Efficient Plant Regeneration from Shoot Tip and Young Leaf in *Rhodiola sachalinensis* A. Bor.

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ABSTRACT

The shoot tip and young leaf of *Rhodiola sachalinensis* were cultured to invest the plant growth regulator condition for callus induction, shoot and root regeneration. When the shoot tip was sterilized in 2.0% of NaOCl for 20min., the contamination rate was the lowest. And the survival rate of the culture material was good in carbenicillin 500mg/L treatment group. Callus was obtained from shoot tip and young leaf segments. NAA 0.1-1.0mg/L and 2,4-D 0.1-0.5mg/L alone treatment were shown to have a good response on callus induction from shoot tip culture. In the case of young leaf culture, NAA and 2,4-D 0.1-0.5mg/L alone treatment were good in callus induction. In culturing shoot tip NAA 0.5mg/L and BA 0.5mg/L, NAA1.0mg/L and BA 0.1mg/L combination treatment was good in shoot regeneration. The regenerated shoots were rooted on MS medium supplemented with NAA and BA combination treatment. Especially, NAA 1.0mg/L and BA 0.1mg/L combination treatment was effective for root regeneration.

Key words **:** *Rhodiola sachalinensis*, Plant growth regulator, Callus, Regeneration

INTRODUCTION

Rhodiola sachalinensis lives in the condition that is not enough of oxygen, full of ultraviolet rays, the difference of temperature is big, and is 3,000 to 5,000 meters above the sea level.

This plant is distributed in western and northern high mountains of Asia, and belongs to Crassulaceae, *Rhodiola*. There are 90 kinds of this plant in the world. There are 70 kinds living in China, and are mainly distributed in Dongbei, Huabei, Xizang and Xinjiang area(Ming *et al.*, 1988). There are four kinds of this plant in Korea: *R. elongata, R.angusta, R. ramosa, R. rosea*(Park, *et al.*,1999).

Rhodiola sachalinensis became well-known for best healthy plant and medicinal plant after discovering *Panax ginseng* and *Acanthopanax senticosus*. This plant could restore people's energy and heal diseases and poison, so it has a nick name, "a plateau ginseng"(Park, *et al.*, 1999). It is a perennial herb, and has rootstock

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that is fleshy which is used for medicine. The research of *Rhodiola sachalinensis* that is ingredient and medical action(Zhang *et al.*, 1991; Qian *et al.*, 1994; Peng *et al.*, 1994, 1996), antioxidative activity(Song *et al.*, 2002), ecology and environment(Wu *et al.*, 1984), seedling culture(Liu *et al.*, 1985), biological characteristic and rhizome propagation(Wang *et al.*, 1986), seed germination(Ahn, 1993), tissue culture(Kirichenko *et al.*, 1993; Krendal *et al.*, 1995) was achieved. Its principle effect is known to the restore people's energy, making people live longer, healing oxygen deficiency, relieving fatigue, enhancing concentration and memory, anticancer, recovering blood pressure, healing diabetic, pneumonia, bruise, and burn(Ming *et al.*, 1988; Qian *et al.*, 1994; Park *et al.*, 1999).

But it is difficult to gather the plant, because it lives in high mountains short of oxygen. It is also difficult to get the plant for propagation. Therefore, this experiment is to research the effect of plant growth control material on specialization of plant by cultivating shoot tip and young leaf. It will be the basic experiment for developing artificial propagation of the plant.

MATERIALS AND METHOD

The material is *Rhodiola sachalinensis* collected from an alpine region of the west-northern China in August, 2000. I germinated its seeds in March, 2001, and used shoot tip grown about 5cm and its young leaf as cultivation materials.

How to disinfect shoot tip

In order to find appropriate disinfection method for the cultivation material, I disinfected shoot tip with sodium hypochlorite (NaOCl) with consitency of 0.1, 1.0, 2.0, and 3.0 for 5, 10, and 20 minutes respectively. Also carbenicillin, an antibiotic, in consistency of 500mg/L and 800mg/L, and mancozed wp., a sterilizer, in consistency of 1%(w/v) were mixed and put into, in order to prevent the material from polluting.

