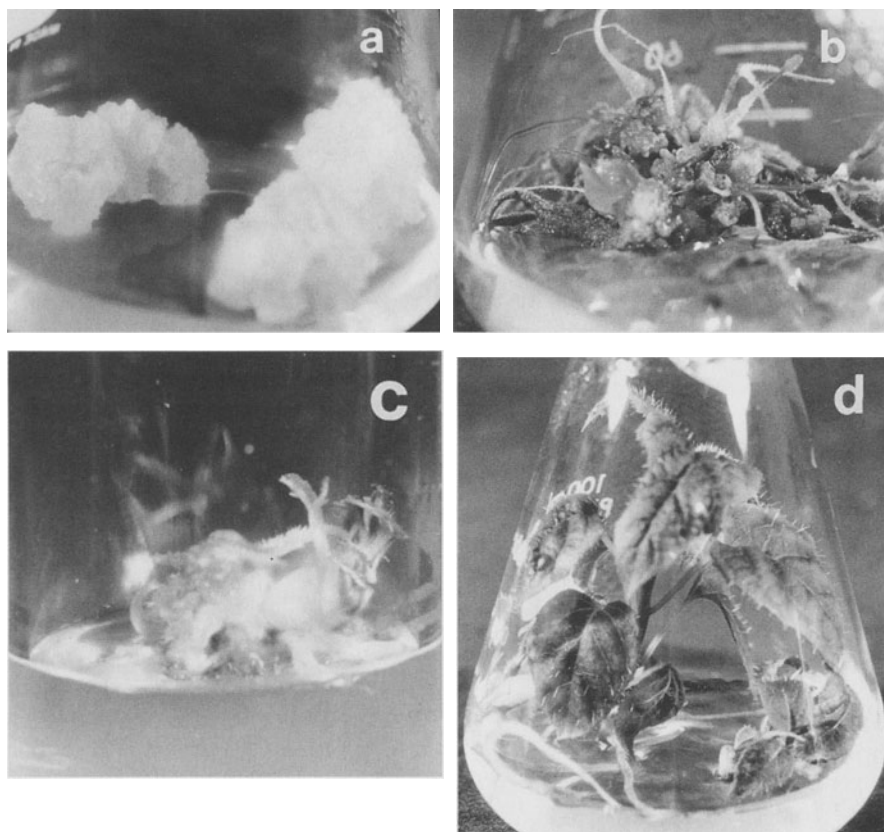
2.1 Callus Formation from Fruit Galls

2.1.1 Materials and Methods

Fruit galls of *A. polygama* were collected from native strains at Sagamiko in Kanagawa Prefecture in Japan. The natural plants, stems, leaves, and roots were collected at Oume in Tokyo. The fruit galls were washed with tap water, and sterilized with 1% NaOCl for 10 min, then with 70% EtOH for 30 s, and finally washed twice thoroughly with sterilized water. The pericarps were removed, and the sarcocarp dissected into a cube (3 mm^3). The basal medium consisted of B5 (Gamborg et al. 1968), LS (Linsmaier and Skoog 1965), and MS (Murashige and Skoog 1962) salt with (in mg/l): myoinositol, 100; nicotinic acid, 0.5; pyridoxine HCl, 0.5; thiamine HCl, 0.1; glycine, 2; sucrose, 30 000; agar, 9000, supplemented with auxins (IBA, NAA), BAP, kinetin, and GA. All culture tubes contained 30 ml of medium adjusted to pH 5.5 before autoclaving. The cultures were exposed to 16 h light, 2000–2500 lx, from cool white fluorescent tubes at 25 ± 1 °C.

At the initiation stage of callus induction, the pericarp segments were cultured for 30 days. In the second stage, callus induced was subcultured for 30 days. Shoot primordia were separated from the callus and transferred to shoot-forming medium and cultured for 30 days. The regenerated shoots were subcultured on the root-forming medium for 30 days.

