Original Paper Plant Biotechnology, **15**(2), 71-75(1998)

Plant Regeneration from the Mass of Shoot Primordia in *Rhododendron pentaphyllum* Maxim. var. *nikoense* Komatsu

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Received 10 June 1997; accepted 20 November 1997

Abstract

A mass of shoot primordia were induced effectively from the shoot apex of *Rhododendron pentaphyllum* Maxim. var. Komatsu on WP media containing 30 μM 2iP+IAA (10, 30, 100 μM). Of the shoot primordia obtained, those cultured on WP medium supplemented with 10 μM IAA and 30 μM 2iP showed the most active proliferation, being 2-4 times in size, after one month of subculture. When shoot primordia obtained were transferred onto the differentiation media, multiple shoots were formed on WP media containing zeatin (1, 10 μM) or 0.1μ M CPPU after one month of culture, irrespective of the presence of IAA. The multiple shoots induced on the medium containing 10 μM IAA and 10 μM zeatin showed active elongation growth. Rooting from the multiple shoots subcultured on the same fresh medium occurred two months after the shoot differentiation.

1. Introduction

Rhododendron pentaphyllum Maxim. var. *nikoense* Komatsu, a deciduous shrub, produces beautiful pink flowers before bud flushing in spring, and is a valuable ornamental plant in gardens and parks in Japan. This species has been most commonly propagated by using seeds. However, the germination rate of the seeds is considerably low, and propagation by cuttings is also difficult due to poor rooting ability [1]. Therefore, application of tissue culture techniques is desired for the propagation of this species.

Most of the studies on the tissue culture of *Rhododendron* species have focused on micropropagation of axillary shoots [2-5]. Successful plant regeneration has also been reported in ovary culture of *R. prinophyllum* [6] and callus cultures induced from shoot tip and stem segment of *R. laetum* x *aurigeranum* [7, 8]. As to the tissue culture of *R. pentaphyllum* Maxim. var. *nikoense* Komatsu, there is only one report of the induction of multiple shoots from shoot apex. However, the multiplication rate in this species was relatively low. No other reports on tissue culture of this species have been provided to date. Selection of explants used for tissue culture seems to be a key factor for the successful propagation with high multiplication rates in the *Rhododendron* species.

The culture of shoot apices is useful for micropropagation of woody plants, because they can be obtained aseptically from field-grown trees. In addition, the shoot apex has an advantage in that it contains few polyphenolic compounds which often inhibit explant growth. A few reports have been published on the induction of a mass of shoot primordia by culturing shoot apex [9-12]. In general, shoot primordia show very active cell division and they can continue to proliferate with chromosomal stability for many years by successively producing new shoot primordia. However, induction of shoot primordia has not yet been reported in the *Rhododendron* species.

In this study, we report an efficient system for micropropagation of this species through the induction of a mass of shoot primordia from shoot apices.

2. Materials and Methods

2. 1 Plant materials

Buds, about 2 cm long, were collected from a mature tree of *Rhododendron pentaphyllum* Maxim. var. *nikoense* Komatsu growing on the campus of Utsunomiya University in late September. They were washed in tap water containing a few drops of neutral detergent for 5 min. After being washed with running tap water for 20 min, they were surface-sterilized for 20 min with 2% sodium hypochlorite solution containing a few drops of Tween 80 in a fume hood, and then rinsed three times with sterile distilled water in a laminar air flow cabinet. After drying on a sterile filter paper, bud scales were removed from the buds. A 4 mm-long shoot apex with five leaf primor-

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dia was excised from the bud to use as an explant.

2. 2 Induction of mass of shoot primordia

After being sterilized, a shoot apex was planted on 10 m*l* of agar-solidified MS medium [14] or WP medium [15, 16] in a 15 m*l* test tube containing various combinations of auxin: indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0, 0.1, 1, 10, 30, and 100 μM), and cytokinin: N^6-(2-isopentenyl)-adenine (2iP) (0, 0.1, 1, 10, and 30 μM). The medium pH was adjusted to 5.8 before addition of agar (0.8%). Shoot apices were cultured at 25°C under the illumination of cool white fluorescent tubes (2,000 lux) for 16 hrs per day for 80 days. Ten explants were cultured in each treatment.

2. 3 Multiple shoot formation from a mass of shoot primordia

A mass of shoot primordia induced from shoot apices were divided into small pieces of about 3 mm in diameter, and then cultured on 12 m*l* of WP agar medium in a 50 m*l* flat-bottomed tube containing various combinations of IAA (0, 10 μM), and cytokinin: 2iP, zeatin, or N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) (0.1, 1, 10 μM). They were cultured at 25°C under the illumination of cool white fluorescent tubes (2,000 lux) for 16 hrs per day. Three masses of shoot primordia were cultured for each treatment. The shoots obtained were subcultured to the same fresh media at one month intervals.

