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Abbreviations

B₅ Gamborg medium (1968)
BAP 6-Benzylaminopurine
2,4-D 2,4-dichlorophenoxyacetic acid
°C Degree Celsius
cm Centimeter (s)
cm² Centimeter square
CPPU N-(2-chloro-4-pyridil)-N'-phenylurea
et al. et alia = and other people
FDA Fluorescein diacetate
Fig. Figure (s)
FW Fresh weight
GA₃ Gibberellic acid
g Gram (s)
g/l Gram per liter
hr Hour
i.e. id est = that is
IAA Indole-3-acetic acid
IBA Indole-3-butyric acid
Kn Kinetin (6-furfurylamino purine)
M Molar
mg Milligram (s)
mg/l Milligram per liter
µM Micro Molar
min Minute
ml Milliliter (s)
mm Millimeter (s)
MS Murashige and Skoog (1962)
MMS₁ Modified MS medium (½ strength of major and full strength of minor salts)
MMS₂ Modified MS medium (¼ strength of major but ½ strength of minor salts)
NAA α-naphthaleneacetic acid
No. Number
pH Negative logarithm of hydrogen ion (H⁺)
TDZ Thidiazuron
viz. videlicet = Namely
v/v Volume per volume
WPM Woody plant medium
w/v Weight per volume
% Percentage
INTRODUCTION

1. Medicinal Plants and Traditional Healthcare

One of the most remarkable uses of plant- and animal-based biodiversity has been medicine. The World Health Organization (WHO) estimates that 4 billion people, 80 percent of the world population, presently use herbal medicines for some aspects of primary health care. WHO (1991) notes that out of 119 plant-derived pharmaceutical medicines, the about 74 percent are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potentials as medicinal value (Bajaj and Williams, 1995).

Herbal medicine is the oldest form of healthcare known to mankind. Herbs have been used by all cultures throughout history. It was an integral part of the development of modern civilization. The plants have been providing food, clothing, shelter, and medicine. So far indeed, well into the 20th century, much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs commonly used today are of herbal origin. (Bajaj and Williams, 1995).

The rich and diverse heritage of traditional medicine systems in sub-continent is increasingly threatened by the interplay of a number of factors: a) About 80.0% of the 1400 or so species used by the indigenous systems are gathered from forests. Rapid deforestation in recent years has caused large-scale losses of plant populations and reduced their distribution in known habitats, b) Trade in this sector is secretive, unregulated and highly exploitative. c) Growing pressure and scarcity also result in the marketing of cheap substitutes and spurious products undermining the health and reputation of the entire traditional medicare system. d) Little or no systematic efforts have been made to extend the availability of these plants through cultivation (Anon, 1994). It is evident that herbal medicine plays an important and even dominant role in some part of the world. There are widespread interests in placing herbal medicines in an appropriate scientific framework by assessing their safety, efficacy, and quality according to a modern standard. These assessments, however, should take the traditional use into account and should therefore avoid any exaggerated claims. The key to the success of primary health care lies in the availability and use of appropriate medicinal plants (Vedavathy, 1994).

2. Micropropagation of Medicinal Plants

During the last few years, the interested in mass propagation in vitro of medicinal plants have distinctly increased for various reasons. Many of these plants, when propagated by conventional methods it takes a long time for multiplication, have a low rate of fruit set and poor seed germination, and are often under protection or threatened with extinction. The pollution of the human environment has been bringing the risk that collecting plants from natural environments may not be safe for health. The alternative to this situation is the rapid in vitro multiplication of plants and their cultivation under special conditions.

The application of tissue culture techniques for micropropagating medicinal plants might give many benefits to the breeders as follows:

a) Increase in the propagation rate of plants.
b) Rapid multiplication of plants which in a particular climate do not give seeds or whose seeds have a low germination ability.
c) Availability of plants throughout the year, i.e., in all the season.
d) Resistance of plants to insects, diseases, and herbicides.
e) Producing of uniform plants from a selected genotype.
f) Production of uniform clones from highly heterozygous plants.
g) Production of plants with changed genotype.
(tetraploids, haploids, hybrids).

h) Conservation of genetic resources of species and threatened plants.

i) Plant improvement by regeneration technique in conjunction with in vitro cell manipulation.