2.1.2 Induction of Callus from Fruit Galls

Callus was induced from fruit galls on the B5 medium supplemented with NAA, BAP, and kinetin (1 mg/l each) or on the LS medium containing 10 mg/l NAA and 1 mg/l kinetin. Callus growth was better on the B5 than on LS medium, as indicated in Table 2. When callus was subcultured on the MS medium containing IBA, growth was favored. The addition of 3 mg/l IBA favored callus growth better than the addition of a higher concentration (Table 2), thus this medium was routinely used in the callus propagation stage (Fig. 3a).

Fig. 3a–d. Propagation system of *Actinidia polygama* by callus culture (Y. Shoyama et al., unpubl.). **a** Callus culture induced from fruit gall segment on MS medium supplemented with IBA (3 mg/l). **b** Formation of shoot primordia-like bud after three subcultures on B5 medium supplemented with NAA, BAP, and kinetin (1 mg/l each). **c** Shoot formation on LS medium supplemented with 2,4-D (0.5 mg/l each). **d** Plantlet formation on hormone-free MS medium

Table 2. Propagation of callus induced from fruit gall of *A. polygama* on different media. (Shoyama et al. 1989; Sashida et al. 1994)

Basal media Hormone ($mg\,l^{-1}$)	Gamborg B5	Linsmaier-Skoog	Murashige-Skoog		
	NAA-BAP-Kin (1:1:1)	NAA-Kin (10:1)	1	IBA 3	5
Fresh weight (mg culture^{-1})	730	240	270	970	390

Culture conditions: $25 \pm 1\,°C$, 16h light, 1 month. NAA: 1-naphthaleneacetic acid, BAP: benzylaminopurine, Kin: kinetin, IBA: indol-3-butyric acid.

2.1.3 Differentiation from Callus

When the callus induced by the medium supplemented with NAA, BAP, and kinetin (1 mg/l each) was subcultured on the same medium for three generations, shoot primordia and adventitious roots appeared on its surface (Fig. 3b). Some of the shoot buds were transferred to LS medium supplemented with 2,4-D and BAP (0.5 mg/l each), resulting in shoot and root regeneration (Fig. 3c). Shoots were subcultured on MS medium supplemented with NAA (1 mg/l), or on hormone-free medium to induce root formation (Fig. 3d).

Chromosome numbers (2n = 58) in the root tip of the plantlet regenerated from callus were the same as in the mother plants (Kitamura and Murata 1979).

2.2 Micropropagation

Shoots of regenerated plantlets on hormone-free MS medium were cut into stem segments having one leaf. The segments were cultured on MS medium supplemented with BAP (0.1, 0.5, 1, or 2 mg/l) for 1 month. The elongated shoots were cut into segments and subcultured on the same medium for 1 month. Stem segments were cultured on hormone-free MS medium for 1 month to produce plantlets, which were transferred to vermiculite and cultured for 3 months.

Shoots differentiated from the callus were transferred to hormone-free MS medium, resulting in plantlets, as indicated in Fig. 3d.

Regenerated shoots were cultured on MS medium supplemented with various concentration of BAP (0.1, 0.5, 1, or 2.5 mg/l). Only lower BAP concentration (0.1 mg/l) was favorable for shoot elongation. The stem of an elongated shoot was cut into six segments each having one leaf. The stem segment regenerated shoots perfectly on hormone-free MS medium, resulting in plantlets (Fig. 3d). Since a single shoot propagates six times in 1 month, the micropropagation system can be used routinely. Young plants were transferred to vermiculite and cultivated for 3 months. The transplantation was perfect, the plantlets growing to 30 cm in height after 6 months of culture in vermiculite.