Carbenicillin was filtered and sterilized with a membrane filter of 0.2㎛ and put into the culture medium, when the temperature was 60℃. Mancozed wp. was melted in sterilized water. And then shoot tip cut in size of 0.5mm was soaked in the solution for some time, and cultivated in a erlenmeyer flask of 200mL by each treatment in group of 10. And this process was repeated ten times.

Contamination rating and survival rating was examined for 30 days after the treatments.

The callus induction, shoot and root regeneration

For sterilizing shoot tip and young leaf used 2.0% sodium hypochlorite solution, which had the best effect in the experiment, as mentioned above(Table 1,2). The materials were deposited in the solution for 20 min. and went through surface sterilization and a lot of cleaning. Then they were put into the solution of tween-20 of 0.01% for 1 min. and shook and cleaned until there were no foam. They were washed with sterilized water in a germ-free room, and deposited in ethyl alcohol(95%) for about 1 to 2 min. As soon as taken out of the alcohol, they were washed with sterilized water three or four times. Then they were cut and inoculate as the cut parts get to the surface of the culture medium.

In order to find out effective culture consistency, a medium for callus induction and shoot regeneration that NAA (0.1, 0.2, 1.0, 2.0, 3.0 mg/L) and 2,4-D (0.1, 0.2, 0.5, 1.0, 2.0 mg/L) are solely added, one that NAA 0.5 mg/L and 1.0 mg/L are added in BA 0.1, 0.5, 1.0, 2.0 mg/L, one that 2,4-D 0.5 mg/L and 1.0 mg/L are added in BA 0.1, 0.5, 1.0, 2.0 mg/L, and one that BA and kinetin of 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 ㎎/L were solely added, were put into the culture medium as plant growth regulators.

MS medium(Murashige and Skoog, 1962) added with carvenicillin 500mg/L was used, and sucrose of 30g/L and agar of 8g/L were added in each culture. pH

of the culture medium was 5.7-5.8, which was controlled before high-pressure sterilization. Erlenmeyer flasks of 200mL were used, and ten flasks of one medium were inoculated ten times.

The source of light was fluorescence, and the culture media got the light of 2,000-2,200lux for about 16 hours a day. The temperature of culture was 24-25℃, and callus induction and regeneration rate of young leaf were examined.

RESULTS AND CONSIDERATION

The sterilizing method of shoot tip

Table 1 is the result of the experiment, which the consistency of NaOCl and deposition time is different, and this is for finding out the sterilizing method appropriate for the experimental materials. When the materials were sterilized in 2.0% NaOCl solution for 20 min., the contamination rate was the lowest as 35%. In 0.1% NaOCl solution, the contamination rate was 95 to 100% regardless of sterilizing time, in 1.0% NaOCl the contamination rate was getting low as the sterilizing time got long. In 3.0% NaOCl, all the materials turned brown and died regardless of sterilizing time.

Table 1. Effect of sodium hypochlorite on contamination of shoot tip cultures of *Rhodiola sachalinensis* after 30 days in culture

NaOCl Conc(%)	Contamination(%)		
	Minutes		
	5	10	20
0.1	100	95	95
1.0	85	85	60
2.0	70	55	35
3.0	D[z]	D	D

[z]all dead.

Therefore, it turned out that just surface sterilization was not enough, so the antibiotic, carbenicillin 500mg/L and 800mg/L, and sterilizer, 1% mancozed wp. was added in the culture medium. Table 2 shows the result that the contamination rate of the six sterilized groups was lower than the control treatment, which was sterilized in 2% NaOCl for 20 min. The carbenicilin 500mg/L group showed the highest survival rate, which was 100%, and the survival rate of other groups was lower than the control treatment. And the more the rate is lower, the more the material turns brown and dies.

So the conclusion was that the culture material of shoot tip has to be sterilized in 2% NaOCl for 20 min. and using a culture medium with carbenicillin 500mg/L was the best condition for survival rate. The contamination rate was 40%, when the shoot tip was deposited in 1.0% NaOCl solution for 20 min. And when carbenicillin of 500mg/L was added, the survival rate was 100%. But when 1% Mancozed wp. was added, the rate was lower(Lee *et al.*, 1994).

