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Plant Regeneration from Leaf Explants of *Primula cuneifolia* var. *hakusanensis*, "Hakusan-kozakura"

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Abstract

Somatic embryos and adventitious shoots were initiated from leaf explants of *Primula cuneifolia* var. *hakusanensis* on the media containing various cytokinins. The best treatment for induction of somatic embryos was on the medium containing 1.0 mg/*l* of TDZ or 5.0–10.0 mg/*l* of zeatin. Kinetin had no effect on the regeneration from leaf explants. Somatic embryos and adventitious shoots grew to plantlets on LS-hormone-free medium. After acclimatization in the growth chamber, the regenerated plantlets flowered and set seeds.

1. Introduction

Primula cuneifolia var. *hakusanensis* (Franch.) Makino is a native perennial plant distributed in the alpine area of Honshu, Japan. Recently the population of the species in the Hakusan area has been decreasing. We have newly established an in vitro propagation system for Hakusan-kozakura as a method to conserve these alpine flowers.

There are a few reports on the tissue culture of *Primuraceae*: Coumans *et al.* (1979) reported that young floral buds of *Primula obconica* proliferated vegetatively when cultured on the MS medium supplemented with benzyl adenine (BA) and naphthalene acetic acid (NAA) [1]. Ohashi and Mii (1988) obtained regenerated plantlets from leaf blades of *P. obconica* and *P. malacoides* cultured on the MS medium supplemented with 1 mg/*l* of IAA and 5 mg/*l* of zeatin [2].

We investigated the effects of plant hormones, especially cytokinins, on the production of somatic embryos and adventitious shoots from leaf explants of *P. cuneifolia*, then development into plantlets and the survival of these plantlets after transplantation in soil.

2. Materials and Methods

Seeds of *P. cuneifolia* var. *hakusanensis* (Franch.) Makino were collected at Midagahara (alt. 2300 m) in Mt. Hakusan from the latter part of August to the middle of September and kept in a refrigerator (4°C). Seeds sterilized with 3% NaOCl solution for 15 min. followed by rinsing three times with sterilized distilled water were sown on LS medium solidified with 0.25% of Gelrite. They were incubated at 20°C under about 3000 lux fluorescent illumination for a 16-h photoperiod.

After a two-month culture, plantlets with 4 to 5 leaves were used as a source of explants. The leaves were cut into pieces of approximately 5 × 5 mm and placed on media containing LS basal medium [3] supplemented with 3% sucrose and various concentrations and combinations of cytokinins, BA, kinetin, zeatin, thidiazuron (TDZ, *N*-phenyl-*N'*-1, 2, 3-thidiazol-5-urea) and *N*-(2-chloro-4-pyridyl-*N'*-phenylurea (4PU), and auxins, NAA and indole acetic acid (IAA) (**Table 1**~**3**). Somatic embryos and adventitious shoots were transferred onto hormone-free LS medium for rooting. Cultures were incubated at 20°C under about 3000 lux in a 16-h photoperiod.

After a two-month incubation the results of the responses of the leaf explants were investigated and the data were calculated by summing the total number of somatic embryos, callus or roots on each medium. More than two replicates of 10 explants each were used for each medium.

Rooted plantlets in test tubes were transplanted to pots containing 2: 1 vermiculite and perlite mixture and maintained at 20°C under a 24-h photoperiod of around 5000 lux.

3. Results and Discussion

3. 1 Effects of cytokinins on the redifferentiation of leaf explants

Since leaf explants turned brown after two months of incubation at 26°C and/or in the dark in the preliminary experiments, the cultures were incubated at 20°C under fluorescent light.

After a 2-month culture on media containing

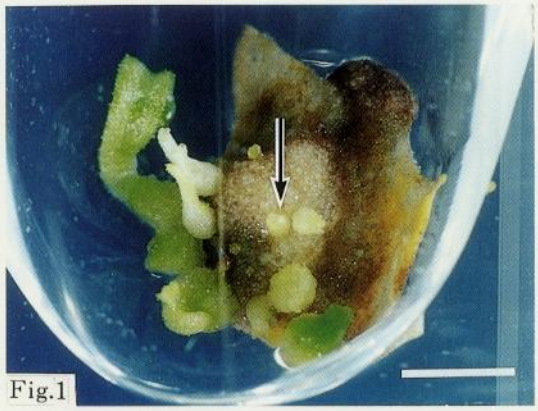

Fig. 1 Somatic embryos in various developmental stages produced on a leaf explant cultured for two months on the zeatin-containing LS medium.

