ISSN : 0974 - 7435 Volume 6 Issue 10

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 6(10), 2012 [317-326]

Plant regeneration through callus-derived protoplasts of *Phellodendron amurense* Rupr.

Mustafa Abul Kalam Azad

Department of Botany, University of Rajshahi, Rajshahi 6205 (BANGLADESH)

E-mail: drmakazad@gmail.com

ABSTRACT

A method for the isolation, culture of the callus-derived protoplasts, and plantlet regeneration in *Phellodendron amurense* is described. In this study, the highest 92.0% viability with the yield of 2.4×10^5 protoplast / g fresh weight was obtained from callus, when the materials were treated for 6 hrs with Cellulase Onozuka R-10 plus Driselase. Sustained cell division and colony formation from the protoplasts were best supported by a 0.2% Gellan gum solidified or liquid medium containing 2.0 μM BAP with 4.0 μM NAA, IBA or 2,4-D, where the plating density was 4×10^5 to 6×10^5 protoplasts/ml. The highest frequency (0.33 ± 0.02%) of colony formation was observed, when the protoplasts were cultured on MS medium supplemented with 2.0 μM BAP and 4.0 μM NAA. The protoplast-derived colonies formed green compact calli on 0.2% Gellan gum solidified MS medium containing 2.0 μM BAP with 4.0 μM NAA or IBA. Adventitious shoots from protoplast-derived callus were induced on MS medium supplemented with 2.0 μM BAP and 1.0 μM NAA or 2.5 μM IBA. Shoot multiplication and elongation occurred on MS medium containing 1.0 μM BAP. The highest 90.0% of *in vitro* grown shoots were rooted on MS medium containing 2.0 μM IBA. Plantlets were transferred to the Kanuma soil and successfully established under greenhouse condition.

KEYWORDS

Protoplast culture;
Plating density;
Plant growth regulator;
Enzyme;
Callus.

INTRODUCTION

Plant protoplasts have been proved to be an excellent tool for *in vitro* manipulation, somatic hybridization, genetic transformation, and for the induction of somaclonal variation. These studies reflected the far-reaching impact of protoplast alternations on agriculture and forest biotechnology. However, the isolation and culture of protoplasts in tree species are very difficult, and there are only a limited number of reports on the successful culture of protoplasts. The isolation efficiency and viability of protoplasts in woody plants are fully depended on the initial plant materials, from which tree protoplasts could be prepared successfully. Plant biotechnology is an important tool for cultivar improvement in conventional breeding,

Phellodendron amurense belongs to family Rutaceae is the best known and almost widely growing

FULL PAPER

in Far East region. The extracts from bark tissues of this tree are used as a crude drug in Japan and in China as an anti-stomachic, for intestinal function control, and as an anti-inflammatory and anti-pyretic agent, cholagogue, and antibacterial medicine[1]. The bark extracts mainly contain berberine derivatives as active constituents.

Most works on *P. amurense* have concentrated on biochemical studies. There is only limited research works in the tissue culture of *P. amurense*[12,15,17,18,20,]. So far, there is no report on shoot regeneration from protoplast culture. In this paper a novel plant regeneration system from protoplasts isolated from stem segment-derived callus is described.

MATERIALS AND METHODS

Plant materials

Plant materials for starting *in vitro* culture were seedlings. Fruits of *Phellodendron amurense* Rupr. were collected from a 50 years old tree growing at the Medicinal Plant Garden of Kumamoto University, Japan. After removing the flesh from fruits, seeds were recovered and surface-sterilized. The sterilized seeds were germinated on 10 ml of MS medium[27] in culture tube without any growth regulators. The cultured seeds germinated within 3 weeks, and gave rise to shoots which developed two to three nodes five to six weeks later. Plants were then propagated by subculturing single-node cuttings at 4-week intervals on MS medium with 2.0 μM BAP, and 2-4 cm long microcuttings were cultured on MS medium supplemented with 2.0 μM IBA. The stem segments were taken from 4-5 week-old *in vitro* grown shoots and used as the sources of protoplasts.

Callus induction

The stem segments (1-1.5 cm long) were incubated on MS medium containing different concentrations (viz. 1.0, 2.0, 4.0, and 6.0 μM) of NAA, IBA or 2,4-D in combination with either 1.0, 2.0, and 4.0 μM BAP, TDZ, or CPPU for testing their effects on callus induction. The media were solidified with 0.2% Gellan gum, and the pH adjusted to 5.7. The explants were cultured on the surface of medium in glass Petri dishes. Each Petri dish contained 10 explants and sealed with Parafilm M (American National Can., USA). All cultures were transferred to the new medium containing the same composition every 4 weeks for callus proliferation. The experiments were repeated 4 times.

Protoplast isolation from callus

Friable calli (about 0.2 g fresh weight) induced from stem segments were cut into small pieces with a surgical blade, and used as materials for protoplast isolation. Twenty-four enzyme combinations were examined with the duration of 24 hrs to select the best enzyme combination. The following enzymes were used: Cellulase Onozuka RS (Yakult, Tokyo, Japan), Cellulase Onozuka R-10 (Yakult, Tokyo, Japan), Driselase (Kyowa Hakko Kogyo, Tokyo, Japan), Hemicellulase (Sigma, St. Louis, Missouri, USA), Macerozyme R-10 (Yakult, Tokyo, Japan), and Pectolyase Y-23 (Seishin Pharm. Co., Tokyo, Japan). All enzymes were dissolved in 0.6 M Mannitol solution and used at 1.0% concentration, except for Pectolyase Y-23 at 0.5%.

