Notes Plant Tissue Culture Letters, **13**(2), 203-206(1996)

Studies on the Medicinal Plant Resources of the Himalayas (1) *In vitro* Regeneration and Alkaloid Contents of *Ephedra gerardiana* Wall.

Takashi WATANABE[a], Kiichiro KAWAGUCHI[a], Takafumi YOSHIKAWA[b], Akihito TAKANO[c], Hiroshi KOHDA[d] and Kuber J. MALLA[e]

Ephedra gerardiana Wall. called "Somlata", a Nepalese crude drug, has recently been a matter of intense interest as a medicinal plant resource of the Himalayan regions. This species is distributed mainly in the Himalayas from Afganistan to Bhutan between alt. 2,400 m and 5,000 m[1], growing on poor, dry soil in the lower alpine zone[2]. Because *E. gerardiana* contains two valuable alkaloids, *l*-(−)ephedrine and *d*-pseudoephedrine, several research groups have investigated the wide range of variation in alkaloid content relating to the differences in habitat, sex[3] and morphological appearance[4] of this plant. Growth[5] and ephedrine production[6] in *E. gerardiana* callus cultures were reported. Moreover, *E. ragilis, E. equisitina, E. gerardiana, E. minima*, and *E. saxatilis* were successfully micropropagated using shoot nodal explants[7]. In this paper, we report on the alkaloid content and characteristics of the regenerated plants obtained from the axillary buds of *E. gerardiana* compared with the cultivated parent plants.

Seeds of *E. gerardiana* collected around Jumla (E82°-1′N29°-2′)[8], Jumla Dist., Karnali Zone, Nepal, alt. 2,900 m to 3,500 m, were provided from the Department of Forestry and Plant Resources in June of 1989; and those, collected around Thimpu (E89°-3′N27°-3′24)[8], Bhutan, alt. 2,500 m to 3,200 m, were provided by the Honorable Consulate of Bhutan in Oct. of 1991.

The young shoots of both *Ephedra* (experimental numbers are NPKS-EG01P-10P and BUKS-EG01P-02P in **Fig. 3**) cultivated in the field of Kitasato University for 3-5 years and aseptically germinated seedlings were used for culture materials. These nodal shoots 5-7 cm in length were prepared by surface sterilizing with 70% ethanol for 20 sec., followed by 2% sodium hypochlorite solution for 60 min. and then washed several times with sterilized water. The meristem tissue was excised from the axillary buds of these aseptic shoots using microscopic technique, and then transferred to Murashige and Skoog's (MS) agar (0.9%) medium supplemented with 0.5-1.0 mg/*l* kinetin (Kin) containing 3% sucrose (Kin 0.5-Kin 1 medium), and cultured at 24±2°C in 3,000-5,000 lx for 16 h with 3-4 weeks culture cycle for shoot formation. The shoots were directly developed in meristem cultures of the axillary buds. Moreover, the shoots were also obtained through adventitious buds, which were developed from the light greenish-brown primordium, when sub-culturing on Kin 0.5 and Kin 1.0 medium (**Fig. 1**). Multiple shoots (**Fig. 2 Right**) were induced on Woody Plant (WP) agar (0.9%) medium supplemented with 5.0 mg/*l* 6-benzylaminopurine (BAP) containing 2% sucrose (WP-5BAP medium) 6-8 weeks after the shoots were transferred from Kin media. The formation of shoots and multiple shoots was most effectively developed on WP-5BAP

[a] *Medicinal Plant Garden, School of Pharmaceutical Sciences, Kitasato University*, Sagamihara, Kanagawa 228, Japan
[b] *School of Pharmaceutical Sciences, Kitasato University*, Minato-ku, Tokyo 108, Japan
[c] *Medicinal Plant Garden, Showa College of Pharmaceutical Sciences*, Machida, Tokyo 194, Japan
[d] *Institute of Pharmaceutical Sciences, School of Medicine, Hiroshima University*, Minami-ku, Hiroshima 734, Japan
[e] *Royal Botanical Garden, Department of Plant Resources*, P.O. Box No. 3708, Godawari, Lalitpur, Nepal

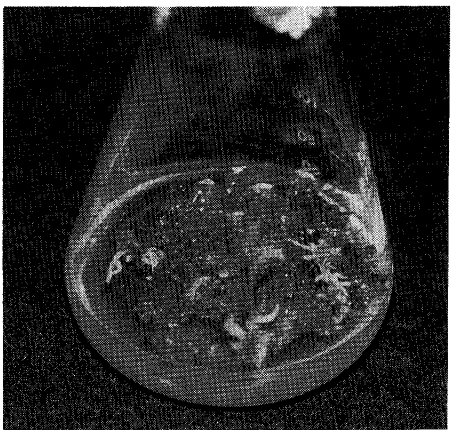

Fig. 1 Shooting developed from adventitious buds.