Also Wang *et al.* (1994) reported that shoot tip culturing of *Haemaria diocolor* in H_3P_4 medium was

Table 2. Effect of antibiotic and fungicide added to the medium contamination in shoot tip cultures of *Rhodiola sachalinensis* after 30 days in culture

Treatments	Carbenicillin(mg/L)	Survival rate (%)	Contamination (%)	Decline of shoot tip(%)
Control	0	65	35	0
0	500	100	0	0
0	800	50	0	50
Mancozed wp.1%	0	35	0	65
Mancozed wp.1%	500	35	0	65
Mancozed wp. 1%	800	30	0	70

better in survival rate than culturing in MS medium. So it can be concluded that the contamination and survival rate of cultruing plants is affected by the kind of culture medium.

The callus induction, shoot and root regeneration

There are many reports about the effect of plant growth regulator on callus induction, shoot and root regeneration(Kayo *et al.*, 1988; Yang *et al.*, 1991; Kirichenko *et al.*, 1993; Lee *et al.*, 1994; Krendal *et al.*, 1995; Han *et al.*, 1997; Eun *et al.*, 1997; Hassanein and Mazen, 2001; Vanegas, 2002). In order to find out how NAA alone treatment influence on callus induction, shoot and root regeneration, I experimented and got the result as table 3. In the case of shoot tip culturing, callus was 100% induced with NAA 0.1, 0.2, 0.5, 1.0mg/L treatment. It was 60% with 3.0mg/L, and the rate was very low with control treatment. With NAA 0.5mg/L shoot the shoot regeneration rate was 40%, but the rates were very low in other treatment group. The shoot tip with control treatment showed better regeneration than the one with NAA 2.0mg/L, or 3.0mg/L.

In the case of culturing of young leaf, the callus induction rate was 100% with NAA 0.1, 0.2, 0.5mg/L treatment. But as the treatment consistency got higher, the callus induction rate got lower and the leaves turned brown and induced.

The regeneration rate of shoot was very low in every treatment group, and the higher consistency was, the lower the rate was. And the shoot wasn't regenerated in the group with control treatment. In most cases auxin such as 2, 4-D, or NAA are used alone, according to circumstances, cytokinin such as BAP, kinetin, or zeatin are used mixed.

The treatment group with NAA 0.5, 1.0mg/L was used for shoot tip culturing added with BA 0.1, 0.5, 1.0, and 2.0mg/L, and the result was that 0.1, and 0.5 mg/L combination treatment group (Table 4, Fig. 1, Fig. 2)

Table 3. Effect of NAA on callus induction, shoot and root regeneration from shoot tip and young leaf of *Rhodiola sachalinensis* after 70 days in culture

Explants	Conc. of NAA(mg/L)	No. of explants cultured	No. of explants forming calli.	No. of explants forming shoots	No. of explants forming roots
Shoot tip	Control	30	8(26.6)[z]	4(13.3)	2(6.6)
	0.1	30	30(100)	6(20)	3(10.0)
	0.2	30	30(100)	7(23.3)	4(13.3)
	0.5	30	30(100)	10(33.3)	4(13.3)
	1.0	30	30(100)	8(26.6)	8(26.6)
	2.0	30	26(86.6)	5(16.6)	5(16.6)
	3.0	30	18(60)	5(16.6)	4(13.3)
Young leaf	Control	30	2(6.6)	0(0)	0(0)
	0.1	30	30(100)	5(16.6)	0(0)
	0.2	30	30(100)	8(26.6)	2(6.6)
	0.5	30	30(100)	6(20)	6(20.0)
	1.0	30	26(86.6)	5(16.6)	5(16.6)
	2.0	30	22(73.3)	5(16.6)	4(13.3)
	3.0	30	14(46.6)	3(10)	2(6.6)

[z]Numbers in parentheses indicate percentage to the number of explants cultured.