3. Results and Discussion

In this experiment, the culture of shoot apex gave little contamination, the rate being only 8% on average. Woody plant media gave shoot elongation, callus formation, or formation of a mass of shoot primordia, whereas MS media failed to form a mass of shoot primordia and gave mostly calli (**Tables 1, 2**). Browning of cultures occurred more frequently on MS media (80.5%)than on WP media (65.8%). Anderson [17, 18] and WP [15, 16] media have been found to be suitable as basal media for micropropagation of *Rhododendron* species. Components of WP medium are very similar to those of MS medium, except for the low concentrations of ammonium nitrate, and potassium and calcium ions, suggesting that cultures of *R. pentaphyllum* do not require large amounts of these components.

Calli were formed frequently from the shoot apices on WP media containing high concentrations of IAA or IBA. In medium containing IBA, addition of 2iP was required for the callus formation (**Table 1**). Two types of calli were formed: a) lumpy, friable white callus, b) compact green callus. White calli were induced effectively at the wide range of auxin concentration irrespective of the presence of 2iP. Most of them showed very slow growth, and subsequently turned brown. However, root formation occurred from these calli cultured on the three WP media containing a high concentration of IBA and a low concentration of 2iP (**Table 1**). On the other hand, green calli were effectively induced on the WP media containing 1-100 μM IAA and 1-30 μM 2iP or on those containing 1-100 μM IBA and 0.1-30 μM 2iP. All of the green calli obtained showed active proliferation. Further research is needed to know the regeneration ability of plantlets from these green calli.

Masses of shoot primordia were induced successfully from the shoot apices cultured on WP media

Table 1. Effects of plant growth regulators on the morphological changes of shoot apex cultured on WP medium.

Cytokinin	Auxin (μM)										
2 ip (μM)	IAA						IBA				
	0	0.1	1	10	30	100	0.1	1	10	30	100
0					W (1)	W (1)					
0.1				W (1)	W (1)	W (2)		S (1)	G,W (1,2)	W(R) (5)	G,W (1,1)
1			G (2)	W (9)	G,W (1,7)	W (6)	S (1)		G,W (1,3)	G,W(R) (2,3)	W(R) (5)
10	S (2)	S (1)	G,W (3,1)	G,W (7,1)	G,W (1,3)	W (4)	S (1)	G,W (3,1)	G,W (3,4)	G,W (2,2)	W (5)
30	S (1)	S (1)	G (1)	Sp,G (2,2)	Sp,G,W (2,3,1)	Sp,G,W (1,1,4)		G (3)	G,W (5,1)	G,W (4,2)	W (6)

S: Shoot elongation, Sp: Shoot primordia formation, G: Green callus formation, W: White callus formation, R: Root formation.

Ten explants were used in each treatment. Values in parentheses indicate the number of explants with callus, shoot or shoot primordia formation. Contamination: 8.5%, Browning: 65.8%.

Table 2. Effects of plant growth regulators on the morphological changes of shoot apex cultured on MS medium.

Cytokinin	Auxin (μM)										
2 ip (μM)	IAA						IBA				
	0	0.1	1	10	30	100	0.1	1	10	30	100
0											
0.1					W (1)	W (3)				W (1)	
1	S (1)			W (2)	W (1)	W (4)			G,W (1,3)	W (1)	W (3)
10			G (3)	G,W (2,1)	G,W (1,3)	W (2)	S (1)	G (3)	G,W (1,3)	W (4)	W (1)
30		S (1)	G (1)	G (2)	G,W (2,1)	W (3)		G (3)	G,W (3,1)	W (4)	

S: Shoot elongation, G: Green callus formation, W: White callus formation.
Ten explants were used in each treatment. Values in parentheses indicate the number of explants with callus, shoot or shoot primordia formation. Contamination: 7.1%, Browning: 80.5%.

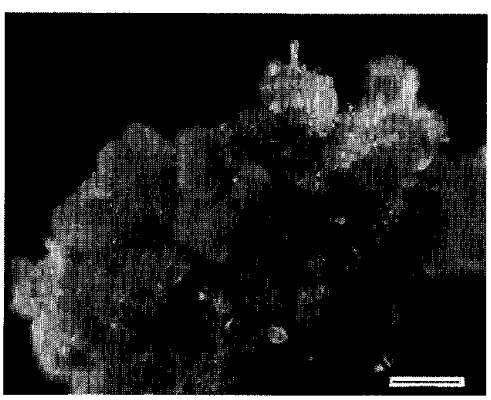

Fig. 1 Shoot primordia formed from shoot apex of *R. pentaphyllum* cultured for 40 days on WP medium containing 10 μM IAA and 30 μM 2iP. Bar=1 mm

