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Plant Regeneration from Leaf Blade Explants of Horseradish (*Amoracia rusticana* L.) through *In Vitro* Culture

Regenerasi Tanaman Horseradish (Amoracia rusticana L.) dari Eksplan Daun Secara in Vitro

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ABSTRACT

Horseradish as an important crop in Japan is difficult to be propagated by using seed. The objective of this research was to overcome such difficulty through *in vitro* culture. The explants used were young leaves and old leaves. The MS basal medium was used and treated with 0; 0.1; 0.3; 0.6; 1.0 and 10 µM 2,4-D combinated with 0; 0.1; and 1.0 µM BA. This experiment showed that a combination of 0.1 µM 2,4-D and 0,.1 µM BA gave the highest plantlet regeneration as compared with 0; 1.0 and 10 µM 2,4-D combinated with 0 and 1.0 µM BA. Plantlet regeneration took place through direct morphogenesis. Sub-culturing calli from 1.0 µM 2,4-D and 0; 0.1 and 1.0 µM BA-supplemented medium to a medium without 2,4-D nor BA was the best sub-culturing method to get optimal plantlet regeneration. Young leaf explants had better response to plant growth regulators compared with old leaf explants.

Key word: Horseradish, Regeneration, Morphogenesis, Calli

INTRODUCTION

Horseradish (*Amoracia rusticana* Gaertn.) is a quite important crop for a material of spice in Japan (Nonnecke, 1989). It grows wildly all over Japan and cultivated in some districts. Unfortunately, in those districts the yield of the horseradish has reduced quite meaningfully because of virus (Sato and Shigeta, 1990). Some diseases that affect the yield were also reported in Illinois, USA (Babadost, 2008). In addition, this plant cannot be propagated by using seed because its sterility. It is usually propagated vegetative by root cuttings (Nonnecke, 1989). As a result, the available root has limited its vegetative propagation considerably. Therefore, research about the vegetative propagation or horseradish *in vitro* should be performed as well as research about other aspects to gain sustainable high yield of horseradish.

As to plant regeneration through tissue culture, Sato and Shigeta (1990) reported the somatic embryogenesis and plantlet regeneration from cell culture in horseradish. It was found that the biggest embryogenic callus was formed on the medium supplemented with 2 ppm 2,4-D and 0,5 ppm Benzyl Adenine (BA). On the other hand, the highest embryogenesis ability was observed on the medium supplemented with 0.1 ppm 2,4-D and 0,1 ppm BA.

To provide more detail information about *in vitro* propagation of horseradish, the presents work was conducted by comparing the effect of more various levels of 2,4-D, BA and condition of leaf blade on plantlet regeneration.

MATERIALS AND METHODS

Experiment I: Morphogenesis from Leaf Explant

Young leaves in June and young and old in October were taken from horseradish plants cultivated in the Experimental Farm of Hokkaido University, Sapporo, Japan. These leaves were

separately washed by running water for 30 minutes. They were surface-sterilized in 70% ethyl alcohol for 10 seconds and 1% hypochlorite solution for 10 minutes, and then they were rinsed by sterile distilled water 3 times for 5 minutes each. In Laminar Air Flow Cabinet, these leaves were cut into small piece (1 x 1 cm^2) and were cultured on the MS basal medium supplemented with different concentrations of 2,4-D and BA as shown in Table 1.

To observe the difference in plant regeneration mechanism among the treatments, histological studies was conducted. The calli in which it was assumed the shoot was about going to come up was fixed in FAA (formalin: acetic acid: ethanol= 5:5:90), dehydrated by using ethanol-buthanol series, and embedded in paraffin. The embedded tissues were cut into 12 µm thick sections, stained with Delafield's hematoxylin.

Experiment II: Regeneration from Subcultured Calli

Green calli formed from young leaf blades on the medium supplemented with 1,0 µM 2,4-D were cut into small blocks (0,5 x 0,5 x 0,5 cm^3) and were transferred to secondary media as shown in Table 4. Plants regeneration was investigated after their subculturing for four weeks.

