Rapid Propagation of *Stephania cephalantha* HAYATA by Tissue Culture

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Young stem explants of *Stephania cephalantha* were cultured on MS agar media (MURASHIGE and SKOOG 1962) supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin). Effects of hormonal concentration on callus induction and shoot development were evident. The medium containing 0.2 mg/l 2,4-D and 0.2 mg/l Kin was suitable for callus growth and shoot development. Since there was a considerable variation in the number of developed shoots (0~11 per callus), callus selection was attempted. Selected high yielding calli produced about twice the number of shoots as compared to the low yielding and non-selected original calli. For rooting, two series of media were tested. In the early phase of root induction, root development (number of roots per shoot and maximum root length) was better in 1/8~1/16 strength MS agar media than in the other media. In the latter phase, hardening period, roots continued to develop and there were no differences in the root development except those grown in 1/32 MS and no nutrient agar medium. From these results, we concluded that 1/8~1/16 strength MS agar media were suitable for rooting, and full~1/4 strength MS agar media was also useful. When the plantlets were transferred to the soil, most of them survived. This culture method will make it possible to rapidly mass propagate *Stephania cephalantha*. Addition of auxin to rooting media was not so effective on root formation. There were no significant differences between male and female except for shoot development on callus inducing media.

KEY WORDS: *Stephania cephalantha*, tissue culture, callus selection, rapid propagation

Introduction

Stephania cephalantha HAYATA (Menispermaceae) is an important medicinal plant. Cepharanthine, a bisbenzylisoquinoline alkaloid commercially extracted from the tuberous roots of this species, is known to have antitumor activity (FUJIWARA *et al.* 1980). Unfortunately it takes several years to produce tuberous roots large enough for extraction materials. Several reports (AKASU *et al.* 1976, SUGIMOTO *et al.* 1988a, 1988b, SUGIMOTO and YAMADA 1989) indicated that calli and roots of this species cultured on media produced some bisbenzylisoquinoline alkaloids, mainly aromoline and berbamine, but not the full spectrum of alkaloids as found in the field grown tuberous roots. Cepharanthine and isotetrandrine, the main alkaloids of the tuberous roots, were not detected from callus tissue and root culture. Furthermore, because of the dioecism of this plant, seed propagation caused variation in the quality of the extracts. In their studies of alkaloids, AKASU *et al.* (1976) induced shoots and roots from a callus cultured on MS agar medium (MURASHIGE and SKOOG 1962) but no reports focusing on propagation of this species by tissue culture have been published. The purpose of this study was to develop a technique for rapid and convenient propagation of this species.

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Materials and Methods

Callus cultures of *Stephania cephalantha* were raised from young stem explants. Young stems (5~10 cm from the stem apex of 80~100 cm plants) were cut from one male and one female plant grown in the field. After removing the leaves, the young stems were initially cut into 3 cm segments, sterilized by soaking in 70% ethanol for 1 minute and 1% sodium hypochlorite solution for 10 minutes, and were finally rinsed three times with sterile distilled water. From these sterilized stems, explants measuring 5 mm in length were removed for culture. MS mineral salt medium with organic constituents at the following concentrations was used as the basal medium :30 g/l sucrose; 10 mg/l thiamine hydrochloride; 5 mg/l nicotinic acid; 10 mg/l pyridoxine hydrochloride and 100 mg/l myo-inositol. For callus induction, 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) were added to the basal medium in different concentrations and combinations as indicated in the results (Table 1). The medium was solidified with 8 g/l agar and adjusted to pH 5.7 before autoclaving. Culture tubes (25 mm × 100 mm) containing 10 ml medium were sealed with aluminium foil. Three tubes were used for each combination of 2,4-D and Kin for both male and female, respectively. Two explants were cultured in each tube.