The rapid multiplication of medicinal and aromatic plants can help to solve some theoretical problems connected with the pathway of biosynthesis of chemical compounds in plants, and the relation between organogenesis and production of secondary metabolites. For example, although flavour compounds in celery tissues were absent in undifferentiated callus, they were produced by embryoids, especially in the torpedo stage (Collin and Watts, 1983).

Various techniques of clonal propagation have been used for the micropropagation of medicinal plants. From the pharmaceutical point of view, the most useful one is propagation from existing meristems (direct), as identical plants with desired traits can be obtained by this method. The plants regenerated form meristems are genetically identical to the donor plants (Hu and Wang, 1983). In a situation where a large number of plants are to be regenerated in a short time, somatic embryogenesis is promising, because somatic embryos can be produced in astounding numbers per gram of callus (Sluis and Walker, 1985). For commercial purposes they can also be used for the production of an “artificial seed” (Redenbough et al., 1986). Two other methods, with which plants can be recovered in large numbers, are the antherogenesis through anther/pollen culture and the culture of isolated protoplasts.

3. A Brief History of Phellodendron amurense Rupr.

3.1. Origin, Distribution, and Description of the Plant

The genus Phellodendron (family Rutaceae) comprises ten species indigenous to the Far East region, including Korea, China, and Japan. P. amurense (Amur cork tree) is the best known and almost widely growing in these area. It grows rapidly and has a broad crown. This species is dioecious, 7-15 m high, and develops a large number of cork tissues in the outer bark. The gray-brown bark becomes fissured and corky with age. Flowers are greenish and very conspicuous. They are borne in panicles of generally five to eight flowers on spurs or lateral branchlets; the ovary contains five carpels. Female trees produce large clusters of fleshy back fruits which remain on the tree into the winter. This tree grows well in the humid climates (Hensley et al., 1991).

3.2. Importance

Trees being harvested for medicinal purposes are usually felled in the spring or autumn, and the bark is removed and dried under the sun light. It is normally used to make aqueous extracts. Extracts of the wood and root bark are used as dye for wool and leather. The crude extracts from the bark of cork trees were well known as antibiotic. Hong et al. (1988) reported that bark extracts were very effective in inhibition of Japanese apple canker caused by Valsa ceratosperma. The bark extracts mainly contain berberine derivatives as active constituents, which will be described in more detail in section 3.4.

The extracts from bark 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, chologogue, and antibacterial medicine (Ikuta et al., 1998). It is also effective for purging heat, detoxifying, clearing damp heat, and lowering blood sugar. It is also a potential source of industrial cork (Ota et al., 1965), important as a nectar-bearing species in bee-keeping areas of the Soviet Far East, and of possible importance for the insecticidal properties of the fruit oils (Schechter, 1943).

This species is frequently planted as an ornamental or a roadside trees, because it is relatively resistant to air pollution. It is also utilized for furniture woods.
3.3. Propagation
This tree species can be propagated from seeds, stem cuttings, and roots. Although propagation by seeds is possible, germination in the field under natural conditions is very poor due to weak dormancy. Stem cuttings have a low rooting rate. The shoot survival rate of a 28-year-old tree cutting decreased markedly to below 10.0%, although treatment with indole-3-butyrac acid (IBA) somewhat improves the survival and rooting rate (Park et al., 1988).

3.4. Alkaloids and Medicinal Compounds
The secondary metabolites include protoberberine alkaloids such as berberine, palmatine, columbamine, and magnoflorine, flavonoids, and terpenoids. Most recently, research has focused mainly on berberine which is found usually in the bark, root, and fruit. This is most abundant in bark tissues, and this single compound can account for up to 5.0% of dry weight (Tosa et al., 1989). Berberine is well known for its anti-inflammatory, antimicrobial, antileukemic, and antineoplastic effects (Otsuka et al., 1981). It has been used as a fluorescent marker in several areas of medicinal research and as a stomach drug in the Orient. The demand for drugs from the cork tree is increasing. In 1992 the demand for the crude materials in Korea was 6000 kg/year: 5000 kg imported from Japan and China, and 1000 kg self-produced in Korea (Seong, 1992).