In all experiment, Murashige and Skoog basal medium with 20 g L^{-1} sucrose was used and 2 g L^{-1} gellan gum were added as supplements. pH was adjusted to 5,8 with 0,1 N NaOH or HCl before autoclaving at 120 °C for 15 minutes. Four explants were cultured in each flask containing 30 mL media. All cultures were maintained at 25 °C under 16 hour photoperiods with 3,000 to 4,000 1ux illumination by fluorescent lamps.

RESULT

Morphogenesis from Leaf Blade Explants

In the blade explants taken in June, shoot and root formation occurred in the medium supplemented with 0,1 µM 2,4-D and any concentrations of BA (Table 2). Shoot developed directly from the explants in the combination between 0,1 µM 2,4-D and 0,1 µM BA, and the highest percentage of plant regeneration was found in that medium.

On the medium containing with more than 0,1 µM 2,4-D, leaf blade explants intended to differentiate calli. In the media supplemented with 1,0 µM 2,4-D, leaf blade explants formed morphogenic calli (Figure 1 a-c). These calli colored green and had a tendency to regenerate into plantlet on the subculture media. Brown calli was formed on 10 µM 2,4-D supplemented media (Table 2). In the young leaf blade explants taken in October, though nine explants formed shoot directly, callus formation was observed from 11 explants in the medium supplemented with 0,1 µM 2.4-D. Plantlets were obtained from the formed calli in the initial medium. In the medium supplemented with more than 0,3 µM 2,4-D, all young explants developed calli, however, only three plants were regenerated from them. Plant regeneration was not observed in the media with more than 0,6 µM of 2,4-D.

Table 1. Treatments of experiments conducted

Month	2,4-D (µM)	BA (µM)	Condition of leaf blade
June	0,0	0,0	Young
	0,1	0,1	
	1,0	1,0	
	10		
October	0,1	0,1	Young
	0,3		
	0,6		Old
	1,0		

Table 2. Effects of 2.4-D and BA on morphogenesis of leaf blade explants of or seradish cultured *in vitro* in June

2.4-D (µM)	BA (µM)	No. of survived leaf blade segments	No. of leaf blade segments showing morphogenic response				
			Shoot formation	Root formation	Regenerated plantlet	Callusing Green	Brown
0	0	0	0	0	0	0	0
0	0,1	3	0	0	0	0	0
0	1,0	2	1	2	1	0	0
0,1	0	7	2	7	2	0	0
0,1	0,1	5	5	5	5	0	0
0,1	1,0	8	2	8	2	0	0
1,0	0	13	0	0	0	13	0
1,0	0,1	15	0	0	0	15	0
1,0	1,0	10	0	0	0	10	0
10	0	16	0	0	0	0	16
10	0,1	15	0	0	0	0	15
10	1,0	16	0	0	0	0	16