The induced calli from the medium containing 0.2 mg/l 2,4-D and 0.2 mg/l Kin were sub-cultured using the same medium (20 ml/100 ml culture flask) every 3~4 weeks. Calli about 5 mm in diameter were transferred to new media. Since there was a considerable variation in the number of developed shoots, two directional selection was attempted. Five calli (about 15 mm in diameter) having many shoots (more than 6 shoots per callus) were selected from 30 calli in each sex. The portion of each callus having developed shoots was dissected out and cut into three parts. Five calli (about 15 mm in diameter) having one or no shoots per callus were selected from 30 calli in each sex. A portion of each callus having no shoots was cut into three parts. These selected calli (about 5 mm in diameter, 15 per sex in each direction) were cultured in the same medium as described above. This same selection procedure was carried out 4 times.

Shoots, 1.5~2.0 cm in length developed from selected calli, were excised and transferred to rooting media. Two series of rooting media were tested. One of which was full to 1/32 strength MS agar media (MS~1/32 MS) and agar with no MS nutrient elements (0). The other was 1/16 MS agar media supplemented with 3-indolebutyric acid (IBA, 0.02, 0.2 mg/l), 3-indoleacetic acid (IAA, 0.02, 0.2 mg/l) and 2,4-D (0.02, 0.2 mg/l). One shoot was placed in a tube containing 10 ml agar medium adjusted to pH 5.7 before autoclaving, and 10 tubes were used for each group. After 30 days of culture, the number of induced roots per shoot was counted and maximum root length was measured.

In all experiments, cultures were grown in a growth room at $27 \pm 1^{\circ}\text{C}$ under continuous illumination using white fluorescent lamps (2,500 lux.).

Both rooted and non-rooted shoots in the rooting media were transferred to pots filled with sterilized vermiculite and maintained under high humidity for the initial 14 days in the greenhouse. After a hardening period of 2 to 3 weeks in the greenhouse, the number of roots and maximum root length were measured. Plantlets were transplanted to the soil. After 40

days of cultivation in the soil, survival rate was determined. As a control, cuttage testing (developed shoots were directly transferred to the sterilized vermiculite) was carried out.

Results

Within 2 weeks, callus tissue developed at both ends of each explant in all concentrations and combinations of 2,4-D and Kin tested. Callus development on the apical end of the explant was delayed by 3~4 days as compared to the basal end. Concentration of 2,4-D and Kin played an important role in callus growth and shoot development (Table 1). After 3 weeks, callus growth on the media with 0.2~2.0 mg/l 2,4-D and 0.02~2.0 mg/l Kin was superior to the others. Induced calli on the media containing 2,4-D at relatively low concentrations (0~0.2 mg/l) were compact and whitish green to green in color. On the other hand, at relatively high concentrations of 2,4-D (2.0~4.0 mg/l) callus color was whitish brown and semitransparent. Shoot development from each callus was observed on the media with low 2,4-D and high Kin concentrations (0~0.2 mg/l 2,4-D and 0.2~4.0 Kin). However, on the 0.2 mg/l 2,4-D medium shoots developed from both male and female calli, on the 0~0.02 mg/l 2,4-D medium only the female calli produced shoots. After approximately one month of culturing, the calli produced shoots 2~3 cm in length with 2~3 leaves.

Callus growth on the subculturing medium was not different between male and female. In both sex, callus diameter became three times as compared to the transferred callus (about 5 mm in diameter) after 3~4 weeks subculturing. The number of developed shoots from original calli varied from 0 to 11. The average number of developed shoots per callus was 3.9 in male and 3.6 in female. The effect of callus selection on shoot yield is indicated clearly in Fig. 1. The average number of developed shoots per callus was 9.0 in male and 12.7

Table 1. Effect of 2,4-D and Kin concentrations on callus induction and shoot development after three weeks culture.