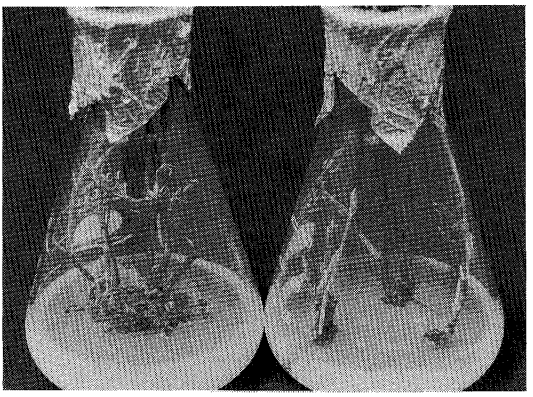

Fig. 2 Left: Regenerated shoots with roots, Right: Multiple shoots developed on WP-5BAP medium after 8 weeks of culture.

Table 1. Effect of phytohormone conditions and various culture media on growing shoots and rooting of *E. gerardiana in vitro*.

phytohormone & media		shoot	multiple shoot	root
MS	Kin 0.5 mg/*l*	+	+	−
	Kin 1.0 mg/*l*	+	+	−
	hormone free	±	−	+
	IBA 5.0 mg/*l*	−	−	++
WP	hormone free	+	−	+
	BAP 5.0 mg/*l*	++	+++	−
1/2 WP	hormone free	±	−	+++

+++ : vigorous, ++ : moderate, + : slight, ± : trace, − : no formation
Abbreviations are as follows. Kin: Kinetin, IBA: Indole-3-butyric acid, BAP: 6-Benzylaminopurine, MS: Murashige-Skoog's medium, WP: Woody Plant medium, 1/2 WP: composition of WP medium in half

medium in comparision to Kin media and WPB medium (**Table 1**). The propagated shoots were divided into bunches of 5-6 shoots each, and were inoculated onto four kinds of agar (0.9%) media for rooting: 1) MS basal medium containing 2% sucrose (MSB medium), 2) MS medium supplemented with 5.0 mg/*l* indole-3-butyric acid (IBA) (5IBA medium), 3) WP basal medium containing 2% sucrose (WPB medium) and 4) a half strength WP medium containing 1% sucrose (1/2 WP medium). As shown in **Table 1**, rooting was most effectively induced on 1/2 WP medium (**Fig. 2, Left**) in comparison to MSB, 5IBA and WPB media.

The regenerated plantlets with healthy rooting were transplanted to two inches pots with Primemix (TKS-No. 2, Sakata Seed Corporation) compost: sand or Perlite (1 : 1). After maintaining for five weeks under a plastic cover in order to avoid desiccation, they were kept in the greenhouse for two months before transplanting to the field. The proportion of surviving plantlets on soil after acclimatization was about 76% (38/50) two month later.

Regenerated plantlets were also obtained from cotyledon and hypocotyle of aseptically germinated seedlings in the same manner.

Fresh nodal shoots of the regenerated plantlets *in vitro* and those of 10-20 cm in lengths of parent plants after 3-5 years cultivation in the garden were prepared for the samples. Dried and powdered

samples were kept in a dessicator. Five hundred mg of the sample was weighed and put into a 50 m*l* screw capped centrifuge tube. Twenty m*l* of H_2O-MeOH (1 : 1) solution was added and the tube was shaken for 30 min. at room temperature (If sample was less than 500 mg, the solvent is reduced in volume.). The solutions were centrifuged for 10 min. at 3000 rpm and filtrated through 0.45 μm filter disc. The filtrate was injected for HPLC analysis. Analytical conditions were as follows: pump; TOSOH CCPM, detector; TOSOH variable wave length UV monitor CCPM UV-8010 (detection; UV 220 nm), column; TOSOH TSK gel ODS-80 TM (4.6 i.d.×150 mm), mobile