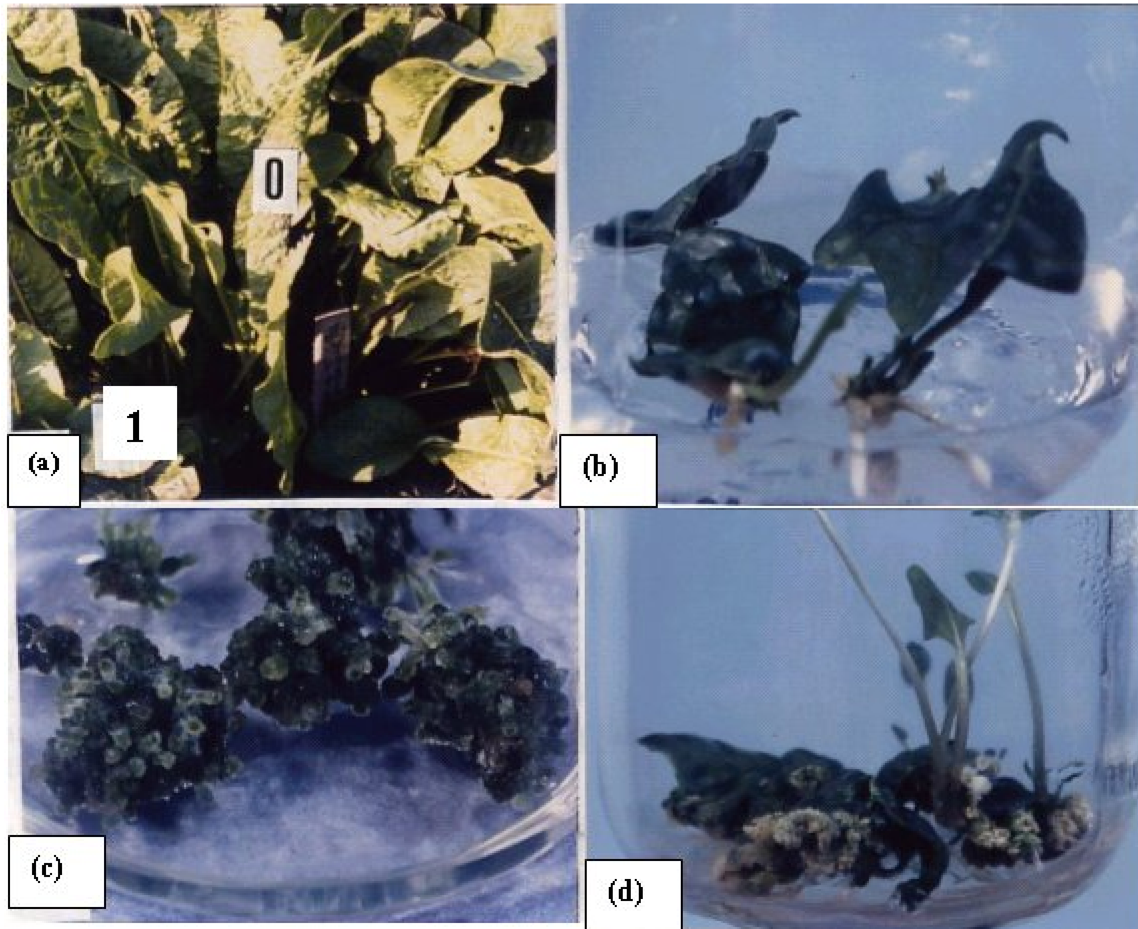

Figure 1. Plant material and morphogenesis of leaf blade explant (a) External appearance of material growing in the field young (0) and old (1) leaves were used, (b) Direct shoot formation, (c) Morphogenic callus, and (d) Differentiation of shoot and root from the callus.

Old leaf blade explants generally developed calli in any concentrations of 2,4-D. There was no direct shoot formation and only one was formed as a result of differentiation from calli. In addition, the number of survived explants was smaller than young leaf explants (Table 3).

Many adventitious buds were formed near the vascular bundle of leaf segment in the media

supplemented with 0,1 µM 2,4-D. These adventitious buds were seen to be connected one another by the vascular bundle. Mesophyll tissues clearly developed into calli (Figure 2-1). On the other hand, in the media supplemented with high concentration of 2,4-D, the leaf blade explants differentiated calli and the vascular bundle continuously was formed in masses of calli. No adventitious bud was formed in that media (Figure 2b).