Purification, yield, and viability of protoplasts

About 0.2 g fresh weight of the friable calli were incubated with 4 ml of enzyme solution in 30 ml sample bottle for different duration (6, 8, 10, 12, and 18 hrs) to find out the optimum treatment duration for protoplast isolation. After the treatment of different duration at room temperature, debris was filtered off with a 40 μm nylon mesh, and the protoplasts were collected by centrifugation at 100 × *g* for 3 min, followed by 3 times washing with 0.6 M mannitol solution. Protoplast yield was measured with a haemocytometer slide (Nitrin, Tokyo, Japan) under an inverted microscope (OLYMPUS IX70-S8F) and calculated by the following equation:

Protoplast yield (protoplasts / ml) = 5 × number of protoplasts × 10^4

To find out the effects of the osmotic potential of enzyme solution on the yield and viability of protoplasts, different concentrations (viz. 0.4, 0.6, and 0.8 M) of mannitol were also tested. After measuring the protoplast yield, protoplasts were stained with 0.01% (w/v) fluorescein diacetate (FDA)[22] in 0.6 M mannitol for 3 min to examine their viability. The protoplasts were observed under a fluorescence microscope with the excitation of blue light (WBV colour combination) us-

ing the excitation filter of Olympus model BA 475. Protoplast viability was calculated as the percentage of the number of viable protoplasts to the number of total protoplasts.

Protoplast culture medium and plating efficiency

Protoplasts obtained from stem segment-derived callus were cultured in liquid or on solid MS, MMS_1 (½ strength of MS Medium) and WPM (Woody Plant Medium) medium solidified with 0.2% Gellan gum (w / v) containing different concentrations (viz. 1.0, 2.0, and 4.0 μM) of BAP or TDZ in combination with NAA, IBA or 2,4-D (2.0 and 4.0 μM), 2.0% sucrose (w / v), and 0.6 M mannitol. Protoplast culture media were adjusted to pH 5.7.

In all the experiments, cell density was adjusted to 2×10^5, 4×10^5, and 6×10^5 protoplasts / ml by adding 2-5 μl of the concentrated protoplast suspension to 50 μl of the medium in a 96-well plate (FALCON, Becton Dickinson and Co., USA). About 10 μl of the sterilized distilled water was dispensed in between the space of every 4 wells to maintain the humidity, and the plate was tightly sealed with Parafilm M. Protoplast suspension (1.5-2.0 ml) was also cultured on the surface of MS solid medium in a Petri dish, where the protoplasts density was 4×10^5 protoplast / ml. Protoplasts were observed at 24 hrs intervals under an inverted microscope during 3 months of culture, by this time the protoplast formed cell colonies. After 2 months of culture, the number of colonies larger than 200 μm in diameter in each well was counted twice. Frequency of colony formation was calculated as follows:

$$\text{Frequency of colony formation (\%)} = \frac{\text{Number of colonies}}{\text{Number of originally plated protoplasts}} \times 100$$

Proliferation of protoplast-derived callus

After 2 months of culture, the microcalli (about 2-3 mm diameter) obtained from stem segment-derived protoplasts were used for callus proliferation. These microcalli were transferred to Petri dishes or 200 ml flasks containing MS solid medium. The medium contained 1.0, 2.0, or 4.0 μM BAP or TDZ in combination with 2.0 or 4.0 μM NAA, IBA, or 2,4-D, and was fortified with 3.0% sucrose (w / v) and 0.2% Gellan gum (w / v) to promote callus proliferation. Microcalli were subcultured every 4 weeks.

Plant regeneration from microcalli

Microcalli were transferred to a sequence of regeneration media supplemented with various cytokinins and auxins for plant regeneration. The calli were transferred to 200 ml conical flask containing 50 ml of MS medium supplemented with different concentrations (viz. 0.5, 1.0, 2.0, or 4.0 μM) of BAP or CPPU in combination with of NAA or IBA (0.5, 1.0, 2.0, or 4.0 μM) for shoot regeneration. For the elongation of shoots, they were transferred to MS medium containing different concentrations (viz. 0.5, 1.0, 1.5, or 2.0 μM) of BAP.

Culture condition

All cultures were maintained in the controlled environment with 25 ± 1°C and16 hrs illumination per day at 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided with cool white fluorescent tubular bulb. Humidity of the growth chamber was not controlled for any experiment.

Rooting of *in vitro* regenerated shoots and *ex vitro* establishment

The elongated shoots were cut into 2-4 cm long and cultured on MS basal medium fortified with either 0.5, 1.0, 2.0, or 4.0 μM IBA, NAA, or IAA for adventitious rooting. Data were recorded on percentage of rooting and the number and length of roots after 4 weeks of transfer onto the rooting media. Rooted plantlets were removed from the culture media after one month and the roots were washed in tap water to remove all traces of Gellan gum. Plantlets were then transferred to plastic pots containing Kanuma soil (Kanuma Bosai Potting Medium Co., Japan) and covered with a small transparent plastic tent (Height 12" × Wide 12") to ensure high humidity during the acclimatization period of 20 days. They were maintained under culture room conditions. The potted plants were irrigated with ¼ MS basal salt solutions devoid of sucrose and myo-inositol every 4 days for 3 weeks. The plastic tents were then removed after three weeks in order to acclimatize plants to laboratory room condition. Acclimatized plants were then transferred to larger pots and maintained in greenhouse.

FULL PAPER

Data analysis

Three replicates were used for the experiments of colony formation. The effects of different media and source of protoplasts were quantified, and the data were analyzed by analysis of variance (ANOVA). Tukey's multiple comparison was used to distinguish differences among treatments.

