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Somatic Embryogenesis and Plant Regeneration from Stem Tissues of *Orostachys japonicus* A. Berger

Min Jung Song[1] and Young Goo Park[2*]

[1]*National Horticultural Research Institute, RDA, Suwon 440-706, Korea*

[2]*Dept. of Forestry, Kyungpook National University, Daegu 702-701, Korea*

ABSTRACT *Orostachys japonicus* A. Berger is a perennial herbaceous plant which has been traditionally used as an anti-inflammatory agent to treat hepatitis and as an anticancer agent. The objective of this study was 1) to establish and proliferate in vitro plant of *O. japonicus,* 2) to induce indirect somatic embryogenesis from *O. japonicus.* General calli and embryogenic calli in all ranges of 2,4-D and BA combination, were induced and were best at 22% (embryogenic cell) in 5.0 mg/L 2,4-D and 0.5 mg/L BA combination. Embryogenic cell line was maintained by subculture at 2 week intervals and transferred to solid and liquid medium for embryo formation. In solid medium culture, globular and heart shaped embryos were observed in MS medium containing 5.0 mg/L 2,4-D and 0.5 mg/L BA combination. The number of embryos was 6.5 per 0.5 g cell, and then the immature embryos transferred to MS basal medium for embryo development. In a suspension culture of embryogenic cells, globular and heart shaped embryos were emerged in MS medium supplemented with 3.0 mg/L 2,4-D and 0.3 mg/L BA combination after 10 days of incubation. The embryo formation rate was about 33% by suspension culture. The ratio of embryo germination was 60.9%, on the other side, the root formation rate was 74.3% in 1/2 MS continuously.

Introduction

Orostachys japonicus A. Berger (Crassulaceae) is a native plant from the orient including Korea, Japan and China. The leaf morphology of *Orostachys* is xerotype with spine and mesophyl chloroplast surrounded by a dense vascular bundle which has a large amount of water storable tissue (Shin et al. 1994). *Orostachys* has been traditionally used as an anti-inflammatory agent to treat hepatitis and also as a hemostatic agent for the treatment of vomiting blood, nose bleeding and blood excrement (Kim et al. 2003). This plant was used as an anti-cancer product against large intestine cancer so it has been used in Korean folk medicine as an anti-cancer agent (Choi et al. 1994). In addition, Moon et al. (1995) reported the anti-mutagenic effects of *O. japonicus* on the aflatoxin B1. The development of efficient plant tissue culture procedures for embryogenic culture induction, maintenance and plant regeneration in *Orostachys* is important for the application of different technologies for crop improvement.

Despite this importance, only a few studies have been reported on tissue culture and somatic embryogenesis of *O. japonicus*. In this study, we describe various combinations of plant growth regulator (PGR) for multiplication and optimization conditions for plant regeneration via indirect somatic embryogenesis from *O. japonicus*.

*Corresponding author Tel 053-950-5747 Fax 053-950-6708
E-mail: ygpark@knu.ac.kr

Materials and Methods

Culture Source

Orostachy japonicus A. Beger was obtained from the campus

of Kyungpook National University, Daegu city, Korea.

Induction of Multi-shoots

Field grown stems with axillary buds of plants were surface sterilized with 1% NaOCl (sodium hypochlorite) with several drops of tween 20 and vigorously shaken for 30 min, then rinsed several times with sterile distilled water. To induce micropropagation, in-vitro grown stem tissues including axillary bud were cultured in MS (Murashige and Skoog, 1962) medium supplemented with 0.1, 0.2, 0.3, 0.4, 1, 2, 3, 4 mg/L BA (6-benzyl amino purine) or 0.01, 0.1, 0.2, 0.3, 0.4 mg/L TDZ (tidiazuron). Tissues were grown under white fluorescent light (15umol $m^{-2}s^{-1}$) at a 16h day photoperiod and 25±1℃. The number of multishoots from one axillary bud were counted.

Somatic Embryogenesis

Embryogenic callus induction and proliferation

Excised stem tissues were placed in 100×15 mm petri dish containing 20 ml semisolid MS medium supplemented with 1.0 ~7.0 mg/L 2,4-D (2,4-dichlorophenoxy acetic acid) and 0.1~ 0.7 mg/L BA and with 3% sucrose and 0.15% gelite for callus induction.