Table 4. Effect of NAA and BA on callus induction, shoot and root regeneration from shoot tip and young leaf of *Rhodiola sachalinensis* after 70 days in culture

Explants	Conc. (mg/L)		No. of explants cultured	No. of explants forming calli.	No. of explants forming shoots	No. of explants forming roots
	NAA	BA				
Shoot tip	Control		30	8(26.6)[z]	4(13.3)	2(6.6)
	0.5	0.1	30	16(53.3)	15(50)	8(26.6)
		0.5	30	19(63.3)	19(63.3)	10(33.3)
		1.0	30	13(43.3)	7(23.3)	2(6.6)
		2.0	30	6(20)	6(20)	0(0)
	1.0	0.1	30	22(73.3)	20(66.6)	18(60)
		0.5	30	12(40)	8(26.6)	8(26.6)
		1.0	30	8(26.6)	7(23.3)	6(20)
		2.0	30	4(13.3)	8(26.6)	0(0)
Young leaf	Control		30	2(6.6)	0(0)	0(0)
	0.5	0.1	30	14(46.6)	10(33.3)	4(13.3)
		0.5	30	13(43.3)	10(33.3)	5(16.6)
		1.0	30	10(33.3)	7(23.3)	2(6.6)
		2.0	30	3(10)	2(6.6)	0(0)
	1.0	0.1	30	14(46.6)	11(36.6)	9(30)
		0.5	30	8(26.6)	7(23.3)	5(16.6)
		1.0	30	8(26.6)	8(26.6)	3(10)
		2.0	30	5(16.6)	4(13.3)	0(0)

[z] Numbers in parentheses indicate percentage to the number of explants cultured.

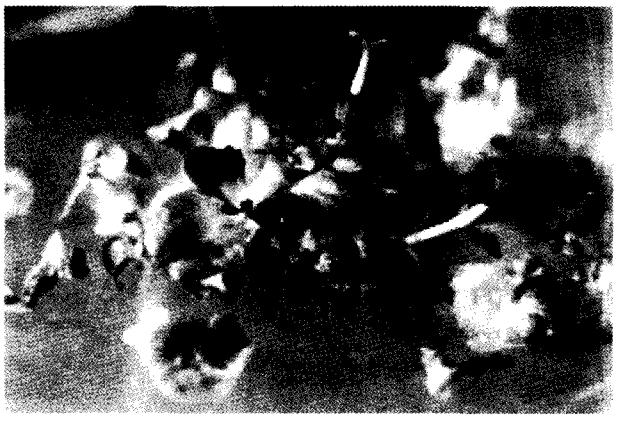

Fig. 1. Multiple shoots regeneration from callus on MS medium added with NAA 0.5mg/L and BA 0.5mg/L.

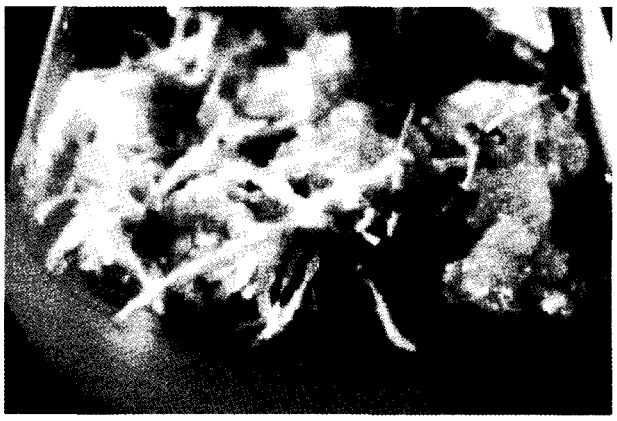

Fig. 2. Shoot and root regeneration from callus on MS medium added with NAA 1.0mg/L and BA 0.1mg/L.

showed 100% of callus induction, and 1.0 and 2.0mg/L one showed low callus induction.

NAA and BA 0.1 and 0.5mg/L combination treatment group showed relatively high percent of shoot regeneration rate, but the higher the consistency rate was, the lower the shoot regeneration rate was.