Table 3. Effect of 2,4-D on morphogenesis of young and old leaf blade segments of horseradish *in vitro*

2,4-D (µM)	Leaf condition	No. of survived explants	Callusing			No. of shoot formation z)		No. of root	No. of plantlet
			No. of formed	Size (cm) 0,5-2	2-3	Direct shoot formation	Differentiation from calli		
0,1	Young	20	11	11	0	9	16	33	25
	Old	4	4	0	4	0	1	10	1
0,3	Young	16	16	8	8	0	3	0	3
	Old	4	4	0	4	0	0	0	0
0,6	Young	18	18	16	2	0	0	0	0
	Old	14	14	8	6	0	0	0	0
1,0	Young	16	16	0	16	0	0	0	0
	Old	18	18	0	18	0	0	0	0

Keterangan : z) the number of explants having average size of callus as indicated

Table 4. Effect of 2,4-D and BA on differentiation of morphogenic calli of horseradish

Primary culture media 2,4-D (µM)	BA (µM)	Subculture media 2,4-D (µM)	BA (µM)	No. of calli transferred	No. of calli with organogenes Shoot formation	Root formation	No. of regenerated plantlet (%)
1,0	0	0	0	11	3	11	2 (18)
1,0	0	0,1	0	9	2	9	1 (11)
1,0	0	0,1	0,1	10	0	10	0 (0)
1,0	0,1	0	0	3	0	0	0 (0)
1,0	0,1	0,1	0	6	2	6	2 (33)
1,0	0,1	0,1	0,1	8	6	8	4 (50
1,0	1,0	0	0	7	7	7	7 (100)
1,0	1,0	0,1	0	7	2	7	2 (28)
1,0	1,0	0,1	0,1	4	1	4	1 (25)

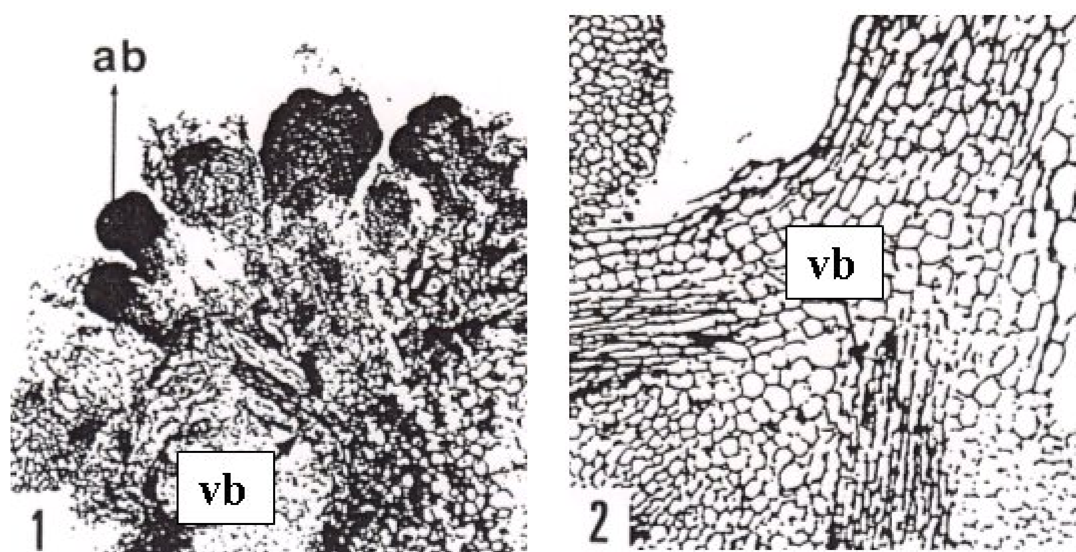

Figure 2. Histological observation (vb) vascular bundle; (ab) adventitious bud.

Regeneration

Development of the morphogenic calli induced on the media with 1,0 μM 2,4-D was affected by the secondary culture media. The highest percentage of plantlet regeneration was found on the calli from 1,0 μM 2,4-D and 1,0 μM BA-treated primary medium subcultured to secondary medium without plant growth regulators (Table 4).

However, if the explants on primary medium containing 1,0 μM 2,4-D and low concentration of BA (0,0 and 0,1 μM) were subcultured to secondary culture without plants growth regulator or low levels of 2,4-D and BA, the percentage of regenerated plantlet were low. If primary medium containing 1,0 μM 2,4-D and 1,0 μM BA and secondary medium supplemented with at least 0,1 μM 2,4-D, the percentage of regeneration reduced as well. Morphogenic calli did not develop to form plantlet in general because shoot induction was low compared with root induction. Root were proliferated in all secondary in high frequency, except calli from primary medium supplemented with 1,0 μM 2,4-D and 0,1 μM BA and subcultured to medium without plant growth regulators.

