Plant Cell, Tissue and Organ Culture **41:** 61–64, 1995.

Vincamine production in multiple shoot culture derived from hairy roots of *Vinca minor*

Nobukazu Tanaka[1], Misato Takao & Takeshi Matsumoto
Research Center, Daicel Chemical Industries, Ltd., Shinzaike 1239, Aboshi-ku, Himeji, Hyogo 671-12, Japan
([1]present address: Center for Gene Science, Hiroshima University, 4-2, Kagamiyama 1, Higashi-hiroshima, Hiroshima 724, Japan)

Received 20 January 1994; accepted in revised form 7 December 1994

Key words: periwinkle, regenerated plant, root-inducing (Ri) plasmid

Abstract

Characteristics of regenerated plants obtained from hairy roots (Ri-transformed plants) of *Vinca minor* L., a producer of a pharmaceutically important indole alkaloid, vincamine, were investigated. A previously established Ri-transformed clone, Vm-101, proliferates rapidly in vitro, displays a high degree of lateral branching and rapid shoot elongation and has a growth index 2.5 times that of an untransformed plant. The addition of 2.2 μM benzyladenine to the culture medium increased the shoot number but did not decrease the growth index. Vincamine content in the leaves of in vitro-cultured Vm-101 was twice that in the cultured untransformed plant. These results suggest that multiple shoot culture of Ri-transformed plants may be an excellent tool for in vitro vincamine production.

Introduction

The Lesser Periwinkle (*Vinca minor* L.), a member of Apocynaceae, is not only an ornamental plant with lilac-blue flowers but also a medicinal plant producing an important alkaloid, vincamine, found in the leaves (Oniscu et al. 1985). Vincamine and its derivatives are marketed by some pharmaceutical companies in Europe and Japan as a cerebral vasodilator. *V. minor* is a perennial herb, but the growth of plants is comparatively slow. Moreover, since the plant is usually propagated by shoot cuttings rather than seeds, the number of plants that may be produced in a given time is limited.

Some attempts have been made to establish a callus culture that produces vincamine in large amounts (Petiard & Demarly 1972; Garnier et al. 1975). Stable, high production rates of vincamine have not been achieved in such unorganized cell culture. Thus, organ cultures, such as adventitious and multiple shoots, are expected to produce vincamine.

Multiple shoot culture is an important tool for micropropagation of useful plants as well as for production of biochemicals. Multiple shoot cultures of *Digitalis lanata* (Hagimori et al. 1982), *D. purpurea* (Lui & Staba 1979) and *Catharanthus roseus* (Hirata et al. 1987) have been established for the production of digoxin, digitoxin and indole alkaloids, respectively. These cultures were maintained for long periods without changes in morphology, growth characteristics or in secondary metabolites production (Hirata et al. 1990). A medium with 4.4 μM benzyladenine had a significant effect on shoot proliferation of *C. roseus* (Hirata et al. 1987). In our preliminary test there was no significant increase in shoot number of *V. minor* cultured on media containing 0 to 22 μM benzyladenine (unpublished results).

Transformed adventitious roots induced by the root-inducing (Ri) plasmid of *Agrobacterium rhizogenes*, viz. hairy roots, can be valuable in biochemical production systems (Toivonen 1993). In some instances, hairy roots have produced whole plants. It is generally recognized that these regenerated plants may display the so-called hairy root syndrome, including various morphogenic and physiological alterations (Tepfer 1984). Little is known about the capacity of these Ri-transformed regenerants for biochemical production. Recently we have obtained a clone of a Ri-

transformed regenerated plant of *Ajuga reptans* var. *atropurpurea* (Tanaka & Matsumoto 1993a, 1993b). The plant was derived from a hairy root line (Matsumoto & Tanaka 1991) selected from more than 20 hairy root clones. It showed not only a high growth rate in culture (Tanaka & Matsumoto 1993a), but also produced more than four times as much 20-hydroxyecdysone as the original field-grown *Ajuga* plant (Tanaka & Matsumoto 1993b). This result suggested that a plant possessing desired characteristics, such as an improved productivity of biochemicals, is selectable from Ri-transformed plants.

Recently, we obtained many Ri-transformed and regenerated *V. minor* plants that displayed various morphologies (Tanaka et al. 1994). However, we did not detect vincamine either in the hairy roots or in the regenerated plants' roots (unpublished results).