4. Review of Relevant Literatures
Commercial exploitation for production and conventional propagation of P. amurense is hampered due to its poor seed viability, low rate of germination, and poor rooting ability of vegetative cuttings (Mukai and Yokoyama, 1985). Alternative propagation methods would be beneficial in accelerating large-scale multiplication, improvement, and conservation of the plant.

There are a few publications concerning in vitro culture of P. amurense, which are related to mass propagation by axillary bud culture and berberine production by cell culture. Ariyoshi (1986) reported successful micropropagation using intrapetiolar buds formed after cutting mature cork trees. Best growth was obtained on liquid WPM (Lloyd and McCown, 1980) containing 0.23-0.64 mg/l BA or 0.02 mg/l IBA. Multiple shoots were formed from lateral buds in the nodal segments on the same medium. Individual shoots were excised and rooted successfully in 30.0-40.0% of cases in the fourth generation. Rooting efficiency was very low, but increased after several generations of in vitro plantlets. Kim et al. (1992) reported mass propagation by using axillary buds from 15-year-old trees. Over 90.0% of in vitro proliferated shoots survived in the greenhouse.

The production of secondary metabolites by cell cultures of the cork tree has been reported (Park et al., 1992b; Choi et al., 1996). Park et al. (1992a) reported that immobilized cells produced 7.0% of the total berberine biosynthesized by suspension cells, and 50.0-70.0% berberine was secreted into the medium from the Ca-alginate beads containing the cells. Continuous light, addition of DMSO, and XAD-2 resin stimulated release of berberine from cells. Choi et al. (1996) concluded that optimization of nutrient components could improve yields of berberine and palmatine.

Park and Choi (1999) demonstrated a micropropagation technique from axillary buds and production of berberine from cell suspension cultures of P. amurense. Axillary shoots were developed on MS medium containing 0.05 μM NAA and 0.44 μM BAP. Elongated shoots were rooted on 500 μM IAA-supplemented MS medium. Best suspension cultures were obtained in MS medium containing 4.52 μM 2,4-D and 0.44 μM BAP. Although berberine was detected in extracts of cultured cells, but the amounts were variable depending on cell line and culture conditions.

Plant protoplasts have been proved to be an excellent tool for in vitro manipulation, somatic hybridization, and 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. Protoplast research in medicinal species is only described briefly here.

*Atropa belladonna*, a medicinal plant with a rich source of some tropane alkaloids, has been extensively studied *in vitro* (Bajaj and Simola, 1991). Complete plants have been regenerated from the cell suspension-derived protoplasts (Gosch *et al.*, 1975) and mesophyll protoplasts (Bajaj *et al.*, 1978), and somatic hybrids have been obtained (Gleba *et al.*, 1988; Bajaj and Simola, 1991).

*Duboisia* spp. is an evergreen woody medicinal plant. Few studies on the isolation and culture of *Duboisia* hipwoodii protoplasts have been reported. Endo *et al.* (1987) fused *Duboisia* suspension cell protoplasts with *Nicotiana tabacum* mesophyll protoplasts and cultured the heterokaryocytes to regenerate calli and shoots of intergeneric somatic hybrids. Intergeneric somatic hybrids of *D. leichhardtii* and *N. tabacum* were also obtained by Endo *et al.* (1991) and Kitamura (1993).