RESULTS

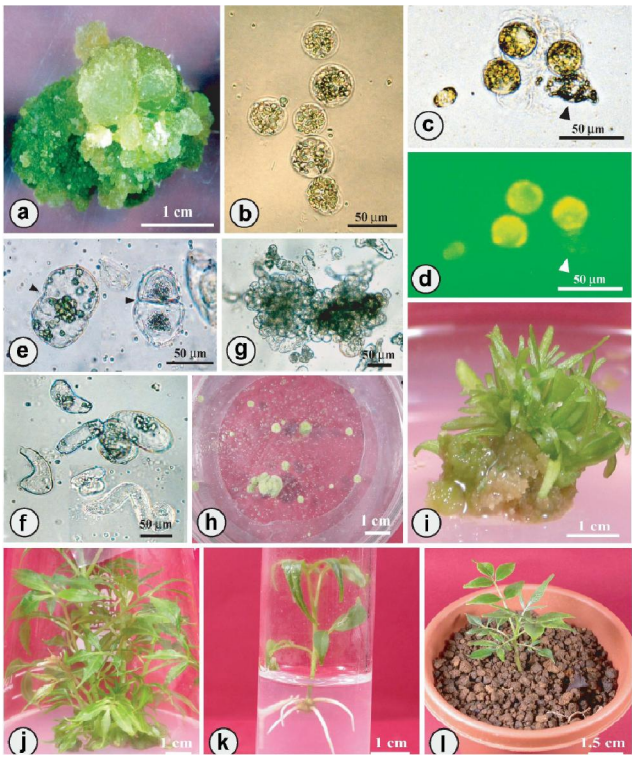
Induction of callus from stem segment explants

Different levels of auxin and cytokinin combinations showed a great variation of the morphology and amount of formed callus. Within 3 weeks of incubation, callus was proliferated from cut margin in different media.

The media containing 2.0 μM BAP, or TDZ in combination with 4.0 M NAA, IBA or 2, 4-D was more effective for callus induction. This combination produced maximum frequency (80.0%) of callus formation (Figure 1a). Callus growth in these media was fairly active, and the callus continued to grow for more than 3 months without browning. In this experiment, it was observed that low (1.0 μM) and high (6.0 μM) concentrations of all auxins in combination with cytokinin gave the lowest frequency (20.0%) of callus formation, and these calli showed browning within 6 weeks. In the present study, it was revealed that 2.0 μM BAP + 4.0 μM NAA, 2.0 μM BAP + 4.0 μM IBA, 2.0 μM TDZ + 4.0 μM NAA, and 2.0 μM TDZ + 4.0 μM 2,4-D were suitable formulation for callus induction in *P. amurense*.

Isolation of protoplasts from callus

In general, selection of digesting enzymes is very important to obtain a high viability and yield of protoplasts. Therefore, 24 enzyme combinations were tested preliminary for obtaining the high viability and yield of protoplasts. In this study, it was found that among the 24 enzyme combinations, Cellulase Onozuka R-10 plus Driselase, Cellulase Onozuka RS plus Driselase, Cellulase Onozuka RS plus Hemicellulase plus Driselase plus Pectolyase Y-23, and Cellulase Onozuka RS plus Pectolyase Y-23 were suitable for protoplast isolation from stem segment-derived callus (data not shown). On the other hand, both Macerozyme R-10 and Hemicellulase were not effective in combination with Cellulase Onozuka R-10 or RS for isolation of callus protoplasts from callus. Protoplasts obtained from callus are shown in Figure 1b.



a: Callus induced from stem-segment explants. b,c: Protoplasts from stem segment-derived-callus. d: Fluorescence microphotograph of protoplasts stained with FDA. Vigorous protoplasts showing yellow-green fluorescence. Arrowheads indicate the damaged protoplasts. c and d indicate the same field. e: Dividing cells after two weeks of culture (arrowheads) in MS medium containing 2.0 μM BAP and 4.0 μM IBA. f: Enlarging and non-spherical cells after two weeks of culture in MS medium containing 2.0 μM BAP and 4.0 μM 2,4-D. g: Colony formation after 5 weeks of culture in MS medium containing 2.0 μM BAP and 4.0 μM IBA. h: Callus formation after 4 months of culture in MS medium containing 2.0 μM BAP and 4.0 μM IBA. i: Shoot differentiation from protoplast-derived calli on MS medium containing 2.0 μM BAP and 1.0 μM NAA. j: Multiplication and elongation of shoots on MS medium containing 1.0 μM BAP. k: Rooting of *in vitro* grown shoots on MS medium containing 2.0 μM IBA. l: Plantlet growing on the Kanuma soil under culture room conditions after 2 weeks of transfer

Figure 1 : Plant regeneration through protoplast-derived callus

Generally, living protoplasts emit yellow-green fluorescence under a fluorescence microscope, when protoplasts were stained with FDA, whereas dead protoplasts do not[22]. In the present study, protoplasts stained with yellow-green fluorescence were judged to be vigorous, and the viability of protoplasts was measured from the number of fluoresced protoplasts (Figures 1c, d). Figure 2 shows the influence of incubation

time from 6 to 18 hrs on the yield and viability of protoplasts. The yield gradually increased as increasing the incubation time. On the other hand, the viability considerably decreased with the increase in incubationtime. In this study, the highest 92.0% viability with the yield of 2.4×10^5 protoplast / g fresh weight was obtained when the materials were treated for 6 hrs with Cellulase Onozuka R-10 plus Driselase. The highest yield of callus protoplasts (10.0×10^5 protoplast / g fresh weight) was obtained by the treatment for 18 hrs with Cellulase Onozuka RS plus Driselase, whereas the protoplast viability was 0%. Among the different enzyme combination and incubation time, Cellulase Onozuka R-10 plus Driselase for 8 hrs was the most suitable for protoplast isolation, the yield and viability of protoplasts were 5.5×10^5 protoplasts / g fresh weight and 90.0%, respectively.