Here, we detail another useful method that utilizes Ri-transformed plants. Secondary metabolites can be produced in multiple shoot cultures derived from hairy roots induced in a medicinal plant. For in vitro production of vincamine, we established a multiple shoot culture from a regenerated plant of Ri-induced *V. minor*.

Materials and methods

Culture of shoots

Stem segments (ca. 3 cm in length), including a terminal bud, excised from *Vinca minor* plants that had been grown in a greenhouse for at least a year were surface-disinfested with sodium hypochlorite solution (0.5% avalable chlorine) and then cultured on Murashige and Skoog's (1962) (MS) medium gelled with 0.2% Gellan Gum (Wako Pure Chemical Industries, Ltd.). Three segments were implanted on 100-ml of medium in 500 ml culture bottles (UM glass sample bottle, Iuchi Seieido Co., Ltd.) closed with TPX (methylpentene) plastic caps with five bottles per treatment. These cultures were maintained at 25°C with a 12-h photoperiod (28 $\mu mol\ m^{-2}\ s^{-1}$, Hitachi Sunlife 37 W white fluorescent tubes). Such in vitro-cultured plants (untransformed shoots) were transferred to fresh MS medium every 30 days.

Ri-transformed *V. minor* plants regenerated from hairy roots, which had been induced by infection with *Agrobacterium rhizogenes* strain DC-AR2 (Tanaka et al. 1993) harboring mikimopine-type pRi 1724 (Tanaka & Oka 1994), have been described in our previous report (Tanaka et al. 1994). The integration of pRi 1724 T-DNA in the genome of these Ri-transformed plants has already been confirmed by Southern-blot analysis (Tanaka et al. 1994). Shoot tips (ca. 2 cm in length, with a single node) of actively growing cultured plants were excised and transferred to MS medium supplemented with various concentrations of growth regulators [benzyladenine (BA), naphthaleneacetic acid (NAA)] or sucrose.

The growth indices were obtained by calculating the quotient of fresh weight of the harvested plantlet divided by the fresh weight of the implanted shoot tip.

Quantitative analysis of vincamine in plant materials

Methods of quantitative analysis of vincamine in plant tissues were as follows: a methanol fraction, extracted from the dried leaves with a sonicator for 3 h, was evaporated to dryness and then dissolved in 1 ml of methanol. Aliquots (10 µl) of each sample were analyzed by high performance liquid chromatography (HPLC: LC-10A, Shimazu) using a Cosmosil C-18 column (25 cm in length, Nacalai Tesque., Inc.); flow rate was 1 $ml\ min^{-1}$, gradient elution was from 50 to 80% methanol in water plus 0.1% ethanolamine and the detector wavelength was 283 nm.

Adaptation and cultivation of cultured plants

Shoot tips, ca. 2 cm in length, were excised from untransformed and Ri-transformed plants and cultured on MS medium with Gellan Gum at 25°C for at least 30 days. Plantlets with sufficient root development were transferred onto moist vermiculite and cultured in a greenhouse. After adaptation for 1 month in humid conditions, they were transplanted into 300 ml of mixed soil (clay : sand : vermiculite = 1 : 1 : 1) in plastic pots (10 cm in diameter) and were further cultivated in the greenhouse for 3 months at 20 to 30°C with a 16-h photoperiod (280–370 $\mu mol\ m^{-2}\ s^{-1}$; if needed, the illuminance was supplemented with Hitachi Sunlife 37 W white fluorescent tubes).

Table 1. Effect of BA or sucrose on shoot number, vincamine content and growth index on Vm-101 plants.

Treatment	Shoot number	Vincamine content (% in dry weight basis)	Growth index
BA (μM)[1]			
0	11.1 ± 2.5	-	10.4 ± 3.3
2.2	15.3 ± 4.3	-	10.2 ± 4.8
4.4	13.4 ± 3.1	-	5.8 ± 1.7
6.7	12.9 ± 2.5	-	6.7 ± 1.2
8.9	11.6 ± 2.1	-	4.9 ± 0.9
Sucrose (%)[2]			
3	-	0.39 ± 0.04	9.9 ± 2.9
4	-	0.38 ± 0.03	10.9 ± 3.7
5	-	0.50 ± 0.10	8.1 ± 2.6
6	-	0.31 ± 0.01	5.2 ± 1.7

[1]The data (mean value ± standard deviations) were calculated from the measurement of eight plants.
[2]The data (mean value ± standard deviations) were calculated from the measurement of four plants.