*Ginkgo biloba* is another woody and dioecious species appreciated for its ornamental value and tolerance to air pollution, and has a source of unique compounds showing interesting pharmacological properties (Carrier *et al.*, 1994). Although various aspects of the *in vitro* culture studies on this plant species have been reviewed earlier, there are only few reports on protoplast culture (Guiller *et al.*, 1996). Protoplasts of *G. biloba* have been enzymatically isolated from both haploid and diploid explants or pure male cell lines derived from single microspores which were cultured at the uninucleate stage. They were inoculated at various densities between $6 \times 10^2$-10$^3$/ml in basal liquid medium of Bourgin and Nitsch (BN; 1967), Murashige and Tucker (MT; 1969), and Gamborg *et al.* (B; 1970) supplemented with osmoticum (0.35-0.60 M mannitol, 0.35 M sorbitol, or 0.15-0.25 M glucose), organic substances, and with or without plant growth regulators and ammonium salts. From microclones, globular, oblong, and heart-stage embryos were achieved (Guiller *et al.*, 1996).

Plants were also obtained from protoplasts of leaf-derived calli and stem segment-derived calli of *Actinidia deliciosa* (Cai *et al.*, 1993) and *A. eriantha* (Zhang *et al.*, 1998). Pan *et al.* (2003) developed a protoplast-to-plant system for the Egyptian medicinal plants *Artemisia judaica* L. and *Echinops spinosissimus* Turra. Micropropagated shoots were used as plant materials for protoplast isolation in that study. Shoot organogenesis from the protoplast-derived callus was induced on MS medium supplemented with 5.0 µM BAP for *A. judaica* and 2.5 µM BAP for *E. spinosissimus*. However, protoplast research on *P. amurense* has not been yet reported.

5. Objectives

The present investigation was undertaken to develop *in vitro* culture techniques for *P. amurense* and to evaluate the morphogenic potentialities of different explants including juvenile as well as mature tissues for most efficient production of plantlets either through precocious axillary or adventitious bud proliferation as well as protoplast culture with the following objectives:

i. Evaluation of *in vitro* response of different types of explant.

ii. Identification and selection of suitable explants(s) for fast response and better regenerative potentialities.

iii. Selection and standardization of medium composition, plant growth regulator requirement, and culture environment for consistently high production of plantlets from primary explants as well as from the subcultured tissues.

iv. Selection of the type and concentration of auxin and culture condition for efficient rooting from *in*
vitro proliferated shoots.

v. Acclimatization and transplantation of in vitro regenerated plantlets onto the soil for growing under field conditions.

vi. Selection of enzyme combination for isolation of protoplasts from different explant sources.

vii. Selection of enzymatic treatment duration for obtaining the highest yield of protoplasts with the highest viability.

viii. Selection of medium and plant growth regulators for protoplast culture and subsequent plant regeneration from protoplast-derived callus.

ix. Evaluation of plant regeneration efficiency from callus by histological studies.

6. Composition of Thesis

This thesis consists of Introduction, Chapter 1-3, and Conclusion.

Introduction

This section describes mainly as to: 1) Medicinal plants and traditional healthcare, 2) Micropropagation of medicinal plants, 3) Brief history of Phellodendron amurense, 4) Review of relevant literature, and 5) Objectives.

In Chapter 1: Establishment of Micropropagation Protocol for Phellodendron amurense Rupr.

P. amurense was studied in order to establish suitable protocols for in vitro plant regeneration. The in vitro responses of different explants, node and shoot tip from the mature plants, and node, shoot tip, hypocotyl, cotyledon, internode, and leaf explants from the in vitro raised seedlings, were studied for regenerating plantlets and establishing protocols in support of large-scale clonal propagation. Among the explants, nodal segments and shoot tips were used for proliferating and developing axillary shoots, while hypocotyl, cotyledon, internode, and leaf segments from the seedlings were used for the induction and growth of callus, and subsequent regeneration of adventitious shoots. The in vitro regenerated shoots were successfully rooted and established under ex vitro conditions.

In Chapter 2: Plant Regeneration from Protoplast of Phellodendron amurense Rupr.

Experiments were carried out for develop a protoplast-derived plant regeneration protocol of P. amurense. This chapter describes the isolation of protoplasts from the leaf- and stem-segment-derived callus, and cell suspension cultures. Best culture condition was found out for isolated protoplasts. This section also describes the differentiation of protoplast-derived callus obtained from different sources of protoplasts. Successful plant regeneration was achieved from protoplast-derived callus, and subsequently regenerated shoots also rooted.