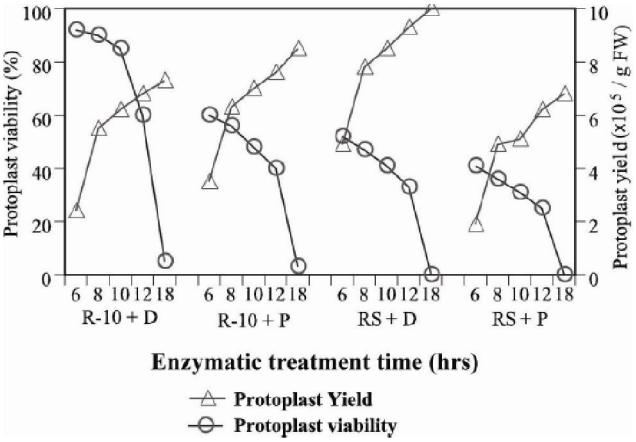


Figure 2 : Effects of enzymatic treatment time on the yield and the viability of protoplasts

Effects of osmotic potential on the yield and viability of protoplasts

The osmotic potential in isolation mixtures also influenced the yield and viability of protoplasts from callus cultures. Generally, mannitol is used as an osmoticum. Different concentrations (0.4, 0.6, and 0.8 M) of mannitol and the enzyme combination of Cellulase Onozuka R-10 plus Driselase were used for examining the effects of osmotic potential on protoplast isolation. The yield of protoplasts increased with increase in mannitol concentration from 0.4 to 0.6 M, and the maximum viability (90.0%) and yield (5.5×10^5 protoplasts / g fresh weight) of protoplasts were obtained with 0.6 M mannitol at 8 hrs enzymatic treatment (Figure 3). It was also noted that higher osmolality induced lower protoplast yield and lower osmolality resulted in more cell burst.

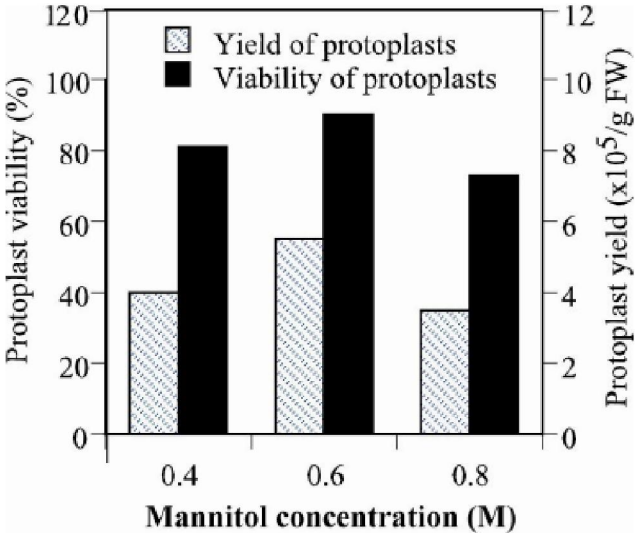


Figure 3 : Effects of mannitol concentration on the yield and viability of protoplasts

Culture of protoplasts

Protoplasts were cultured in MS liquid medium supplemented with different combinations and concentrations of cytokinins and auxins at three different cell densities i.e. 2×10^5, 4×10^5, and 6×10^5 protoplasts / ml. After one week of culture in same media, there was no change in protoplast growth. It seemed that the cell wall regeneration had commenced only after one week of growth. However, the first cell division was observed after 2 weeks of culture in the media containing high concentration (4.0 μM) of NAA, IBA, or 2,4-D with 2.0 μM BAP (Figure 1e), suggesting that the time required for the cell wall regeneration was about 2 weeks. In the MS media containing BAP with NAA or IBA, protoplasts developed spherical cells with cell division, whereas in the media containing BAP plus 2, 4-D they showed both spherical and non-spherical cell enlargement (Figure 1f).

The colony formation from the protoplasts was observed in the MS liquid media containing 0.6 M mannitol in combinations of BAP or TDZ (1.0, 2.0, or 4.0 M) and NAA, IBA, or 2,4-D (2.0 or 4.0 μM) after 5 weeks of culture (Figure 1g). The colonies actively proliferated after one month of culture, and grew up to 1 mm in diameter. Among the different densities of protoplasts examined here, the plating density of 4×10^5 protoplasts / ml effectively induced the active growth

FULL PAPER

and cell division of protoplasts (Figure 4).

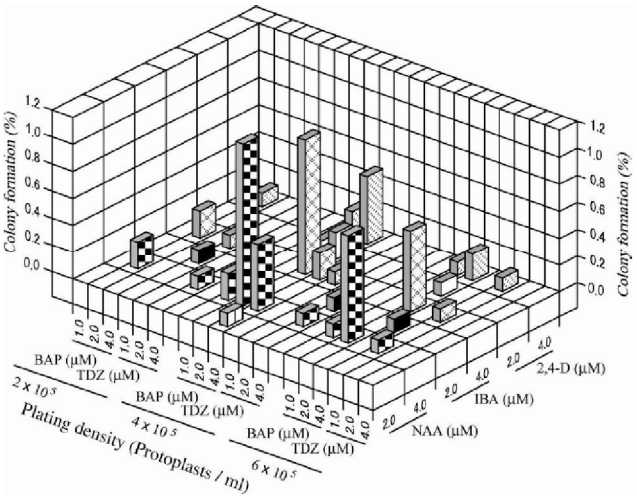


Figure 4 : Effects of different combinations and concentrations of cytokinin and auxin, and plating densities on colony formation in protoplast culture

After 2 months of culture, the obtained microcolonies developed into the microcalli larger than 2 mm in diameter, and then they were transferred to Petri dishes containing 30 ml of MS solid medium of the same components without mannitol. After 4 months of culture in this medium, the microcalli 4 mm in diameter and their proliferation were observed (Figure 1h). Among the different media composition, MS media containing 2.0 μM BAP with 4.0 μM NAA or IBA were suitable for the proliferation of stem segment-derived callus, when protoplasts were cultured at the density of 4×10^5 protoplasts / ml.