After 4 weeks, the yellowish and friable calli or white and compact embryogenic calli which developed on the cutting plane of the explants were transferred to new medium. The two types of calli (embryogenic or non-embryogenic calli) were maintained on the MS medium supplemented with 5.0 mg/L 2,4-D and 0.5 mg/L BA in the dark at 25±1℃ and sub-cultured at 2 week intervals. Embryogenic calli were induced 2 months after callus induction so, embryogenic sectors from the initial callus were cut and separated from non-embryogenic parts. Embryogenic cell line was kept in same MS medium supplemented with 5.0 mg/L 2,4-D and 0.5 mg/L BA in solid condition.

Induction of somatic embryos through suspension culture

To obtain somatic embryos, cell suspension culture were executed in 100ml of liquid MS medium containing 10% (v/v) packed embryogenic cell and various concentration of plant growth regulators, such as 1~7 mg/L 2,4-D and 0.1~0.7 mg/L BA and with 3% sucrose in a 250 ml Erlenmeyer flask at 25± 1℃. Cell suspension cultures were grown in a dark condition on a gyratory shaker at 120 rpm. Cultures were filtered through 125 μM stainless steel sieves to separate individual cells and small cell clumps. Cells from the suspension were observed with a microscope during the culture period, and the growth rate of cells was evaluated for the first 7 days by determining the packed cell volume (PCV) of samples from 3 replicates. Primary somatic embryos were formed after 2 weeks and subsequently used to initiate secondary somatic embryos.

Induction of somatic embryos through solid medium culture

Embryogenic calli were subcultured in MS medium supplemented with 1.0~7.0 mg/L 2,4-D and 0.15% gelite for 2 weeks and then transferred to auxin free MS basal solid medium. Globular or heart shaped somatic embryos were formed in the 2,4-D supplemented solid medium.

Formation of Secondary Embryos

For induction of secondary somatic embryogenesis, well-developed single somatic embryos were placed into 250 mL Erlenmeyer flasks containing 50 mL of liquid MS medium supplemented with various plant growth regulators such as 0~ 3.0 mg/L zeatin or 2.0 mg/L abscisic acid (ABA) or 5.0 mg/L 2,4-D and 0.5 mg/L BA. The cultures were kept on rotary shaker at 120rpm in the dim light, with subculturing every 2 weeks.

Secondary somatic embryos (SSE) were directly induced from single somatic embryos and then the number of SSE counted separately. The efficiency of secondary somatic embryogenesis was determined after 3 weeks of culture. After 1 month of culture SSE were transferred onto solid germination medium for plant recovery.

Germination of the Somatic Embryos

Immature somatic embryos were transferred and cultured for 2 to 3 weeks on MS basal medium at 25±1℃ under dim light.

Some of the immature somatic embryos were transferred to MS solid medium supplemented with different combinations of 0.5～2.0 mg/L BA or 0.5～2.0 mg/L 2ip (iso-pentenyl amino purine) or 0.1～1.0 mg/L zeatin, and the others were kept continuously in MS basal medium.

RAPD Analysis

Genomic DNA was isolated from germinated shoot leaves through somatic embryos of *Orostachys japonicus* by CTAB (1% CTAB, 50 mM Tris-HCl, pH 8.0, 70 mM NaCl, 10 mM EDTA) Murray et al. method (1980). Twenty decamer oligonucleotide primers (OPC1～20, Operon technologies inc., Alameda CA. USA.) were used for screening somatic variations. Amplification was conducted in 25 $\mu\ell$ reaction mixture containing 10 ul reaction buffer, 10 mM dNTPs, 10 pmole random primer, 25 mM $MgCl_2$, 1 unit Taq DNA polymerase (Promega), and 20 ng template DNA. Reaction was performed for 1 cycle of 94℃ for 3min, followed by 35 cycles of 92℃ for 1 min, 36℃ for 1 min, 72℃ for 1 min 30 sec, and finally incubated at 72℃ for 5 min.

Statistical Analysis

Analyses of variance and mean separations were carried out using Duncan's Multiple Range Test (DMRT).