2.3 Production of Monoterpenes and Triterpenoids in Callus Culture and Regenerated Plantlet (Shoyama et al. 1989; Sashida et al. 1994)

2.3.1 Materials and Methods

The fresh callus propagated on MS medium containing IBA (3 mg/l) was extracted with MeOH. The MeOH solution was evaporated in vacuo. The extract was reextracted with ethylether. This crude terpenoid fraction was subjected to GC-MS.

The callus was extracted with hot EtOH several times, and the EtOH solution was evaporated in vacuo. The extract was suspended in H_2O. The suspension was extracted with EtOAc and BuOH, successively. The EtOAc soluble part was repeatedly chromatographed on a silica gel column ($CHCl_3$-Me_2CO and $CHCl_3$-EtOAc system) to distinguish individual triterpenoids.

Structures were determined for these triterpenoids for their IR, EIMS, FABMS, ^{1}H-, and ^{13}C-NMR data (Cheung and Tokes 1968; Sakakibara and Kaiya 1983; Kikuchi et al. 1984; Kojima and Ogura 1986; Kojima et al. 1987; Bhandari et al. 1990; Sashida et al. 1994).

2.3.2 Results

Figure 4 shows the GC-MS spectrum of the crude terpenoid fraction obtained from callus culture. Although the GC spectrum indicated that many volatile compounds were contained in the crude terpenoid fraction, only dihyronepetalactone (or isodihydronepetalactone), which is a major constituent (Fig. 4, arrow), was determined. The stereochemistry of this compound is still unknown.

Figure 5 shows the structure of triterpenoids isolated from callus culture and fruit gall. Table 3 shows the distribution of triterpenoids in callus tissue, regenerated plantlets, in vivo plants, and fruit galls reported previously (Sashida et al. 1992). It became clear that the triterpenoid contents in both in vivo and regenerated plants are the same, in agreement with the fact that they have the same chromosome number (2n = 58) (Kitamura and Murata 1979), indicating that no variation occurred in callus culture. 3β,24-dihydroxyurs-12-en-28-oic acid and 2α,3β,24-trihydroxyurs-12-en-28-oic acid are found only in callus tissue, and this is the first example isolated from natural sources. 3β,24-dihydroxyurs-12-en-28-oic acid may be an important product in the oxidation process between delta-12 and delta-11 triterpenoid, because hydroxylation of C-13 may occur through the epoxide on C-12 and C-13. This introduction of a hydroxyl group on C-12 of triterpenoids is variable for the transformation of various triterpenoids. 2α,3β,24-trihydroxyurs-11-en-13β,28-olide, 3β,24-dihydroxyurs-12-en-28-oic acid, 2α,3α,24-trihydroxyurs-12-en-28-oic acid, 2α,3β,24-trihydroxyurs-12-en-28-oic acid, and 3β-(trans-p-coumaroyloxy)-2α,24-dihydroxyurs-12-en-28-oic acid, which are contained in callus tissue, have the 4-β-CH_2OH group. On the other hand, 2α,3α,23-trihydroxyurs-12-en-28-oic acid, 2α,3β,23-trihydroxyurs-12-en-28-oic acid, and 3β-(trans-p-coumaroyloxy)-2α,23-dihydroxyurs-12-en-28-oic acid having the 4-CH_2OH group are not found in callus tissue. This clearly shows that the hydroxylation ability of the C-4 dimethyl group in natural plants, regenerated plants, and fruit galls is nonspecific, but that in callus tissue is specific. Therefore, it is speculated that the selective hydroxylation ability of callus tissue can be used for the selective biotransformation of the cyclohexane ring having the C-4 dimethyl group. The result that fruit galls indicated the intermediate triterpenoid pattern between the in vivo plants