Effects of culture medium on colony formation

The effects of components and conditions of medium on colony formation from cultured protoplasts were examined. Isolated protoplasts were cultured in liquid or solidified (0.2% Gellan gum) medium with each of three different media, MS, MMS_1, and WPM. The liquid media contained 3.0% sucrose, 0.6 M mannitol, 2.0 μM BAP, and 4.0 μM NAA, and solidified media had no mannitol. In this study, the plating density was 4×10^5 protoplasts / ml. After one month of culture, data were recorded and are presented in TABLE 1. Both medium and type of culture, and their interaction had a significant effect on colony formation. Colony formation of protoplasts induced on MS solid medium was significantly higher (0.33%) than other media. This fact revealed that MS solid medium was the best for callus-derived-protoplast culture of *P. amurense*, whereas MS liquid medium was less effective.

TABLE 1 : Effects of culture medium on colony formation from protoplast culture

Medium	Type of culture	Colony formation (%)
MS	Solidified medium	0.33 ± 0.02*a*
	Liquid medium	0.26 ± 0.01*b*
MMS_1	Solidified medium	0.27 ± 0.01*b*
	Liquid medium	0.20 ± 0.01*c*
WPM	Solidified medium	0.14 ± 0.01*d*
	Liquid medium	0.11 ± 0.01*d*

All media contained 2.0 μM BAP and 4.0 μM NAA, and plating density was 4×10^5 protoplasts / ml. Values represent means ± standard errors of three replicates per treatment in three repeated experiments. Data were recorded after 4 weeks of culture. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level

Proliferation of protoplast-derived callus

The protoplast-derived microcalli (about 2-3 mm in diameter) were cultured on MS medium supplemented with different combinations and concentrations of a cytokinin and an auxin. TABLE 1 revealed that liquid media were less effective for colony formation or proliferation of callus. In contrast, solid medium stimulated the callus proliferation. The callus cultured on the MS medium, solidified with Gellan gum, containing 2.0 μM BAP and 4.0 μM NAA or IBA exhibited active growth for 3 months. The media containing 1.0 μM TDZ and 4.0 μM NAA, IBA, or 2,4-D showed active callus growth for 2 months, and after that, they gradually became brown. In *P. amurense,* BAP with NAA or IBA seemed to be more effective for the callus proliferation and culture of green callus, compared with TDZ and 2,4-D considering that in general green callus is physiologically active and suitable for differentiation of shoots.

The maximum 80.0% of callus proliferation was obtained from the protoplast-derived microcalli on MS medium containing 2.0 μM BAP and 4.0 μM NAA or IBA (Figure 5). BAP or TDZ in combination with 2,4-D produced white-greenish callus with the highest proliferation rate of 60.0%. The light conditions also influenced the callus proliferation. In general, the dark condition is effective for the growth of callus. In this experiment, the callus grew actively in the light and remained

green, whereas the dark condition inhibited the callus proliferation and caused browning of callus after one month. In the present study, successful proliferation of callus through the protoplast culture was obtained.

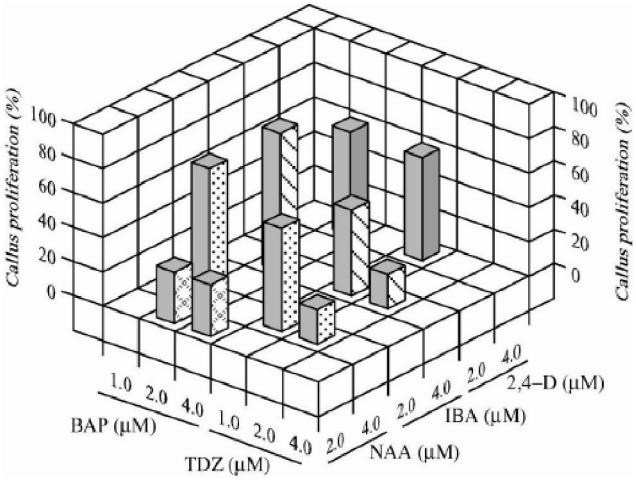


Figure 5 : Effects of plant growth regulator on proliferation of protoplast-derived callus

Regeneration of plantlets from protoplast-derived callus

For plant regeneration, protoplast-derived calli were transferred to a sequence of regeneration media supplemented with various cytokinins and/or auxins. In this study, MS solid media containing 0.5, 1.0, 2.0, and 4.0 μM of BAP or CPPU in combination with 0.5, 1.0, 2.5, and 5.0 μM NAA or IBA were used.

The adventitious shoots were successfully induced from the protoplast-derived green calli (Figure 1i), whereas root differentiation occurred from the white-greenish calli on MS medium containing 0.5 μM BAP and 1.0 μM IBA. The green calli were soft compact, while white-greenish calli were hard compact. Among the different plant growth regulators, 2.0-4.0 μM BAP plus 0.5-1.0 μM NAA, and 2.0-4.0 μM BAP plus 1.0-2.5 μM IBA in MS medium produced adventitious shoot regeneration from the green calli. The maximum 80.0% of shoot proliferation was obtained on MS medium after 5 weeks of culture, when 2.0 μM BAP plus 1.0 μM NAA was used as plant growth regulators (data not shown). In this study, 1.0 μM CPPU and 1.0-2.5 μM NAA developed only green callus, but these failed to produce any shoots. For the multiplication and elongation of shoots, the proliferated shoots were transferred to MS medium containing 0.5, 1.0, 1.5, and 2.0 μM BAP. Among the different concentrations tested, 1.0 μM BAP showed the highest length and maximum number of shoots (Figures 1j, 6).