Results

Induction of Multiplication

The multishoot development was best in the MS medium containing 3mg/L BA, and the number of shoots from one axillary bud was 21 (Figure 1). The shoots were formed 3 weeks after node culture, and the shoots were transferred to MS basal medium in a mass or separately. After 3 months, the mass of multishoots was numerously propagated.

Somatic Embryogenesis

Induction and proliferation of embryogenic callus

Callus proliferation from stem tissues could be observed after 3 weeks of incubation but the embryogenic callus was initiated 2 months later. Callus was initiated from the stem cutting end, but only in a few cases did non-wounded surfaces responded. Non-embryogenic calli were induced in most media containing both auxin and cytokinin. In stem tissue cultures, the highest value of callus induction was 4.83 g (mean weight of 5 stems) at 5 mg/L 2,4-D and 0.5 mg/L BA combination. And also, the highest percentage of embryogenic callus formation was 22±5.88 % in 5 mg/L 2,4-D plus 0.5 mg/L BA (Table 1). At a higher concentration of PGR than 5mg/L 2,4-D and 0.5 mg/L BA combination, the callus induction rate was decreased. The use of 1 mg/L 2,4-D, 0.1 mg/L BA combination gave rise to lower percentages of non-embryogenic and embryogenic callus induction. Frequencies of callus proliferation from stem segments scored after 4 weeks. The embryogenic calli were obtained from the range of 3.0 mg/L 2,4-D and 0.3 mg/L BA combination to 5.0 mg/L 2,4-D and 0.5 mg/L BA combination, but the other concentration of auxin plus cytokinin had no response (Table 1). Embryogenic calli were induced 2 months later after callus induction. Then, embryogenic sectors were cut and separated from non-embryogenic parts. Embryogenic cell line was maintained in the same MS with 5.0 mg/L 2,4-D and 0.5 mg/L BA solid medium and some of them were also maintained by suspension culture.

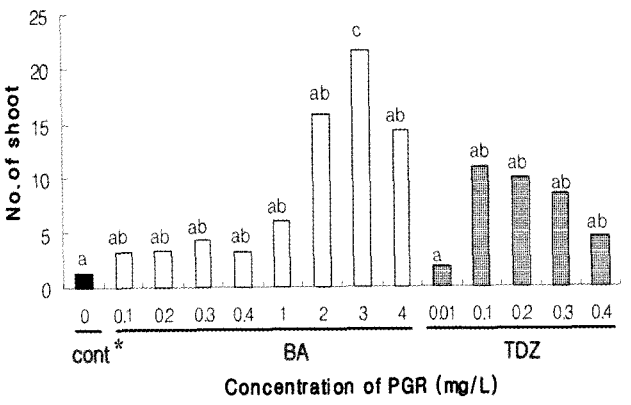

Figure 1. Shoot multiplication from stem segment including one axillary bud from in vitro-grown *O. japonicus*. The number of multi-shoots from one axillary bud was counted. The values followed by the same letter are not significantly different at p=0.05 according to the Duncan test. *cont: control

Table 1. Effects of plant growth regulator concentration on embryogenic callus induction from the stem tissues of *O. japonicus*

PGR* (2,4-D/BA mg/L)	Fresh callus weight (g)	Embryogenic callus (%)
1.0 / 0.1	1.67±0.26 c	NR**
2.0 / 0.2	2.52±0.38 bc	NR
3.0 / 0.3	3.43±0.38 bc	6.70±3.85 a
4.0 / 0.4	4.42±0.56 b	4.40±1.89 a
5.0 / 0.5	4.83±0.31 a	22.0±5.88 b
6.0 / 0.6	3.20±0.50 bc	NR
7.0 / 0.7	2.40±0.51 c	NR

The values followed by the same letter are not significantly different at p=0.05 according to the Duncan test. *PGR: plant growthregulator **NR: no respons

Somatic embryo induction through solid medium culture

Embryogenic calli were transferred to MS medium supplemented with various concentration and combination of 2,4-D and BA. About 0.6 mm diameter embryogenic calli were placed onto solid medium, and the calli were grown to double their size within 2 weeks. Active division and growth of cells were observed in 3.0 mg/L 2,4-D and 0.3 mg/L BA combination until the 5th day of culture. After 7 days, the cells showed two morphologically distinct cells namely, spherical and elongated cells. The pro-embryo divided further in multiple planes which resulted in the formation of globular stage embryos within one week. The embryos in globular stage differentiated into heart-shaped embryos within 10 days, which later developed into torpedo-shaped embryos. The transparent pre-globular stage of embryos and globular, heart-shaped embryos were formed well in combination of 5.0 mg/L 2,4-D and 0.5 mg/L BA (Fig. 2). The immature embryos were developed into cotyledonary stage after being transferred to MS basal medium. Embryos continuously cultured in MS or 1/2 MS medium without plant growth regulators and germinated into normal plants but the rates was low (Figure 2).