Table 2. Content of vincamine in untransformed and Ri-transformed plants of *V. minor*.

Plant	Source of tissue	Vincamine content in leaf[1] (% in dry weight basis)
Untransformed[2]	Tissue culture	0.24 ± 0.06
Ri-transformed[2]	Tissue culture	
Vm-101		0.44 ± 0.10
-102		0.33 ± 0.05
-103		0.30 ± 0.12
-104		0.18 ± 0.10
Untransformed[3]	Greenhouse	0.20 ± 0.10
Ri-transformed[3]	Greenhouse	
Vm-101		0.42 ± 0.11

[1]The data (mean values ± standard deviations) were calculated from the measurement of three plants.
[2]Cultured at 25°C with a 12-h photoperiod for 30 days.
[3]Cultivated for 3 months after a month adaptation in a greenhouse.

Results and discussion

Effect of BA on shoot number of untransformed plant

Stapfer & Heuser (1985) have reported that cytokinin effectively promotes shoot proliferation of *V. minor*, especially the cytokinin BA. Before investigating the proliferation of Ri-transformed plants, we investigated the proliferation of untransformed plants on MS medium with concentrations of BA ranging from 0 to 66.7 μM, and with NAA ranging from 0 to 10.7 μM, and with combinations of the two. The addition of BA did not improve shoot proliferation with the number of shoots per explant ranging between one and four in all BA concentrations examined. Combining BA with 0.11, 1.1, or 10.7 μM NAA did not increase the number of shoots. The addition of BA was not, therefore, a useful technique in establishing a multiple shoot culture from the untransformed *V. minor* plant material used in this study.

Shoot number and growth index on Ri-transformed plant

From more than 10 clones of Ri-transformed plants, we obtained one actively growing clone, Vm-101, with a high proliferation activity and a growth index of ca. 10 (Tanaka et al. 1994) as compared with growth index of ca. 4 for the untransformed plant. This clone displayed reduced apical dominance but did not demonstrate shorter internodes. As a result, the clone showed an increased shoot number and the elongation of shoots was rapid in culture. In an attempt to improve shoot proliferation, we examined the effect of BA on shoot number and on growth index. As shown in Table 1, an average of more than 11 shoots emerged from one shoot tip on growth regulator-free MS medium. The addition of 2.2 μM BA increased the average numbers of shoots to 15, but further increases in BA concentration up to 8.9 μM did not increase number of shoots above the control. The growth index was ca. 10 on medium without growth regulators or with 2.2 μM BA and decreased with higher BA concentrations. Thus, a culture medium supplemented with 2.2 μM BA was selected for clonal propagation.

Comparison of vincamine content among untransformed and Ri-transformed plants

HPLC traces of alkaloid fractions isolated from the leaves of cultured untransformed and Ri-transformed plants were similar to that from the original plant (data not shown). The vincamine content of each type of plant examined is summarized in Table 2. It is of particular interest that the vincamine content of the clone Vm-101 is twice that of the untransformed plant in tissue culture. Since the vincamine content of Ri-

transformed plants was variable, the high content of Vm-101 was not a general characteristic of regenerants derived from hairy roots. Apparently Vm-101 is a superior clone that produces vincamine in vitro, having both a high growth rate and a high productivity of vincamine.

To find a suitable culture condition for the production of vincamine by Vm-101, the shoot tips were cultured on medium containing 2.2, 4.4, 6.7 or 8.9 μM BA; however, the content tended to decline as BA concentration increased (data not shown) in a manner similar to that observed in cultures of untransformed plants (unpublished data). We found previously that shoot cultures derived from the untransformed *V. minor* plant and cultured on MS medium containing 5–6% w/v sucrose showed an increase in the growth index and also in the vincamine content (unpublished data). To examine whether sucrose concentration would have a similar effect on Vm-101, shoot tips were cultured on MS medium supplemented with 3 to 6% sucrose. As shown in Table 1, the maximum content of vincamine, 0.5% in dry weight basis, was observed in the shoot culture grown on a medium with 5% sucrose, while the growth index at 5% sucrose did not differ from that at 3%. Clearly, the Ri-transformed plant had a similar response to sucrose although the degree of response was small.