Kin (mg/l)	sex	2,4-D (mg/l)				
		0	0.02	0.2	2.0	4.0
0	♂	2.0±0.0 ¹⁾	4.0±0.0	3.8±0.5	4.0±0.0	3.3±0.5
	♀	2.0±1.0	3.3±0.5	3.5±0.5	4.2±0.4	4.3±0.5
0.02	♂	1.8±0.5	2.3±0.5	4.5±0.6	4.3±0.8	4.3±0.5
	♀	2.8±0.4	2.8±0.8	4.0±0.0	4.8±0.5	4.5±0.5
0.2	♂	2.0±0.0	2.8±0.8	4.8±0.4 (66.7)	4.2±0.4	3.3±0.5
	♀	2.3±0.5	2.7±0.5	4.3±0.5 (33.3)	5.0±0.0	4.0±0.6
2.0	♂	1.7±0.6	2.5±0.5	4.0±0.0 (50.0)	4.5±0.5	3.8±0.4
	♀	3.3±1.0 (75.0)	3.8±0.5 (75.0)	4.7±0.5 (50.0)	3.7±0.8	3.8±0.4
4.0	♂	1.3±0.6	2.5±0.7	3.3±0.5 (66.7)	3.3±0.5	3.6±0.5
	♀	2.0±0.8	2.4±0.5 (20.0)	3.3±0.5 (66.7)	3.8±0.5	3.5±0.7

Six explants were used in each combination of 2,4-D and Kin.

Degree of callus growth was scored on a scale of 1 (explant enlarged, but formed no callus) to 5 (callus rapidly grew, and covered explant).

¹⁾: Mean ± standard deviation of score.

(): Percentage of callus developed shoots, and blanks indicate 0%.

in female after the first selection for high yield. During the experiment the average number of shoots developed from high yielding selected calli was about two times as great as that from low yielding or original calli. There was a significant difference between high and low yielding calli at the 0.1% level in each step. But the average number of developed shoots gradually reduced. Callus growth was not different between both types of calli and growth rate was maintained during the experimental period. However at the beginning of this selection test 30 calli were used for each sex, finally selected calli were derived from one callus originally used except in one case. Low yielding selected calli in male were derived from two original calli.

Selected high and low yielding calli were shown in Fig. 2. There was no difference in the surface view of them. On the high yielding callus, axillary buds elongated from the nodes formed near the callus surface. Adventitious buds as shown in Fig. 3 were observed on the peripheral region of both low yielding and high yielding calli. Irregular vascular tissue also developed in both types of calli.

For rooting, two series of media were tested (Table 2, Table 3). After 12 days of culturing, roots were formed in 1/4~1/16 strength MS agar media without plant growth regulators. In the early phase of culture, 30 days of culture on agar medium, 65.0~77.5% of shoots rooted in 1/8 and 1/16 strength MS media. Root development (number of roots per shoot and maximum root length) was better in 1/8~1/16 strength MS media than in the other media. In the media containing relatively high concentrations of MS nutrient elements (full~1/4 MS), a small callus (2~4 mm in diameter) with red spots was formed at the base of each shoot within 2 weeks (Fig. 4). In the latter phase, hardening period, roots continued

to develop and there were no differences in the roots development except those grown in 1/32 MS and no nutrient agar medium (Fig. 5). When the plantlets

Fig. 1. Effect of callus selection on number of developed shoots.

◆: Mean $\pm$ standard error

Note: Since there was no significant difference between male and female the data were lumped together.

Fig. 2. Selected calli of low and high yield.

a: Low yielding callus with no developed shoots.

b: High yielding callus with many developed shoots.

were transferred to the soil, most of them survived (Table 2). After approximately 80 days of cultivation in the soil, young plants which had small tuberous roots (diameter 5~7 mm) were successfully established and transplanted to the field.

As shown in Table 3, addition of auxin to rooting media was not so effective on root formation, but promoted callus growth. Diameter of roots formed in the medium containing auxin was somewhat greater than that in the medium without auxin. Relatively high levels of auxin, especially 2,4-D, suppressed the root elongation in the latter phase. When the developed shoots were directly transferred to sterilized vermiculite, root development was not observed during the experimental period.

Discussion

AKASU *et al.* (1976) induced shoots from the callus using MS agar medium supplemented with 1 mg/l IAA and 1 mg/l Kin. In our preliminary experiment (data

Fig. 3. Inner view of the low yielding selected callus. Paraffin section, 10 μ m in thickness, stained by Delafield's haematoxylin. a: Adventitious bud formed in the peripheral region. b: Irregular vascular tissue.