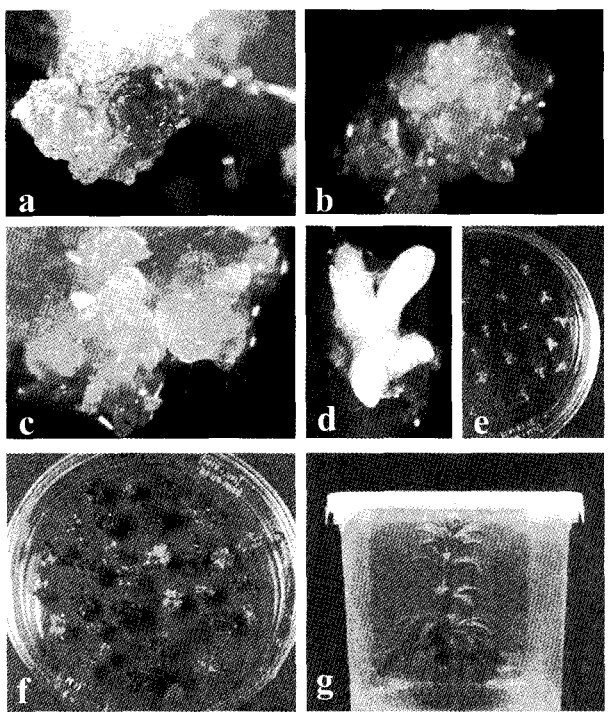

Figure 2. Formation and germination of somatic embryos a: pre-globular stage, b: globular stage, c: heart shaped embryos, d-e: embryo germination after 2 weeks under dark condition, f: normal shoot formation after 2 months under light condition, g: 10cm height plant after 5 months

Induction of somatic embryo by suspension culture

Primary somatic embryos were formed after 2 weeks from embryogenic callus and subsequently used to initiate secondary somatic embryos. In suspension culture, somatic embryos from embryogenic cell clumps were developed in lower concentrations of 2,4-D and BA combination compared with solid medium culture. 3.0 mg/L 2,4-D and 0.3 mg/L BA combination was the best for embryogenesis in suspension culture at 33.2% but 5.0 mg/L 2,4-D and 0.5 mg/L BA combination was the best in

Table 2. Effects of plant growth regulators on the somatic embryo induction by culture in solid and liquid medium

PGR (2,4-D/BA) (mg/L)	Solid medium	Liquid medium		
	No. of SE* per 0.5 g callus	PCV** after 1 week	PSEV***	Percentage of somatic embryo (%)
1.0 / 0.1	NR***	9.66±0.33 ab	1.33±0.17 bc	13.8 c
2.0 / 0.2	1.1 a	19.00±0.58 d	4.16±0.44 d	21.9 de
3.0 / 0.3	0.9 a	30.60±0.88 g	10.16±0.44 f	33.2 g
4.0 / 0.4	3.0 b	28.30±0.88 f	6.83±0.60 e	24.1 f
5.0 / 0.5	6.5 c	21.30±0.88 e	4.17±0.60 d	19.6 de
6.0 / 0.6	1.0 a	12.00±0.58 c	0.67±0.17 ab	5.6 b
7.0 / 0.7	NR	9.33±0.33 ab	0.16±0.17 ab	1.7 a

The values followed by the same letter are not significantly different at p=0.05 according to the Duncan test. *SE: somatic embryo, **PCV: packed cell volume (mL), ***PSEV: packed somatic embryo volume (mL), ****NR: no response

solid medium culture at the number of 6.5 per 0.5 g callus (Table 2).