Vincamine content in Ri-transformed plant cultivated in a greenhouse

After acclimatization, the plants were cultivated in the greenhouse for 3 months. Since these cultivated Ri-transformed plants had shorter internodes and reduced apical dominance, the rapid shoot growth observed with the shoot cultures may be a property only displayed in vitro.

The vincamine content in the leaves of Ri-transformed plant was double that in the untransformed one, and these values were similar to those in the tissue cultured plant materials (Table 2). Thus, the result indicates that the capacity for vincamine production associated with cultured clones was stably maintained in greenhouse cultivation. On the other hand, the vincamine content of leaves of untransformed plants was only about one-fourth that of the content of leaves of the original untransformed plant cultivated for at least 3 years in the field (ca. 0.8% in dry weight basis) [unpublished data]. The difference in vincamine content between the two plants may be attributable to the age of the leaves and/or the period of cultivation. The environmental stress associated with production of *V. minor* plants (temperature and irradiation effect) may also be a factor. Thus, the Ri-transformed clone, Vm-101, cultivated in the field may have a higher vincamine content than that of the original untransformed plants.

References

Garnier J, Kunesch N, Siou E, Poisson J, Kunesch G & Koch M (1975) Etude des cultures de tissus de *Vinca minor* isolement d'un lignane, le lirioresinol B. Phytochemistry 14: 1385–1387

Hagimori M, Matsumoto T & Obi Y (1982) Studies on production of *Digitalis* by plant tissue culture. Plant Physiol. 69: 653–656

Hirata K, Yamanaka A, Kurano N, Miyamoto K & Miura Y (1987) Production of indole alkaloids in multiple shoot culture of *Catharanthus roseus* (L). Don. Agr. Biol. Chem. 51: 1311–1317

Hirata K, Horiuchi M, Ando T, Miyamoto K & Miura Y (1990) Vindoline and catharanthine production in multiple shoot cultures of *Catharanthus roseus*. J. Ferment. Bioeng. 70: 193–195

Lui J H C & Staba E J (1979) Effect of precursors on serially propagated *Digitalis lanata* leaf and root cultures. Phytochemistry 18: 1913–1916

Matsumoto T & Tanaka N (1991) Production of phytoecdysteroids by hairy root cultures of *Ajuga reptans* var. *atropurpurea*. Agr. Biol. Chem. 55: 1019–1025

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Oniscu C, Macoveanu M, Horoba E, Cojocaru M & Potorac E (1985) The study of the extraction process of vincamine. Rev. Roumaine Chem. 30: 807–815

Petiard V & Demarly Y (1972) Mise en evidence de glucosides et d'alcaloides dans les cultures de tissue vegetaux. Ann. Amélior. Plantes 22: 361–374

Stapfer R E & Heuser C W (1985) In vitro propagation of periwinkle. Hort. Science 20: 141–142

Tanaka N & Matsumoto T (1993a) Characterization of *Ajuga* plant regenerated from hairy roots. Plant Tiss. Cult. Lett. 10: 78–83

Tanaka N & Matsumoto T (1993b) Regenerants from *Ajuga* hairy roots with high productivity of 20-hydroxyecdysone. Plant Cell Rep. 13: 87–90

Tanaka N, Takao M, Matsumoto T & Machida Y (1993) Transformation of *Agrobacterium rhizogenes* strain MAFF03-01724 by electroporation. Ann. Phytopath. Soc. Jpn. 59: 587–593

Tanaka N, Takao M & Matsumoto T (1994) *Agrobacterium rhizogenes*-mediated transformation and regeneration of *Vinca minor* L. Plant Tiss. Cult. Lett. 11: 191–198

Tanaka N & Oka A (1994) Restriction endonuclease map of the root-inducing plasmid (pRi1724) of *Agrobacterium rhizogenes* strain MAFF03-01724. Biosci. Biotech. Biochem. 58: 297–299

Tepfer D (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. Cell 37: 959–967

Toivonen L (1993) Utilization of hairy root cultures for production of secondary metabolites. Biotechnol. Prog. 9: 12–20