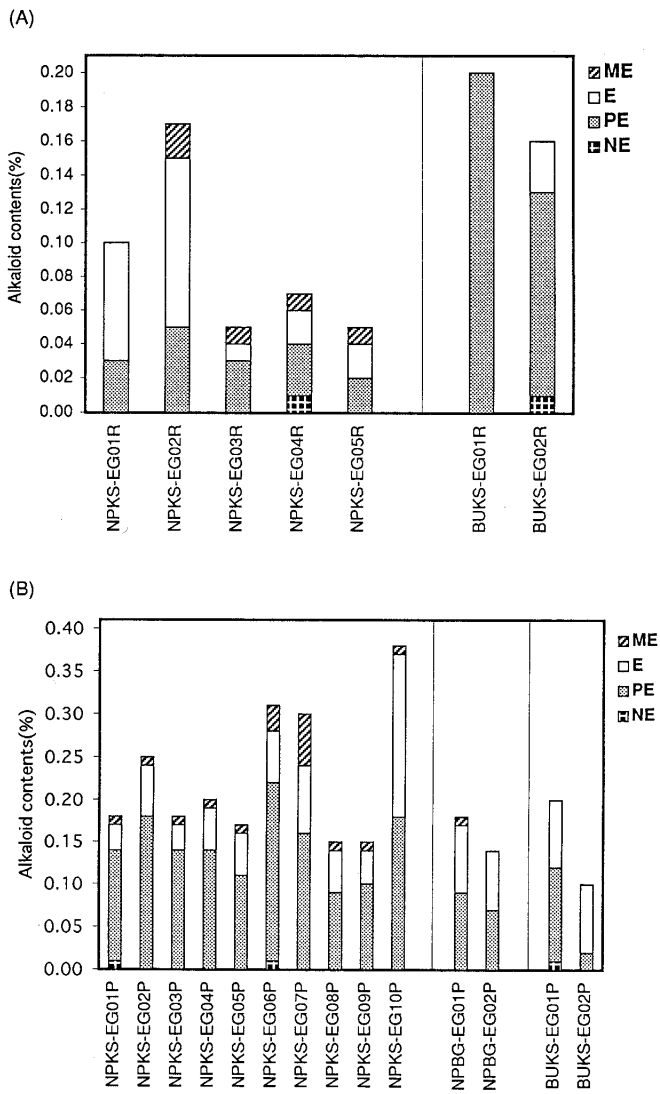

Fig. 3 Alkaloid contents in *E. gerardiana*; multiple shoot, regenerated plantlets (*in vitro*) and cultivated parent plants.
A: Regenerated multiple shoots and plantlets.
B: Parent plants cultivated in the gardens.
NE: nor-ephedrine/PE: *d*-pseudoephedrine/E: *l*-(-)ephedrine/
ME: methyl-ephedrine, Parent: 3-5 years plant in the garden.
NPKS-EG (01P-10P) : cultivated in Kitasato Univ., introduced from Nepal.
NPBG-EG (01P-05P) : cultivated in Royal Botanical Garden of Nepal.
BUKS-EG (01P-02P) : cultivated in Kitasato Univ., introduced from Bhutan.

phase; purified water/acetonitrile/phosphoric acid (600 : 400 : 1, v/v/v) with 5 g of SDS (Sodium rauril sulfic acid), flow rate; 1.0 m*l*/min. at room temperature.

Ephedrine alkaloid contents in the materials were analyzed as shown in **Fig. 3** with authentic samples; nor-ephedrine (NE), *d*-pseudoephedrine (PE), *l*-(−) ephedrine (E) and methylephedrine (ME). Using the seeds collected in Nepal, multiple shoots without root (NPKS-EG01R, 02R; PE< E) and the regenerated plantlets *in vitro* (NPKS-EG03R-05R; PE≧E) could synthesize alkaloids though a lower level of total alkaloids (0.10-0.17, 0.04-0.06%) compared with the cultivated plants at Kitasato University [(NPKS-EG01P-10P; 0.15-0.38% : PE≫E except NPKS-EG 10 P) and the plants in Nepal (NPBG-EG01P, 02P; 0.14-0.18%; PE≧E)]. On the other hand, using the seeds of Bhutan, the total alkaloids were almost the same levels as the regenerated plantlets (0.16-0.20%; PE>E) and the cultivated plants at Kitasato University (0.10-0.20%; PE≷E).

The variation of total alkaloids and of their patterns were observed not only in the multiple shoots and plantlets *in vitro* but also in the plants cultivated from seeds. So, It is necessary to select the desirable character of *E. gerardiana*, for example high alkaloid content (NPKS-EG10P), before micropropagation of them *in vitro*.

Acknowledgement

We are grateful to the Royal Botanical Garden, Department of Plant Resources, Nepal and Mr. N. Sasaki (Japan Golf Promotion Inc., Honorable Consulate of Bhutan) for providing the seed samples of *Ephedra gerardiana* Wall. Particularly we wish to express our deep thanks to Dr. S. B. Malla (former Director general, Department of Medicinal Plants, Nepal), Dr. T. K. Rajbhandari (Director general, Department of Plant Resources, Nepal) for their collaboration in the study of the medicinal plant resources in the Himalayas. A part of this study was supported by a Grant-in-Aid from the foundation of the supporting organization of Japan Overseas Cooperation Volunteers belonging to Japan International Cooperation Agency. (Accepted January 18, 1996)

References

1) Polunin, O., A. Stainton, 1984. In "Flowers of the Himalaya", Oxford University Press, Oxford.
2) Ohba, H., S. B. Malla, 1988. In "The Himalayan Plants (Vol. 1)", p. 351-352. University of Tokyo Press, Tokyo.
3) Ramawat, K. G., H. C. Arya, 1979. Indian J. Exp. Biol., **17**: 106-107.
4) Namba, T., M. Mikage, A. Takano, H. Jin, T. Tomimori, 1987. Shoyakugaku Zasshi, **41**: 209-214.
5) Ramawat, K. G., H. C. Arya, 1979. Indian J. Exp. Biol., **17**: 227-228.
6) Ramawat, K. G., H. C. Arya, 1979. Phytomorphology, **29**: 16-25.
7) O'Dowd, N. A., D. H. S. Richardson, 1993. J. Horticul. Sci., **68**: 1013-1020.
8) Kanai, H., 1984. In "Index Gazetteer of the Himalaya (ed. 4)", Japan Nepal Society, Tokyo.