GC-MS

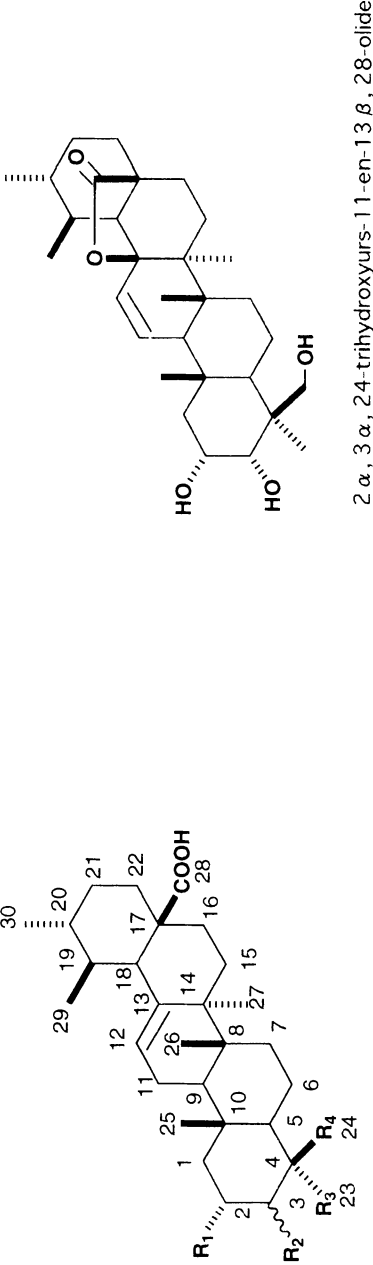2% OV-17(1.1 m x 2.6 mm),
He: 40 ml/min, 152°C

250°C
70eV
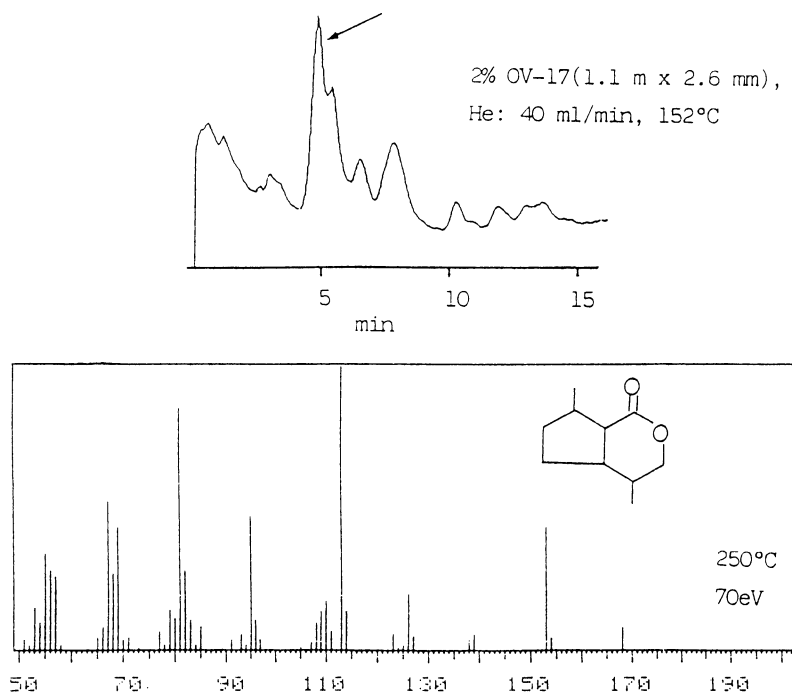
Fig. 4. Analysis of monoterpenoids by gas chromatography-mass spectrometry. (Shoyama et al. 1989). GC-MS spectrometry was carried out by the Shimadzu QP-1000. GC and MS conditions were indicated in individual spectrum, respectively. Peak indicated by *arrow* analyzed by MS spectrometry in MS spectrum indicating that the molecular peak was *m/e* 168 in the MS spectrum

and callus tissue shows that fruit galls may possess greater metabolic ability than callus tissue.