In Chapter 3: Morpho-Histological Studies of Shoot Regeneration System from Callus of Phellodendron amurense Rupr.

A morphological and histological study was undertaken to gain a better understanding of cellular changes in callus tissues through out the plant regeneration. This chapter also describes the effects of plant growth regulators on formation of different types of callus, and identification of cellular origin, cell characterization, and development pattern of generative callus. Cell wall development and a sequential shoot developmental stage were also described in this chapter.

In Conclusion, the results obtained in the present investigation strongly suggested potential application for fast multiplication of shoots. Furthermore, it was concluded that the culture system of protoplasts and plant regeneration via protoplast culture provided the effectiveness of using protoplasts as a tool to create novel medicinal germplasm as well as to evaluate genetic control of the synthesis of secondary metabolites.
Chapter 1: Establishment of Micropropagation Protocol for *Phellodendron amurense* Ruhr.

1. INTRODUCTION

Two aspects of the biotechnology of medicinal and aromatic plants are of immediate application: (a) Micropropagation under controlled germ-free conditions enables their fast multiplication and availability throughout the year, irrespective of external environment - this is especially useful for elite and rare plants. (b) A large-scale culture and low-temperature storage of cells enables retention of their biosynthetic potential for the production of important secondary metabolites, medicines, flavours, and other pharmaceutical products.

If a particular plant variety is a high level chemical line, it could be produced in large numbers maintaining it quality fully by micropropagation, which is difficult under conventional cultivation methods. A number of medicinally important species of plants are under threat for various reasons. Therefore, they need to be protected and conserved. Some other species are in great demand in medicinal industry. Conventional cultivation methods are both slow and inadequate to meet the demand. Establishment of micropropagation is also the answer for these problems (Patil and Jayanthi, 1997).

Cultivation of medicinal and aromatic plants is encouraged by the European Common Agricultural Policy, which places a particular emphasis on alternative agricultural products, including medicinal plants (Nuvoli, 1996). Furthermore, one must take into account that pollution of the environment by human means that it is no longer completely safe for man to use wild plants. The heterogeneity of wild plant species also means that supply is not constant and continuous or in homogenous quality (Magherini, 1988). Thus, it would be advisable to cultivate the highest possible number of plants used in medicine.

Some plant species are very difficult to propagate by seeds, stem cuttings, or grafting. In such cases, *in vitro* production of clones of single cells or explant tissues, from which whole plants can be raised in a very large number, is a major area of research. A number of medicinally important species have been successfully experimented for mass production by micropropagation for a source of many alkaloids (Kameswara, 2000). Alternative propagation methods would be beneficial in accelerating large-scale multiplication, improvement, and conservation of the plants. Some chemical analysis has been done on *P. amurense* (Choi et al., 1996; Ikuta et al., 1998). To date few studies have been reported on micropropagation of *P. amurense* through axillary buds, which yielded low number of shoots (Ariyoshi, 1986). This method may not be useful for large-scale production and cultivation of the elite clones. It has been shown that the shoot organogenesis from callus cultures can be used as an effective method for multiplication of medicinal plants (Castillo and Jordan, 1997).

In the present study, I have also chosen axillary shoot proliferation and adventitious shoot regeneration from hypocotyl, cotyledon, leaf, and internode as alternative methods to achieve a higher rate of shoot multiplication.

2. MATERIALS AND METHODS

2.1. Materials

In the present study, attempts were made to regenerate plantlets under *in vitro* conditions for clonal propagation of *Phellodendron amurense*. The study was carried out by the following two different approaches. First, explants from field-growing mature plants of *P. amurense* were collected and cultured under aseptic *in vitro* conditions, and then plantlets were regenerated. Second, seeds were collected from fruits, and were germinated on MS medium containing 2.2 μM BAP for raising aseptic seedlings, and then complete plants were regenerated from different parts of the seedlings.