Table 2. Effect of medium strength on root development and survival rate.

Medium ¹⁾	On the rooting medium				After hardening period			
	Number of shoots used	% of shoot rooted	No. of roots per shoot	Maximum root length (mm)	% of shoot rooted	No. of roots per shoot	Maximum root length (mm)	Survival rate ²⁾
MS	20	7.5	0.5	0.5	80.0	4.8	57.7	77.5
1/2 MS	20	52.5	2.5	1.7	85.0	5.9	53.0	82.5
1/4 MS	20	52.5	3.2	9.6	80.0	4.6	49.2	65.0
1/8 MS	20	77.5	4.3	13.2	90.0	6.0	55.3	82.5
1/16 MS	20	65.0	5.1	18.8	87.5	5.9	57.6	82.5
1/32 MS	20	42.5	2.9	8.6	70.0	4.4	44.5	60.0
0	20	30.0	2.3	8.1	42.5	3.3	30.9	17.5

Note: Since there was no significant difference between male and female, the data were lumped together. Ten shoots of each sex were used, and the experiment was repeated twice.

• : Significant at the 5% level. Transformed data ($\arcsin \sqrt{\%}$) were used for statistical analysis of percentage.

¹⁾ : Full ~ 1/32 strength MS agar media and agar medium with no MS nutrient elements (0)

²⁾ : (No. of finally survived plantlets / No. of shoots used) $\times$ 100.

Fig. 4. Root formation on the medium with no plant growth regulators after 30 days culture (early phase of root induction).

Full(1) ~ 1/32 strength MS agar media and agar medium with no MS nutrient elements(0).

Fig. 5. Developed roots during the hardening period (latter phase of root induction).

Numbers refer to respective media, full(1) ~ 1/32 MS and agar(0), of early root induction phase.

was not shown), combination of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) was also effective to develop shoots from the callus as shown by SUGIMOTO *et al.* (1988a). The shoots developed on the medium containing NAA and BA were thinner than those from the 2,4-D and Kin medium. These results suggested that callus induction and shoot development of this species does not depend upon a specific kind of auxin and cytokinin. The ability to develop shoots was different between male and female in three combinations of 2,4-D and Kin (relatively low concentrations of 2,4-D) (Table 1). In order to determine the

Table 3. Effect of auxins on root development and survival rate.

Auxin (mg/l)	Number of shoots used	On the rooting medium				After hardening period			
		% of shoot rooted	No. of roots per shoot	Maximum root length (mm)	% of shoot rooted	No. of roots per shoot	Maximum root length (mm)	Survival rate ²⁾	
H.F ¹⁾	20	70.0	5.3	23.7	80.0	7.0	57.2	70.0	
IBA { 0.02 0.2	20	90.0	3.4	11.8	90.0	6.1	46.4	70.0	
	20	90.0	4.5	13.9	95.0	6.6	44.6	85.0	
IAA { 0.02 0.2	20	65.0	4.8	9.7	75.0	6.7	36.1	50.0	
	20	25.0	1.8	13.6	60.0	4.8	33.8	35.0	
2,4-D { 0.02 0.2	20	85.0	3.9	12.6	85.0	6.5	43.4	50.0	
	20	65.0	1.5	1.8	10.0 ³⁾	4.0	10.0	0	

Note: Since there was no significant difference between male and female, the data were lumped together. Ten shoots of each sex were used, 1/16 strength MS was used as a basal medium.

• : Significant at the 5% level. Transformed data ($\arcsin \sqrt{\%}$) were used for statistical analysis of percentage.

¹⁾ : Hormone free. ²⁾ : (No. of finally survived plantlets / No. of shoots used) $\times$ 100.

³⁾ : Most of the shoots rooted on the rooting medium were dead during hardening period.

difference in shoot developing ability between male and female, further experiments are needed.