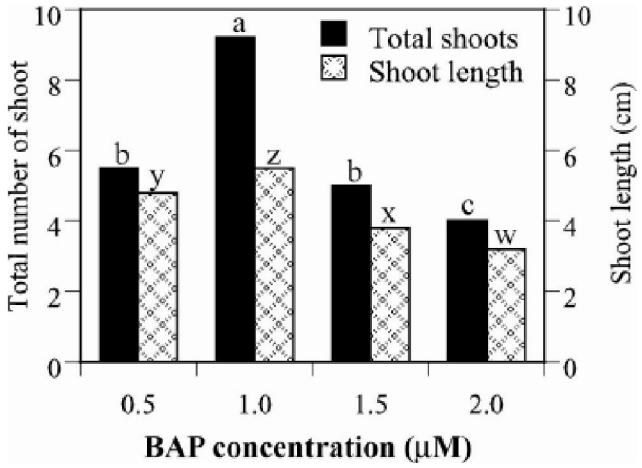


Figure 6 : Effects of different concentrations of BAP on multiplication and elongation of shoots induced from protoplast-derived callus

Rooting and establishment of *in vitro* proliferated shoots

The microcuttings (2-4 cm long) were prepared from elongated shoots induced from callus, and were transferred to MS medium containing different concentrations of IBA or NAA for rooting. One week after inoculation, root formation was noticed from the basal cut portion of shoots. There were a satisfactory result in rooting: 90.0% shoots, 4.2 total number of roots, and 4.6 cm average length could be obtained on MS medium containing 2.0 μM IBA (Figure 1k), while 80.0% root formation, 2.5 maximum number of roots, and 3.0 cm average length were obtained with 1.0 μM NAA.

Twenty plantlets were removed from culture tubes, and the Gellan gum was washed away from the roots thoroughly with running tap water, then they were transferred to small plastic pot containing Kanuma soil for hardening under diffuse light (16 hrs photoperiod). Normal growth of the potted plants was observed after one week of transfer. Two months later, they were moved to another larger pot containing the same soil and transferred to greenhouse. The *in vitro* grown plantlets were gradually acclimatized and successfully established on the Kanuma soil under greenhouse condition with a survival rate of 90% (Figure 1l), where they showed no morphological variation with mother plants.

FULL PAPER

DISCUSSION

The most significant finding of this study was the development of protocols for plant regeneration from protoplasts of a medicinal tree *P. amurense*. Before starting protoplast culture, it should be needed to find out essential culture conditions and culture environment of callus induction from the experimental explants. To find out their callus induction efficiency, stem segment explants were cultured on MS medium containing different combinations and concentrations of a cytokinin and an auxin. Friable callus was formed on MS medium supplemented with 2.0 μM TDZ and 4.0 μM 2,4-D. Li-Chun *et al.*[9] reported the same results for callus production from stem in *Cinnamomum camphora*. Stem segment explants produced highly proliferating friable callus showing green colour on the medium containing 2.0 μM BAP with 4.0 μM NAA, IBA, or 2,4-D. Schween and Schwenkel[8] reported the similar results for callus production in *Primula* spp. Ikuta *et al.*[1] reported that 0.1 μM Kn with 1.0 μM 2,4-D was better for callus induction from the stem segment in *P. amurense*. In the present study, it was revealed that 2.0 μM BAP or TDZ with 4.0 μM NAA, IBA, or 2, 4-D were the best formulations, and stem segment was a suitable explant for callus production in *P. amurense*. The results are similar to those of callus induction in *Astragalus adsurgens*[13]. No adventitious embryos were induced from the subcultured callus during the prolonged culture periods in this study.

Considering protoplast yield and viability the enzymatic treatment with Cellulase Onozuka R-10 and Driselase for 6-8 hrs was relatively better among the 24 enzyme combinations used. The better yield and viability in combination enzymatic treatment might be due to their synergetic effects. Wakita[32] reported that Cellulase Onozuka R-10 with Driselase was better for highest yield and viability of leaf protoplasts in *Betula platyphylla* and *Alnus firma*. So far, there is no report on protoplast isolation of *P. amurense*. This study, therefore, is the first experiment of protoplast isolation in this plant species.

Either mannitol or sorbitol as an osmotic stabilizer for protoplasting of both green plant materials and cell suspension cultures has been used generally. The osmotic potential in isolation mixtures also influenced protoplast isolation of *P. amurense* were used to isolate protoplasts from stem segment-derived callus. Among the three concentrations (0.4, 0.6, and 0.8 M) of mannitol the yield of protoplasts improved when mannitol concentration was increased from 0.4 to 0.6 M, but the yield decreased at 0.8 M. Poor yield of protoplast at 0.4 and 0.8 M concentrations might be due shrinkage and bursting of protoplast at lower (0.4 M) and higher (0.8 M) concentration respectively. Similar results were reported in *Prunua amygdalus*[26], *Ginkgo biloba*[14], *Alnus firma*[32], *Artemisia judaica*, and *Echinops spinosissimus*[33].

A minimal number of protoplasts per ml is always required to ensure cell divisions[24]. It was found in this study that protoplasts could divide from a plating density as low as 2×10^5 protoplasts / ml, and an optimum of 4-6 $\times 10^5$ protoplasts / ml. In order to optimize colony formation, the incubation period had to be limited to 6 hrs despite a maximal protoplast yield recorded after 12-18 hrs. Similar observation was found in *Platanus orientalis*[34]. Among the different combinations and concentrations of a cytokinin and an auxin, 2.0 μM BAP with 4.0 μM NAA or IBA showed the best performance of colony and microcallus formation, and microcallus proliferation in MS liquid medium from callus protoplasts. This agrees with reports for other woody species, such as *Gentiana scabra*[31] and *Ginkgo biloba*[14].

Apparently, the enzymes frequently show some toxicity. Light is necessary during enzyme incubation, as darkness is shown to increase protoplast mortality. Therefore, light may contribute to protoplast defence against enzyme toxicity and osmotic stress[7]. The importance of light for protoplast culture was demonstrated in the present study, since more colonies were formed under this condition. This result is different from the procedure reported so far[24], in which the protoplast culture of the first week was performed in the dark.