An arrow shows a somatic embryo in the globular stage. Bar=5 mm.

Fig. 2 Plantlets regenerated by transferring somatic embryos onto LS hormone-free medium.

Fig. 3 Flowering plants regenerated through somatic embryos derived from leaf explants.

Table 1.
Effect of zeatin combined with IAA and NAA on the redifferentiation of leaf explants.

Hormones (mg/*l*)			No. explants cultured	% explants producing		
Zeatin	IAA	NAA		embryos	roots	callus
0		2.0	20	0	35.0	90.0
0.2	0.2		20	0	0	0
0.2	2.0		20	0	5.0	15.0
0.2		0.2	32	3.1	6.3	59.4
0.2		2.0	32	6.3	6.3	71.9
2.0	0.2		20	15.0	0	20.0
2.0	2.0		20	5.0	5.0	50.0
2.0		0.2	31	16.1	6.5	41.9
2.0		2.0	31	0	19.4	83.9
5.0			25	76.0	0	0
5.0	0.2		25	44.0	0	64.0
5.0	2.0		25	36.0	0	68.0
5.0		0.2	69	15.9	0	55.1
5.0		2.0	44	0	11.4	77.3
10.0			25	80.0	0	0
10.0		0.2	70	10.0	5.7	68.6
10.0		2.0	43	2.3	9.3	51.2

Table 2.
Effect of BA combined with IAA and NAA on the redifferentiation of leaf explants.

Hormones (mg/*l*)			No. explants cultured	% explants producing			
BA	IAA	NAA		embryos	shoots	roots	callus
0		2.0	20	0	0	35.0	90.0
0.2	0.2		20	0	0	0	15.0
0.2	2.0		20	5.0	0	0	10.0
0.2		0.2	62	1.6	0	1.6	53.2
0.2		2.0	57	0	0	14.0	56.1
2.0	0.2		19	10.5	0	0	10.5
2.0	2.0		20	5.0	0	0	10.0
2.0		0.2	53	0	1.9	0	17.0
2.0		2.0	60	0	0	5.0	43.3

Table 3.
Effect of kinetin combined with IAA and NAA on the redifferentiation of leaf explants.

Hormones (mg/*l*)			No. explants cultured	% explants producing		
Kinetin	IAA	NAA		embryos	roots	callus
0		2.0	20	0	35.0	90.0
0.2	0.2		20	0	5.0	60.0
0.2	2.0		20	0	30.0	100
0.2		0.2	40	0	15.0	65.0
0.2		2.0	38	0	7.9	63.2
2.0	0.2		20	0	0	5.0
2.0	2.0		20	0	0	85.0
2.0		0.2	40	0	2.5	20.0
2.0		2.0	38	0	0	50.0

Table 4.
Effect of TDZ combined with IAA and NAA on the redifferentiation of leaf explants.

Hormones (mg/*l*)			No. explants cultured	% explants producing			
TDZ	IAA	NAA		embryos	shoots	roots	callus
0		2.0	20	0	0	35.0	90.0
0.02			62	21.0	0	0	0
0.02	0.2		66	33.3	0	3.0	1.5
0.02	2.0		66	31.8	0	4.5	27.3
0.02		0.2	83	19.3	0	12.0	72.3
0.02		2.0	82	0	0	35.0	90.0
0.2			62	56.5	0	0	0
0.2	0.2		66	54.5	0	0	0
0.2	2.0		66	59.0	0	1.5	18.2
0.2		0.2	83	44.6	1.2	6.0	80.7
0.2		2.0	83	1.2	1.2	7.2	96.4
1.0			82	74.4	0	0	11.0
1.0	0.2		65	66.0	0	0	10.8
1.0	2.0		66	68.2	0	0	27.3
1.0		0.2	62	87.1	0	0	71.0
1.0		2.0	62	6.5	0	1.6	96.8

Table 5.
Effect of 4PU combined with NAA on the redifferentiation of leaf explants.