As shown in Fig. 1, callus selection was effective for shoot production. Selected high yielding calli produced about twice the number of shoots as compared to the low yielding and non-selected original calli. Low yielding calli developed no shoots also had adventitious buds formed in the peripheral region (Fig. 3). From these results, it is reasonable to consider that about one half of the newly induced shoots on the high yielding calli were developed from callus tissue and the rest were formed by the elongation of axillary buds. AKASU *et al.* (1976) also reported the ability to develop shoots was retained through successive subculturing over 3 years. In this experiment, the average number of developed shoots per callus was gradually reduced in both high and low yielding selections. Therefore it is fruitful to use newly induced calli for mass production of shoots.

Root growth was better in 1/8~1/16 strength MS agar media in the early phase of root induction, but no difference was observed in the latter phase except for 1/32 MS and no nutrient agar medium. It seemed that root primordia (red spots in the small callus formed at the base of each shoot) were formed in the early phase of root induction and grew into roots in the latter phase. According to SUGIMOTO *et al.* (1988b), reduction of ammonium sulfate, potassium nitrate and magnesium sulfate to half that of original B5 medium (GAMBORG *et al.* 1968) increased root growth. But elimination of ammonium sulfate was not so effective

on root growth. So, the concentration of potassium nitrate and magnesium sulfate seemed to have an important role for root elongation in the early phase of root induction. Furthermore, no roots developed from the shoots directly transplanted to sterilized vermiculite. From these results, we concluded that 1/8~1/16 strength MS agar media were suitable for rooting, and full~1/4 strength MS agar media were also useful. The former method is slightly better than the latter for the reason that phenomena of root induction and growth can be confirmed in the medium. This culture system will make it possible to rapidly mass propagate *Stephania cephalantha*.

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組織培養によるタマザキツツラフジ (*Stephania cephalantha* HAYATA) の増殖

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タマザキツツラフジ(ツツラフジ科)の塊根に含まれるCepharanthineは、抗腫瘍活性を有することが知られている(藤原ら 1980)。本種は雌雄異株植物のため、種子繁殖を行った場合変異の拡大が予想される。そこで、組織培養による大量増殖法について検討した。雌雄それぞれ1株より若い茎を採取し、滅菌後、長さ約5 mmの茎を置床した。カルス誘導とシュート分化には、MS培地(MURASHIGE and SKOOG 1962)に0, 0.02, 0.2, 2.0, 4.0 mg/lの2, 4-dichlorophenoxyacetic acid (2, 4-D)とkinetin (Kin)を添加した25種類の培地を用いた。培養条件は $27 \pm 1^\circ\text{C}$, 明条件(2,500 lux.)とした。

培養3週間後にほとんどの区でカルスが形成され(Table 1), 以後カルス増殖, シュート分化ともに良好でホルモン濃度の比較的低い0.2 mg/l 2, 4-D, 0.2 mg/l Kinで継代した。

継代中にカルス当りのシュート数が0~11とばらついたので、分化シュート数による選抜を試みた。4回の選抜を行ったところ、シュート数の多いカルスの部分を継代することによって、約2倍のシュートが得られることが明らかとなった(Fig. 1)。これは、カルスより新たに分化するシュートの他に、シュート基部の側芽が伸長するためと考えられる(Fig. 2, Fig. 3)。

発根については、濃度の異なるMS培地(MS~1/3 2 MSと寒天のみの培地)と1/16 MS培地にauxinを添加した培地について検討した。Auxin無添加のMS培地では、培養30日後に顕著な差異が認められ、1/8~1/16 MS培地で発根は最も良好であった。しかし、MS~1/4 MS培地に置床したものも馴化中にほとんど発根した(Table 2)。これは、発根培地中でシュート基部に形成された根の原基(シュート基部の小さなカルス中に赤色の点として確認できる)が、馴化中に伸長したためと考えられる。一方、発根培地へのauxinの添加は、あまり効果がなかった(Table 3)。シュート分化において雌雄間で差が見られたが、選抜によるシュート数の増加、発根、活着については、性による差異が認められなかった。