3 Conclusion

The procedure described here can be used as a simple method of micropropagation, thereby helping to produce rapid strains of *A. polygama*. Moreover, since the chromosome numbers of *A. polygama* in the root tip of plantlets regenerated by callus culture were the same as those of the mother plants (Kitamura and Murata 1979), this system can be utilized to supply a homogeneous population of this plant. Although the sex of regenerated plantlets has not been determined, it may be possible to propagate either female or male plantlets by this system.

2α, 3α, 24-trihydroxyurs-11-en-13β, 28-olide

	R_1	R_2	R_3	R_4
ursolic acid	OH	β-OH	CH$_3$	CH$_3$
3-O-acetylursolic acid	H	β-OAc	CH$_3$	CH$_3$
2α, 3β-dihydroxyurs-12-en-28-oic acid	OH	β-OH	CH$_3$	CH$_3$
3β, 24-dihydroxyurs-12-en-28-oic acid	H	β-OH	CH$_3$	CH$_2$OH
2α, 3α, 24-trihydroxyurs-12-en-28-oic acid	OH	α-OH	CH$_3$	CH$_2$OH
2α, 3β, 24-trihydroxyurs-12-en-28-oic acid	OH	β-OH	CH$_3$	CH$_2$OH
3β- (trans-p-coumaroyloxy) -2α, 24-dihydroxy-urs-12-en-28-oic acid	OH	β-p-Coumaroyloxy	CH$_3$	CH$_2$OH
2α, 3α, 23-trihydroxyurs-12-en-28-oic acid	OH	α-OH	CH$_2$OH	CH$_3$
2α, 3β, 23-trihydroxyurs-12-en-28-oic acid	OH	β-OH	CH$_2$OH	CH$_3$
3β- (trans-p-coumaroyloxy) -2α, 23-dihydroxy-urs-12-en-28-oic acid	OH	β-p-Coumaroyloxy	CH$_2$OH	CH$_3$

Fig. 5. Structures of triterpenoids isolated from *Actinidia polygama* callus. (Reprinted from Sashida et al., copyright 1994, Phytochemistry 35:377–380, with kind permission from Elsevier Sciences Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK)

Table 3. Distribution of the triterpenoids in callus, regenerated plants, in vivo plants, and galls of *A. polygama*. (Sashida et al. 1994)

Compound	Callus	Whole plant		Fruit gall
		Regenerated[a]	Natural[a]	
Ursolic acid	+	+	+	+
3-*O*-acetylursolic acid	+	+	+	+
2α, 3β-dihydroxyurs-12-en-28-oic acid	+	+	+	+
2α, 3α, 24-trihydroxyurs-11-en-13 β, 28-olide	+			
3β, 24-dihydroxyurs-12-en-28-oic acid	+			
2α, 3α, 24-trihydroyurs-12-en-28-oic acid	+	+	+	+
2α, 3β, 24-trihydroyurs-12-en-28-oic acid	+	+	+	+
3β-(*trans-p*-coumaroyloxy)-2α, 24-dihydroxy -urs-12-en-28-oic acid	+	+	+	
2α, 3α, 23-trihydroyurs-12-en-28-oic acid				+
2α, 3β, 23-trihydroyurs-12-en-28-oic acid		+	+	+
3β-(*trans-p*-coumaroyloxy)-2α, 23-dihydroxy -urs-12-en-28-oic acid				+

[a] Triterpenoids in these specimens were detected by TLC.

Since *A. polygama* produces interesting secondary compounds which are specifically attractive to Felidae species, especially for cats, the callus culture was analyzed by GC-MS, but proved, however, to produce only a low amount of attractant for cats. On the other hand, the callus culture usually formed various kinds of triterpenoids. The oxidation process of these triterpenes suggested that the hydroxylation ability of the C-4 dimethyl group in callus culture favored beta, while the natural plants, fruit galls and regenerated plantlets had nonspecific hydroxylation ability against the C-4 methyl group. Therefore, this ability may be useful in selective biotransformation of cyclohexane ring triterpenoids possessing a C-4 dimethyl group.

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