To obtain large amount of somatic embryos, we used standard form of balloon type bubble bioreactor after small volume of flask suspension culture. Embryogenic cell clumps were inoculated MS supplemented with 3.0 mg/L 2,4-D and 0.3 mg/L BA combination, 100mg/L myo-inositol and 3% sucrose. After 10 days, the cell clumps were developed to somatic embryos, and the cell volume was increased as double (Figure 3).

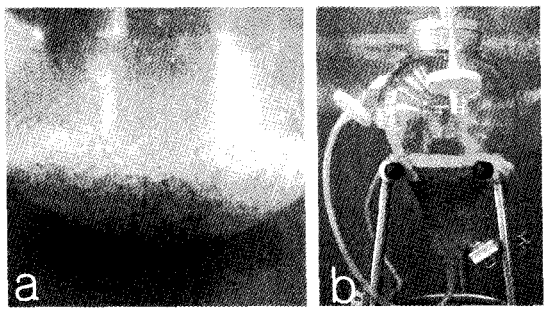

Figure 3. Somatic embryogenesis by cell suspension culture of *Orostachys japonicus*. a: Somatic embryos were formed in MS liquid medium with 2,4-D and BA combination. b: Mass propagation of somatic embryos by standard form of balloon type bubble bioreactor

Induction of secondary somatic embryos

The primary somatic embryos were transferred onto MS medium without growth regulators. A few primary embryos germinated and rooted in the MS basal medium but embryos showed no further secondary growth.

The secondary embryo formation was best at MS medium containing zeatin 3.0 mg/L and the frequency was at 52.3% (Table 3). The lowest secondary embryo development was obtained in MS basal medium at 7.2%, and with treatment of 2,4-D or ABA, there was no response at all (Table 3). Embryogenic cell proliferation showed in 5.0 mg/L 2,4-D and 0.5 mg/L BA combination, and cell browning occurred in MS medium containing ABA.

Table 3. Effect of plant growth regulators on initiation of secondary somatic embryos from primary embryos in MS medium

Rates of secondary embryo induction	
PGR (mg/L)	No. of SSE* / No. of primary SE**×100 (%)
0	7.17 ± 1.45 a
2,4-D 5.0 + BA 0.5	NR***
ABA 2.0	NR
Zeatin 0.5	30.17 ± 1.69 bc
Zeatin 1.0	29.40 ± 2.21 bc
Zeatin 2.0	48.03 ± 1.55 d
Zeatin 3.0	52.33 ± 5.21 e

The values followed by the same letter are not significantly different at p=0.05, according to the Duncan test. *SSE: secondary somatic embryo, **SE: somatic embryo, ***NR: no response

Germination of the Somatic Embryos

The globular-shaped somatic embryos obtained indirectly from stem tissues were cultured on MS medium without plant growth substances, and matured within 2 to 3 weeks. After embryo maturation, the embryos were transferred to 1/2MS, MS basal medium and MS supplemented with BA, 2ip, and zeatin medium, and they gave rise to plantlets within 3 weeks. Abnormal structures (for instance vitrified shoots) were found at a very low frequency (Table 4). In this experiment, we obtained the best germination of somatic embryos by subsequent subcultures of the somatic embryos. The second step of treatment, incubation of MS containing 0.5 mg/L BA after MS basal

Table 4. Effect of plant growth regulators on the germination of somatic embryos and formation of roots in germinated plants

PGR (mg/L)	No. of germinated SOE / No. of SOE (%)	Rates of root formation
1/2MS	33.17±3.66	74.33±4.70 k
MS	39.50±2.18	61.33±1.86 j
MS+BA 0.5	60.90±1.12	35.67±2.33 fgh
MS+BA 1.0	14.40±3.02	30.33±1.45 ced
MS+BA 2.0	20.57±2.46	31.00±2.08 ced
MS+2ip 0.5	50.50±6.14	35.00±3.21 fgh
MS+2ip 1.0	25.33±2.03	36.33±2.33 fgh
MS+2ip 2.0	16.43±2.09	44.00±3.06 i
MS+zea* 0.1	31.17±2.54	31.00±2.52 cde
MS+zea 0.5	22.73±1.97	24.00±3.21 ab
MS+zea 1.0	6.10±1.71	25.00±5.00 ab

The values followed by the same letter are not significantly different at p=0.05, according to the Duncan test. *zea: zeatin