Plant regeneration was determined by the concentration of 2,4-D and BA in the medium and condition of leaf blade segments. From the present data, three pathways were recognized as to plant regeneration from leaft blade segments.

The first way was the direct plantlet regeneration without callusing. For the direct shoot formation from leaf blade segments, the medium with 0,1 μM 2,4-D and 0,1 μM BA was supposed to be the most suitable. In addition, the young leaf explants were able to form shoot directly, while old leaf explants could not.

The second way was callus formation and subsequent shoot and root diferentiation without subculturing. The response was observed only in young explants taken in October.

There was a little difference in the direct shoot formation between the explants taken in June and October. All of the young leaves were taken in June and October. All of the young leaves taken in June development to form shoot directly, while axplants in October showed that shoot formation accured not only through direct shoot formation but also via callusing that subsequently developed to form plantlet. Since there was no other different aspect but season, maybe that occured because leaf blade taken in June and October could have physiologically different metabolism which resulted in different response to the same concentration of 2,4-D and BA supplemented to media. The season of the year can effect callus initiation from explants, especially when the donor plant is field grown, because seasonal variations may affect the concentration of endogenous auxins (Evans *et al.,* 1983; Pierik, 1987).

The third way was indirect plantlet regeneration through callus induction and subculturing calli to a new medium. Higher concentration of 2,4-D than 0,1 μM accelerated the explants to form callus. Morphogenic callus was induced in the media suplemented with 1,0 μM 2,4-D or less, which was indicated by a large number of adventitous buds on its surface. Shoot proliferation was recognized after morphogenic calli were transferred to secondary medium. Plantlet regeneration was best to occur from morphogenic calli on media supplemented with 1,0 μM 2,4-D and 1,0 μM BA which were subcultured to media without growth regulator. Other squential media caused reduction in the number of plantlet regeneration due to reduction in shoot formation. Instead, root formation was almost perfectly induced from all calli. It happened probably because the proportion of 2,4-D was still high compared with BA, so that shoot induction was rather inhibited (Pierik, 1987). The condition of leaf culture showing the same result of organogenesis was studied from Petunia (Rao *et al.,* 1973b), mangosteen (Goh *et al.,* 1994), Apple (James *et al.,* 1984), Annona (Nair *et al.,* 1984), and Garlic (Nagasawa and Finer, 1988).

Previously Sato and Shigeta (1990) found that medium suplemented with 0,1 ppm 2,4-D and 0,1 ppm BA gave highest percentage of embryogenesis ability from petiole tissue. Most studies having examined the effect of BA and 2,4-D indicated that BA gave rise on shoot formation (Goh *et al.,* 1994; Pence *et al.,* 1984; Kurtz *et al.,* 1983) and 2,4-D caused extensive cell proliferation (Rao *et al.,* 1973a; James *et al.,* 1984).

Comparing the effects of the condition of leaf explants on plantlet regeneration, young leaf explants gave significant difference from old leaf explants. Young leaf explants supposed to be more sensitive to exogenous plant growth regulators and they formed more callus, shoot, and root, corresponding with the concentration of 2,4-D and BA. It was an expected result because young tissues usually consist of actively divided cells and have beter regeneration ability than old tissues (Pierik, 1987; Marubashi *et al.,* 1989).

CONCLUSION

Plant regeneration of horseradish through tissue culture could be better by using young leaf blade explants compared with old leaf explants.

The formation of plantlets of horseradish were higher by using media treated with 0,1 µM 2,4-D combinated with 0; 0,1; or 1,0 µM BA.

The higher concentration of 2,4-D than 0,1 µM, the calli tended to be formed.

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