In the case of culturing of young leaf, NAA and BA 0.1 and 0.5mg/L combination treatment group showed 100% of callus induction, but as the treatment amount got higher, the induction rate got lower. NAA and BA 0.1 and 0.5mg/L combination treatment group the shoot regeneration rate was in the middle, but if the treatment consistency was higher, the rate was low. NAA 1.0mg/L with BA combination treatment showed lower callus induction rate and shoot regeneration rate than NAA 0.5mg/L treatment. The result was that NAA 0.5 and 1.0mg/L with BA 0.1 and 0.5mg/L showed the best callus induction rate, and the result was the same as shoot tip regeneration and young leaf.

That is, in the case of callus induction rate, NAA alone treatment showed good response, and NAA with BA combination treatment was good for shoot and root regeneration than NAA alone treatment.

When plants are cultured *in Vitro*, the kinds of growth regulator, the amount of their treatment, culture condition affect on callus induction, shoot and root regeneration.

There is a report that if NAA and BA are mixed, it's effective on callus induction, shoot and root regeneration. The report mentioned that all the treatment groups of sweet potato's leaves and stem culture with added NAA and BA were induced and callus growth was good. And shoot regeneration was increased, if they're cultured in primary culture (Carswell and Locy, 1984). This report showed similar result as this experiment except callus induction, and this could be contributed to the culture material difference.

Kayo *et al.* (1988) said that if NAA and BA is added in culturing the stem of *Cephaelis*, shoot tip grow fast. Yang *et al.* (1991) argued that NAA and BA low consistency treatment is effective for shoot regeneration in the hypocotyl tissue culture of *B. carinata*, and if the NAA consistency is over 0.01mg/L, the regeneration gets lower. Besides Bejoy and Hariharan (1992) claimed that in the the hypocotyl tissue culture of

Table 5. Effect of 2,4-D on callus induction, shoot and root regeneration from shoot tip and shoot tip of *Rhodiola sachalinensis* after 70 days in culture

Explants	Conc. of 2,4-D(mg/L)	No. of explants cultured	No. of explants forming calli.	No. of explants forming shoots	No. of explants forming roots
Shoot tip	Control	30	8(26.6)[z]	4(13.3)	2(6.6)
	0.1	30	30(100)	6(20)	6(20)
	0.2	30	26(86.6)	8(26.6)	7(23.3)
	0.5	30	26(86.6)	13(43.3)	10(33.3)
	1.0	30	20(66.6)	7(23.3)	7(23.3)
	2.0	30	23(76.6)	7(23.3)	4(13.3)
	3.0	30	16(53.3)	4(13.3)	2(6.6)
Young leaf	Control	30	2(6.6)	0(0)	0(0)
	0.1	30	30(100)	7(23.3)	4(13.3)
	0.2	30	30(100)	7(23.3)	5(16.6)
	0.5	30	24(80)	5(16.6)	5(16.6)
	1.0	30	21(70)	6(20)	6(20)
	2.0	30	18(60)	4(13.3)	3(10)
	3.0	30	18(60)	4(13.3)	2(6.6)

[z] Numbers in parentheses indicate percentage to the number of explants cultured.

Annona muricata the treatment group with mixed NAA and BA showed the best regeneration rate. But if the consistency of NAA and BA is higher, the rate gets lower, and low consistency good shoot regeneration rate.

The treatment group added with 2,4-D 0.1mg/L had the best callus induction rate, but shoot and root regeneration rate(Table 5) was low. 0.2 and 0.5mg/L group's callus induction rate was 86.6%, and shoot regeneration rate was 8 and 13%, which was better than the first one. In the case of 2,4-D 1.0, 2.0 and 3.0mg/L treatment group's callus induction rate were lower than 0.1, 0.2 and 0.5mg/L, and the shoot regeneration rate was lower than 0.5mg/L treatment group.

So I could conclude that the lower the consistency is, the better the callus induction rate is. And in 0.5mg/L treatment group the shoot regeneration rate was the highest, but in other groups it was lower.

In the case of culturing young leaf, the callus induction rate was very low with control treatment, and shoot wasn't regenerated. The 2, 4-D 0.1, 0,2mg/L treatment had the best callus induction rate, but shoot regeneration rate were low. Higher 2, 4- D consistency is not effective for callus induction and shoot regeneration.