2.1.1. Collection of plant materials

Young twigs (Fig. 1.1A) of *P. amurense* were collected from 20-year old mature trees growing in the private
forest of Tochigi Prefecture, Japan, and fruits (Fig. 1.1B-1) were collected from a 50-year-old tree growing at the Medicinal Plant Garden of Kumamoto University, Japan. Seeds were germinated aseptically in the laboratory (Fig. 1C). The primary explants collected from mature fruits as well as aseptically grown seedlings were used to conduct experiments for establishment of culture system in the present investigation. In the subsequent experiments, different segments of in vitro grown cultures were used as explants for sub-culturing. Names of the specific explant for specific purposes are mentioned below:

A. From field-growing mature plants, the following parts were used as explants for axillary shoot proliferation:
   i) Nodal segment.
   ii) Shoot tip segment.

B. From in vitro grown seedling, the following parts were used as explants for axillary and/or adventitious shoot proliferation:
   i) Nodal segment.
   ii) Shoot tip segment.
   iii) Cotyledon segment.
   iv) Hypocotyl segment.
   v) Internode segment.
   vi) Leaf.

2.1.2. Chemicals and sources
The chemical compounds including macro- and micro-nutrients, agar, gellan gum, growth additives, sucrose etc. used in the present study were the reagent grade (Plant Tissue Culture Tested) products of either Wako Pure Chem. Indus. Ltd. or Kanto Chem. Co. Inc., Japan. The vitamins and plant growth regulators were mostly products of Wako Pure Chem. Indus. Ltd or Sigma Chem. Co., U.S.A.

2.1.3. Sterilant and surfactant
In the present investigation, sodium hypochlorite solution (5.0% available chlorine in sodium hypochlorite [NaOCl], product of Wako Pure Chem. Indus. Ltd., Japan) was used as sterilizing agent, while washing detergent, Lion Co. Ltd. Japan, was used as detergent cum surfactant. Seventy percent ethyl alcohol was also used for surfactant.

2.1.4. Culture media
In the present study, mainly the MS (Murashige and Skoog, 1962) salt-based formulation were used as either original or modified forms in addition to WPM (Woody Plant Medium) (Lloyd and McCown, 1980) to fulfill the special need of the experiments, which are mentioned in appropriate places. Detailed chemical compositions of the medium formulations used are mentioned in the Appendix 1-1. However, the following culture media were used in the present study for various purposes as stated against each.

2.1.4.1. For seed germination and in vitro raised seedlings
Semi-solid MS media with different concentrations of BAP or hormone-free were used.
2.1.4.2. For differentiation and multiplication of shoots

Semi-solid MS (full strength of MS salts and vitamins), MMS: (½ strength of macronutrients, but full strength of micronutrients and organic nutrients), and WPM with different supplementation of auxin and cytokinin were used (See Appendix 1, 2, & 4).

2.1.4.3. For regeneration of roots

MS, MMS, and MMS; semi-solid media with different combinations and concentrations of auxin were used (See Appendix 1-3). Agar and gellan gum were used as source of gelling agents.

2.1.5. Plant growth regulators

The following plant growth regulators were used at different stages of the present study for regenerating complete plantlets of *P. amurense*.

**Auxin:** The following four type auxins were used to fulfill the experimental purpose:
- Indole-3-butyric acid (IBA).
- Indole-3-acetic acid (IAA).
- \( \alpha \)-Naphthaleneacetic acid (NAA).
- 2,4-Dichlorophenoxyacetic acid (2,4-D).

**Cytokinin:** The following cytokinins were used in these experiments:
- 6-Benzyl amino purine (BAP).
- 6-Furfuryl amino purine (Kinetin, Kn).
- \( N \)-\((2\)-chloro-1-pyridyl\)-\(N\)-phenyleurea (CPPU).
- Thidiazuron (TDZ).