More colonies were produced in MS solid medium without mannitol than in liquid medium with mannitol, whereas MMS_1 and WPM medium showed less performance. Under these conditions, protoplasts and colonies grew mainly in the upper part of the medium, where gas and light are easily accessible. In liquid medium containing mannitol, colonies frequently fell to the bottom of the dish, where gas and light accessibility seem

to be probably lower. The beneficial effects of solid medium on protoplast culture have been previously reported[10]. However, the beneficial effects varied with the nature of the gelling agent[2]. For *P. amurense*, gellan gum was the most efficient one. This result could be explained either by the presence of toxic compounds in the gelling agent[3] or by the influence on nutrients or growth regulator availability[19].

Individual groups of 20-25 protoplast-derived microcalli (approx. 2-3 mm in diameter) were transferred to Petri dishes or flasks including gellan gum-solidified MS medium, without mannitol, containing 1.0-4.0 μM BAP or TDZ with 2.0-4.0 μM NAA, IBA, or 2,4-D. In this study, BAP and NAA or IBA were the most effective plant growth regulators for proliferation of protoplast-derived callus. Same results were found in many other woody species, such as: *Gentiana scabra*[31], *Ginkgo biloba*[14], and *Rosa hybrida*[25] .

This experiment revealed that the ratio between low concentration of auxin and high concentration of cytokinin enhanced adventitious bud formation, i.e. adventitious buds could be induced by transferring callus from the medium containing 0.5-1.0 μM NAA or 1.0 μM IBA to the medium containing 2.0-4.0 μM BAP under 16 hrs photoperiods. In addition, the nitrogen concentration of the medium appeared to affect the regeneration of adventitious buds from protoplast-derived calli in *P. amurense*. MS medium was more effective for adventitious bud formation than MMS_1 or WPM medium. Similar results were reported in *Actinidia deliciosa*[23], *Rosa* species[4], and *Diospyros kaki*[21].

For further development of shoots, protoplast-derived adventitious shoot buds were transferred to MS medium containing different concentrations of BAP. The subculture of the small buds on the medium supplemented with 1.0 μM BAP gave better growth and development of the shoots. Similar observation was found in *Solanum khasiamum*[5].

In vitro rooting was achieved successfully when the microshoots were cultured on MS media containing auxins, IBA or NAA. Success of *in vitro* rooting of microshoots excised from proliferating cultures depends frequently on the species and clone used, on the media combination, and on the concentration of the auxin used[28]. In general, low salt media stimulated root number and length in *Phellodendron*[16]. Kim *et al*.[12] reported that 500 μM IAA was best for root induction of *P. amurense* microshoots, while both IBA and NAA failed to produce any roots. In the present study, 2.0 μM IBA showed the best result for root induction and the rooted shoots were successfully established on the Kanuma soil and grew up gradually in the greenhouse conditions.

REFERENCES

[1] A.Ikuta, T.Hakamura, H.Urabe; Indolopyridoquinazoline, furoquinoline and canthinone type alkaloids from *Phellodendron amurense* callus tissues. Phytochem., **48**, 285-291 **(1998)**.

[2] A.Saito, M.Suzuki; Plant regeneration from meristem-derived callus protoplasts of apple (*Malus* x *domestica* cv. 'Fuji'). Plant Cell Rep., **18**, 549-553 **(1999)**.

[3] B.J.Nairn, R.H.Furneaux, T.T.Stevenson; Identification of an agar constituent responsible for hydric control in micropropagation of radiata pine. Plant Cell Tissue Org.Cult., **43**, 1-11 **(1995)**.

[4] D.Matthews, J.Mottley, K.Yokoya, A.V.Roberts; Regeneration of plants from protoplasts of *Rosa* species (Roses), In: Y.P.S.Bajaj (Ed); Biotechnology in Agriculture and Forestry, Plant Protoplasts and Genetic Engineering, Springer-Verlag Berlin Heidelberg, **29**, 146-160 **(1994)**.

[5] D.Sihachakr, I.Serraf, M.H.Chaput, I.Mussio, Rossignol, G.Ducreux; Regeneration of plants from protoplasts of *Solanum khasianum* C.B. Clark and *Solanum laciniatum* Ait., In: Y.P.S.Bajaj (Ed); Biotechnology in Agriculture and Forestry, Plant Protoplasts and Genetic Engineering, Springer-Verlag Berlin Heidelberg, **34**, 108-120 **(1995)**.

[6] E.C.Cocking; A method for the isolation of plant protoplasts and vacuoles. Nature, **187**, 962-963 **(1960)**.

[7] G.Hahne, H.Lorz; Release of phytotoxic factors from plant cell walls during protoplast isolation. J.Plant Physiol., **132**, 345-350 **(1988)**.

[8] G.Schween, H.G.Schwenkel; Effect of genotype on callus induction, shoot regeneration, and phenotypic stability of regenerated plants in the greenhouse of *Primula* spp. Plant Cell, Tissue and Org.Cult., **72**, 53-61 **(2003)**.

[9] H.Li-Chun, H.Bau-Liang, T.Murashige; A micropropagation protocol for *Cinnamomum camphora*. In Vitro Cell Dev.Biol-Plant, **34**, 141-146 **(1998)**.

FULL PAPER

[10] H.Lörz, P.J.Larkin, J.Thomson, W.R.Scowcraft; Improved protoplast culture and agarose medium. Plant Cell Tissue Org.Cult., **2**, 217-226 **(1983)**.

[11] I.Takebe, Y.Otsuki, S.Aoki; Isolation of tobacco mesophyll cells in intact and active state. Plant Cell Physiol., **9**, 115-124 **(1968)**.

[12] J.H.Kim, G.H.Goo, M.S.Choi, Y.G.Park; Micropropagation and soil adjustment of cork tree (*Phellodendron amurense* Rupr.) through *in vitro* culture. Korean J.Plant Tissue Cult., **19**, 37-42 **(1992)**.