These results show that low 2,4-D consistency is effective for callus induction, but not for shoot regeneration. Therefore, in the case of 2,4-D alone treatment, low 2,4-D consistency is effective for callus induction, but not for shoot and root regeneration.

To this 2,4-D treatment BA was added, and the result(Table 6) in shoot tip was that the callus induction rate was 76.6% and 66.6% in BA 0.1 and 0.5mg/L combination treatment groups, and 10.0 and 6.0% in BA1.0 and 2.0mg/L combination treatment groups.

The shoot regeneration rate was almost the same as the callus induction rate, but better than the control treatment.

In the case of young leaf, the callus induction rate of 2,4-D and BA 0.1, 0,5mg/L combination treatment groups were 73.3 and 80.0%, and the shoot regeneration rate were almost the same as the shoot tip culture.

So it could be concluded that in the case of 2,4-D alone treatment, the callus induction rate is very effective for callus induction, but 2,4-D and BA combination treatment is not so effective for callus induction, effective for shoot and root generation. Also shoot tip was better effective than young leaf for callus induction and shoot and root regeneration.

There were some reports that shoot and root regeneration response of 2,4-D with kinetin was good in *B. carinata* hypocotyl tissue culture, and shoot growth was better than the one of NAA with BA (Yang *et al.*, 1991). And regeneration of marigold was good on MS basal medium containing BA with IAA(Vanegas *et al.*, 2002).

In this experiment the shoot regeneration rate of 2,4-D with BA treatment was better than 2,4-D alone treatment group, but lower than BA with NAA treatment group. The callus induction of 2,4-D alone treatment was more effective than 2,4-D with BA treatment group. Shoot tip culture was better than young leaf culture for callus induction.

Han *et al.*(1997) reported that shoot growth was good in BA with IAA treatment group in *Delphinium* culturing, low consistent of auxin treatment was effective for shoot growth.

There were reports that auxin and cytokinin combination treatment is effective for shoot and root regeneration, but it depends on the kinds of BA with auxin and cultured plants. The report of Yang *et al.* was that for shoot regeneration BA treatment is effective, and for regeneration 2,4-D and kinetin combination treatment is effective. Another report said that BA with IBA treatment is effective for root regeneration (Hassanein and Mazen, 2001), but these reports show different results.

Table 6. Effect of 2,4-D and BA on callus induction, shoot and root regeneration from shoot tip and shoot tip of *Rhodiola sachalinensis* after 70 days in culture

Explants	Conc. (mg/L)		No. of explants cultured	No. of explants forming calli.	No. of explants forming shoots	No. of explants forming roots
	2,4-D	BA				
Shoot tip	Control		30	8(26.6)[z]	4(13.3)	2(6.6)
	0.1	0.1	30	13(43.3)	10(33.3)	7(23.3)
		0.5	30	14(46.6)	10(33.3)	4(13.3)
		1.0	30	10(33.3)	5(16.6)	2(6.6)
		2.0	30	7(23.3)	6(20)	0(0)
	0.5	0.1	30	8(26.6)	8(26.6)	7(23.3)
		0.5	30	10(33.3)	10(33.3)	3(10)
		1.0	30	6(20)	6(20)	4(13.3)
		2.0	30	5(16.6)	6(20)	0(0)
Young leaf	Control		30	2(6.6)	0(0)	0(0)
	0.1	0.1	30	11(36.6)	8(26.6)	4(13.3)
		0.5	30	11(36.6)	10(33.3)	3(10)
		1.0	30	9(30)	8(26.6)	3(10)
		2.0	30	5(16.6)	5(16.6)	1(3.3)
	0.5	0.1	30	10(33.3)	10(33.3)	8(26.6)
		0.5	30	7(23.3)	7(23.3)	4(13.3)
		1.0	30	7(23.3)	6(20)	4(13.3)
		2.0	30	4(13.3)	5(16.6)	0(0)

[z] Numbers in parentheses indicate percentage to the number of explants cultured.

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