containing 30 μM 2iP and 10-100 μM IAA, but not on MS media (**Tables 1, 2, Fig. 1**). No shoot primordia were formed on media containing IBA. Development of shoot primordia on media containing IAA was as follows. Within 3 to 4 weeks from the initial culture, a mass of green callus formed at the base of the shoot apex. Subsequently, the green callus actively proliferated, and then within one month of culture shoot primordia appeared around the surface of the green callus. The shoot primordia obtained showed dark green color, and were globular in shape with many protuberances resembling "moss" in appearance. Histological observation of the callus revealed a difference in color between the surface and core tissues, dark green and pale yellow, respectively. Judging from the active proliferation, the surface tissues seemed to be embryogenic. The surface tissues of callus masses were easily divided with sterilized tweezers into small pieces, showing a characteristic shoot primordia. Among the shoot primordia obtained, only those cultured on WP medium supplemented with 10 μM IAA and 30 μM 2iP showed the most active proliferation. The shoot primordia were selected for the following experiments and maintained by subculturing at one-month intervals for more than 2 years each with 2-4 times proliferation. These results suggest that the addition of 10-100 μM IAA to WP medium is effective for the induction and proliferation of shoot primordia in the presence of 30 μM 2iP in *R. pentaphyllum* var. *nikoense*.

It seems that lower concentrations of IAA and 2iP in the medium differentiated shoots, while higher concentrations of both phytohormones produced calli, and that the intermediate concentrations formed shoot primordia. In fact, shoot primordia obtained on WP medium containing high concentrations of IAA (100 μM) and 2iP (30 μM) subsequently developed into calli after the subculture. It is considered that shoot primordium is physiologically in a threshold state between callus and shoot [11], indicating that shoot primordium has the abilities of both proliferation and differentiation. This fact suggests that shoot primordium can be expected to be induced by using intermediate concentration levels of the auxin and cytokinin in other *Rhododendron* species.

Shoot primordia obtained on WP medium containing 10 μM IAA and 30 μM 2iP were subcultured on the various WP media with combinations of IAA and one of the 3 cytokinins, 2iP, zeatin, and CPPU, to induce the adventitious shoots (**Table 3**). At the beginning of culture, active proliferation of shoot primordia occurred on WP media containing various concentrations of zeatin (1, 10 μM) and CPPU (0, 1, 10 μM), irrespective of the presence of IAA. While in the media containing 2iP and 0.1 μM zeatin shoot primordia failed to proliferate, and finally died. After one

Table 3. Shoot formation from a mass of shoot primordia.

	Plant growth regulators (μM)																	
	IAA																	
	0									10								
	Cytokinin																	
	2 ip			Zeatin			CPPU			2 ip			Zeatin			CPPU		
	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10
Callus formation									3								2	3
Proliferation of SP								3									1	
Shoot formation*					1	3	3							2	3	3		
Browning or necrosis	3	3	3	3	2					3	3	3	3	1				

* Shoot formation was observed after one month of culture. Three mass of shoot primordia were used for each treatment. Sp: Shoot primordia.

Fig. 2 Multiple shoots induced from shoot primordia on WP medium containing 10 μM IAA and 10 μM zeatin. Bar=1 cm

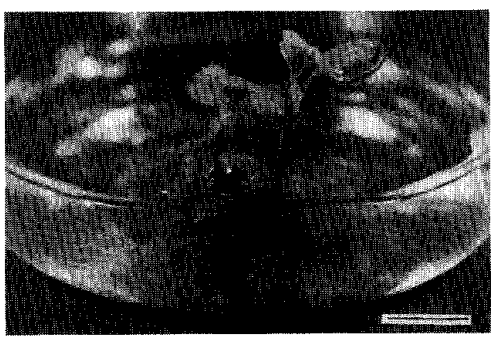

Fig. 3 Rooted multiple shoots two months after shoot differentiation. For clear observation of the roots produced, plantlets were transferred into water after removing agar medium attached to roots. Bar=1 cm

month of culture on media containing zeatin (1, 10 μM) or 0.1 μM CPPU, multiple adventitious shoots were formed irrespective of the presence of IAA (**Fig. 2**). On media containing 1 or 10 μM CPPU, however, shoot primordia developed into green calli instead of forming adventitious shoots.

It is noteworthy that shoot differentiation from shoot primordia depends on cytokinin activities. In this experiment, two media containing high concentrations of zeatin (10 μM) developed multiple shoots irrespective of the presence of IAA, while two media containing low concentrations of zeatin (1 μM) differentiated only one adventitious shoot. On the other hand, on media supplemented with CPPU, multiple shoots formed only at the concentration of 0.1 μM, irrespective of the presence of IAA. Masubuchi [19] and Iwanade and Masubuchi [20] suggested that addition of GA_3 is required for the shoot elongation in *R. pentaphyllum*. In this experiment, however, multiple shoots induced on a medium containing 10 μM IAA and 10 μM zeatin showed active elongation growth by subculture on the same medium without the addition of GA_3. Rooting from the multiple shoots occurred two months after the differentiation of shoots and leaves during the subculture on the same fresh medium (**Fig. 3**). This fact indicates that the supplementation of zeatin to the medium is effective for the differentiation and elongation of multiple shoots from shoot primordia, while that of IAA is effective for rooting in *R. pentaphyllum* Maxim. var. *nikoense* Komatsu.

Acknowledgment

The authors would like to thank Kyowa Hakko Co. Ltd. for providing CPPU.

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