[13] J.P.Luo, J.F.Jia; Callus induction and plant regeneration from hypocotyl explants of the forage legume *Astragalus adsurgens*. Plant Cell Rep., **17**, 567-570 **(1998)**.

[14] J.Trémouillaux-Guiller, D.Laurain, J.C.Chénieux; Direct embryogenesis in protoplasts *Ginkgo biloba* (Maidenhair Tree), In: Y.P.S.Bajaj (Ed); Biotechnology in Agriculture and Forestry, Plant Protoplasts and Genetic Engineering, Springer-Verlag Berlin Heidelberg, **38**, 33-47 **(1996)**.

[15] K.Ariyoshi; Plantlet formation by interpetiolar bud culture of *Phellodendron amurense* Rupr. Forest Tree Breed., **138**, 24-26 **(1986)**.

[16] L.Maene, P.C.Degergh; Liquid medium additions to the established tissue culture to improve elongation and rooting *in vitro*. Plant Cell, Tissue and Org.Cult., **5**, 23-33 **(1985)**.

[17] M.A.K.Azad, S.Yokota, T.Ohkubo, Y.Andoh, S.Yahara, N.Yoshizawa; Micropropagation of plantlets through callus in Kihada (*Phellodendron amurense* Rupr.). J.SHITA., **16**, 122-130 **(2004)**.

[18] M.A.K.Azad, S.Yokota, T.Ohkubo, Y.Andoh, S.Yahara, N.Yoshizawa; *In vitro* regeneration of the medicinal woody plant *Phellodendron amurense* Rupr. through excised leaves. Plant Cell Tissue and Org.Cult., **80**, 43-50 **(2005)**.

[19] M.H.Brand; Agar and amonium nitrate influence hyperhydricity, tissue nitrate and total nitrogen content of serviceberry (*Amelanchier arborea*) shoots *in vitro*. Plant Cell Tissue Org.Cult., **35**, 203-209 **(1993)**.

[20] M.S.Choi, D.III.Shin, Y.G.Park; Berberine production by cell suspension culture of cork tree (*Phellodendron amurense* Rupr.) Kor. J. Pharmacogn., **27**, 32-36 **(1996)**.

[21] M.Tamura, R.Tao, A.Sugiura; Regeneration of plants from protoplasts of *Diospyros kaki* L. Japanese Persimmon, In: Y.P.S.Bajaj (Ed); Biotechnology in Agriculture and Forestry, Plant Protoplasts and Genetic Engineering, Springer-Verlag Berlin Heidelberg, **34**, 43-54 **(1995)**.

[22] P.J.Larkin; Purification and viability determinations of plant protoplasts. Planta (Berl.), **128**, 231-216 **(1976)**.

[23] Q.G.Cai, Y.Q.Qian, S.Q.Ke, Z.C.He; Regeneration of plants from protoplasts of kiwifruit (*Actinidia deliciosa*). In: Y.P.S.Bajaj (Ed); Biotechnology in Agriculture and Forestry, Plant Protoplasts and Genetic Engineering, Springer-Verlag Berlin Heidelberg, **23**, 3-17 **(1993)**.

[24] Q.Yang, S.Laliberté; Cell division and microcolony formation in protoplasts from mature material of hybrid larch (*Larix* x *eurolepis* Henry). Plant Sci., **117**, 159-165 **(1996)**.

[25] R.Marchant, M.R.Davey, J.B.Power; Isolation and culture of mesophyll protoplasts from *Rosa hybrida*. Plant Cell, Tissue and Org.Cult., **50**, 131-134 **(1997)**.

[26] S.C.Wu, A.H.Kuniyuki; Isolation and culture of almond protoplasts. Plant Sci., **41**, 55-60 **(1985)**.

[27] T.Murashige, F.Skoog; A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol.Plant., **15**; 473-497 **(1962)**.

[28] V.Chalupa; Micropropagation of European mountain ash (*Sorbus acuparia* L.) and wild service tree [*Sorbus torminalis* (L.)Cr.]. In: Y.P.S.Bajaj (Ed); Biotechnology in Agriculture and Forestry, High Tech and Micropropagation II, Springer-Verlag Berlin Heidelberg, New York, **18**, 211-216 **(1992)**.

[29] Y.G.Park, M.S.Choi, S.H.Son; Anthocyanin synthesis in cell cultures of *Populus alba* × *P.glandulosa*. Korean J.For.Soc., **81**, 183-190 **(1992)**.

[30] Y.Otsuki, I.Takebe; Isolation of intact mesophyll cell and their protoplasts from higher plants. Plant Cell Physiol., **10**, 917-921 **(1969)**.

[31] Y.Takahata, H.Jomori; Plant regeneration from mesophyll protoplasts of gentiana (*Gentiana scabra* Bungei). Plant Tissue Cult.Let., **6**, 19-21 **(1989)**.

[32] Y.Wakita; Plant regeneration from protoplasts of broad-leaved trees. Bull Utsunomiya Univ.For., **33**, 55-106 **(1997)**.

[33] Z.G.Pan, C.Z.Liu, S.J.Murch, M.El-Demerdash, P.K.Saxena; Plant regeneration from mesophyll protoplasts of the Egyptian medicinal plants *Artemisia judaica* L. and *Echinops spinosissimus* Turra. Plant Sci., **165**, 681-687 **(2003)**.

[34] Z.M.Wei, Z.H.Xu; Regeneration of plants from protoplasts of *Platanus orientalis* (Plane Tree), In: Y.P.S.Bajaj (Ed); Biotechnology in Agriculture and Forestry, Plant Protoplasts and Genetic Engineering, Springer-Verlag Berlin Heidelberg, **34**, 101-107 **(1995)**.