Hormones (mg/*l*)		No. explants cultured	% explants producing			
4PU	NAA		embryos	shoots	roots	callus
0	2.0	20	0	0	35.0	90.0
0.1	0.1	70	15.7	4.3	8.6	65.7
0.1	1.0	70	0	0	14.3	77.1
1.0	0.1	70	32.9	0	5.7	78.6
1.0	1.0	69	0	0	7.2	68.1
5.0	0.1	30	26.7	3.3	6.7	53.3
5.0	1.0	30	6.7	0	6.7	80.0
10.0	0.1	29	17.2	6.9	0	65.5
10.0	1.0	30	0	0	0	7.0

different cytokinins in combination with IAA or NAA, leaf explants showed different reactions to the kind of cytokinin as shown in **Tables 1** to **5**.

On LS medium containing high concentrations (over 2 mg/*l*) of zeatin, leaf explants produced somatic embryos on the surface of leaf explants (**Fig. 1**), while on the medium containing IAA or NAA they produced callus and/or roots (**Table 1**). Although on the media containing BA, TDZ and 4-PU in a high concentration somatic embryos were also produced on the leaf explants, a few adventitious shoots emerged at low frequencies, from 1.2% to 4.3% (**Table 2, 4,** and **5**). The media containing 5 or 10 mg/*l* of zeatin, 0.2 or 1.0 mg/*l* of TDZ, or 1.0 mg/*l* of TDZ in combination with 0.2 mg/*l* of NAA were most effective for somatic embryogenesis, showing that over 70% of explants produced somatic embryos (**Table 4**). Average number of somatic embryos on a leaf explant was from 2 to 10 and varied with the plant hormones contained in the media; the highest number of embryos was produced on the medium containing 1.0 mg/*l* of TDZ and 0.2 mg/*l* of IAA. On the other hand, no somatic embryos or adventitious shoots were observed on the medium containing kinetin (**Table 3**). Kinetin and 4-PU stimulated the callus induction.

Zeatin and BA stimulated somatic embryogenesis of *P. malacoides* and *P. obconica* and adventitious shoot formation in combination with auxin [1, 2]. In this experiment on *P. cuneifolia,* somatic embryos were formed on the media containing BA and zeatin, even in the absence of auxin. IAA and NAA rather suppressed the somatic embryogenesis, while a high concentration of auxins promoted callus proliferation and root redifferentiation.

TDZ was effective for the somatic embryogenesis and by increasing the concentration (0.02-1.0 mg/*l*)

the frequency of somatic embryo formation was increased. TDZ is the most effective cytokinin available for tissue culture of many woody species [4]. Recently TDZ has been reported to promote the adventitious shoot regeneration of various herbaceous species, such as white clover [5], peanut [6] and *Saintpaulia ionantha* [7].

3. 2 Regenerated plantlets

Around 50% of somatic embryos grew shoots and roots, after they were transferred onto the LS medium in the absence of plant hormones (**Fig. 2**). Almost all adventitious shoots also generated roots on the medium containing no plant hormones. The plantlets were transplanted to pots containing a vermiculite: perlite (2: 1) mixture and acclimatized under high humidity conditions. Almost all plants survived and after 4 months, some of them flowered in the growth chamber (**Fig. 3**). Regenerated plants set seeds by artificial crossing with heterostyly plants.

A few regenerated plants exhibited morphological variation, such as serrate leaves and double flowers by petaloid stamens. Some variations seemed to be caused by the physiological disturbances of *in vitro* conditions and some from somaclonal variations. Genetic analysis of the variants is needed.

The regenerated plants flowered within 6 months after the start of leaf explant culture. This method of culturing leaf explants on the zeatin- or TDZ-containing LS medium is effective for the micropropagation of *P. cuneifolia*. Development of this culture system may contribute to the conservation of alpine flowers.

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