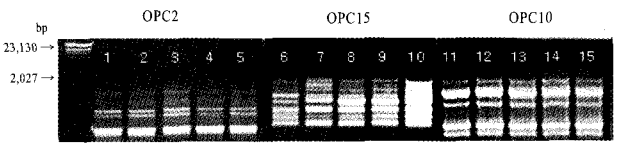

Figure 4. The band pattern of RAPD products with the primer OPC2, OPC15 and OPC10. lane 1: λDNA/HindIII marker; no. 1, 6, 11: field grown plant, no; 2, 7, 12: in vitro grown plant; no. 3, 4, 5, 8, 9, 10, 13, 14, 15: plant derived from somatic embryos

medium treatment, induced 60.9% germination from somatic embryos (Table 4). After 2 months of continuous culture on each two-step medium, plantlets rooted the best at 74.3%.

RAPD Analysis

Any genetic variation was not found between plants derived from somatic embryos in field and in vitro growth via RAPD analysis (Fig. 4).

Discussion

Choi et al. (1994) reported that the callus was not induced from leaf tissues of *O. japonicus*, but in this study, we obtained a little calli from leaf tissues through 2,4-D and BA combination. The white and friable non-embryogenic calli and yellowish and compact embryogenic calli with pre-globular stage were developed on the cutting planes of the stem tissues. The embryogenic callus and non-embryogenic callus formation was significantly affected by plant growth regulator concentration. In this experiment, we didn't obtain any results in the treatment of 1.0 mg/L of 2,4-D and 0.1 mg/L of BA combination and 7.0 mg/L 2,4-D and 0.7 mg/L BA combination. In somatic embryo induction by solid medium culture, we found something interesting compared with suspension culture. The optimum medium for somatic embryo formation was MS solid medium with 5.0 mg/L 2,4-D and 0.5 mg/L BA combination, but the low concentration of PGR treatment at 3.0 mg/L 2,4-D and 0.3 mg/L BA was the best in liquid medium culture. This auxin requirement was similar to that of mono cotyledonous plants, such as Sorhum bicolor L. (Wernicke and Brettell, 1980) and Allium fistulosum L. (Kim and Soh 1996). Embryogenic callus derived from the stem of *O. japonicus* has been described as proembryogenic masses (Button et al., 1974) rather than non-differentiated cell masses (Gavish et al., 1991). Embryo developmental arrest and subsequent swelling and callusing in suspension, as described by Chee & Cantliffe (1989), was circumvented by improving the nutritional environment allowing the formation of mature torpedo and cotyledonary embryos. Somatic embryos developed normally up to the globular stage, but subsequent development was suppressed and they eventually became callus. In plant growth regulator free medium, globular somatic embryos formed at a very low rate. Embryos at different stages of development were separated and subcultured in MS liquid medium with different 2,4-D concentrations. After one week of subculture, globular, heart shaped embryos were formed. Secondary embryos were obtained from liquid MS basal medium and MS medium supplemented with zeatin. Similarly, in cultivated sunflowers, growth regulators are required for the induction of secondary somatic embryogenesis (Finer 1987, Pelissier et al. 1990). As in most plant species, the production of embryos by secondary embryogenesis was higher than in primary embryogenesis (Raemakers et al. 1995). The embryos are difficult to develop. Therefore, successful plant regeneration can be claimed only if normal plantlets are ultimately achieved (Visessuwan et al. 1997). After embryo maturation, the embryos gave rise to plantlets within 3 weeks. Abnormal structures (for instance, vitrified shoots) were found at a very low frequency. It is note worthy that several plantlets developed somatic embryos from axillary or apical buds. Michaux-Ferriere et al. (1992) have reported that meristems or meristemic cell clusters can give rise to somatic embryos of multicellular origin. In many reports on somatic embryogenesis, the induction medium yields a mixture of embryos in different stages of development, but these embryos need to be subcultured on a different medium in order to germinate. This has been reported by the *Myriciaria* (Litz 1984) and Eucalyptus (Muralidharan et al. 1989). The lack of phenotypic variability between field grown and regenerated plants in our experiment allowed us to consider these methods for repetitive and secondary embryogenesis in *O. japonicus* as efficient tools for the regeneration and propagation of plants of this species with high embyogenic potential.

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