**Gibberellin:** Only one grade of gibberellin was used in these experiments.
- Gibberellic acid grade III (GA₃)

2.2. Methods

The methodological steps followed in this investigation for carrying out the experiments are described under the following headings and sub headings:

2.2.1. Preparation of culture media

The first step in the preparation of culture medium was the preparation of the stock solutions. Different constituents of the medium were made into stock solutions for ready use during the preparation of media for different experiments. As various constituents were required in different concentrations, stock solutions of macronutrients, micronutrients, , and plant growth regulators were prepared separately and stored in a refrigerator at 4-6°C or freezer, while the stock solution of organic nutrients stored at below 0°C for several weeks.

2.2.2. Culture techniques

The following methods were employed in the present experiment for primary establishment and regeneration of complete plantlets, which are discussed under separate headings.

2.2.2.1. Surface sterilization of explants for establishing shoots culture

Different explants (shoot tips and nodal segments of mature plants) of *Phellodendron amurense* were collected, washed with detergent for 15 min and then rinsed thoroughly with running tap water for 20 min. After the explants were surface-sterilized with 70.0% ethanol (EtOH) for 3 min, they were transferred to a sterilized conical flask in a laminar-air-flow cabinet. Subsequent surface disinfection was done with 3.0% (v/v) NaOCl solution for 10, 15, 20, 25, 30, 35, and 40 min. To remove any trace of sterilant, the explants were then washed with at least three changes of sterile distilled water. Shoot segments (1-1.5 cm) containing one node were excised from the surface sterilized materials, and cultured on different media supplemented with various concentrations of cytokinins for induction of axillary buds and multiplication of shoots. The data were collected as percentage of contamination-free explants, shoot proliferation, and death of explants after 28 days of culture.
2.2.2.2. Surface sterilization of seeds for raising aseptic seedlings

Seeds were collected from the ripe fruits of *P. amurense* Rupr. and surface-sterilized by the techniques as mentioned in explant surface sterilization. The pre-washed seeds (Fig. 1.1C-2) were surface-sterilized with 70.0% EtOH for 5 min; they were transferred to a sterilized conical flask in a laminar-air-flow cabinet. Subsequent surface disinfection was done with 3.0% (v/v) NaOCl solution for 10, 15, 20, 25, 30, 35, and 40 min. To remove any trace of sterilant, the seeds were then washed with at least three changes of sterile distilled water. All subsequent operations were carried out under aseptic conditions. After that, seeds were implanted on MS medium with 2.2 μM BAP. Five to six weeks old in vitro grown seedlings were used as the source of explants for different experiments. The data were collected as percentage of contamination-free seeds after 8 days and germination of seeds after 28 days of culture.

2.2.2.3. Maintenance of proliferating cultures of axillary shoots

Proliferating cultures of axillary shoots were established on suitable medium from the explants with pre-formed meristems. The nodal segments were found to be the best for axillary shoot formation on agar-gelled MS medium with 2.0 μM of BAP. On this medium, axillary buds showed sprouting within 10 days of incubation. The usable shoots (>1.5 cm long) were excised from the proliferated cultures and transferred individually to the rooting media. Some of the shoots after removing leaves were cut into small pieces with axillary buds, and recultured to freshly prepared medium for multiplication of axillary shoots. These cultures again produced usable axillary shoots (>1.5 cm long) within 4 weeks of subculture. The process was repeated 4 times in order to ensure continuous production of axillary shoots.

2.2.2.4. Maintenance of callus and proliferating cultures of adventitious shoots

Hypocotyl, cotyledon, internode, and leaf explants produced callus and subsequently adventitious shoots on a suitable medium within 6-8 weeks of culture. At this stage, the proliferating cultures were subcultured in the same medium composition in order to enhance caulogenesis. The usable shoots (>1.5 cm long) were excised from the caulogenic cultures and transferred individually to the rooting media. Further multiplication of adventitious shoots could be achieved by subculturing the callus. Proliferation of axillary shoots could also be achieved using nodal segments of the adventitiously regenerated shoots on suitable medium. Multiplication cycles were repeated at regular intervals. The process was repeated several times in order to establish a callus culture with continuous production of shoots.

2.2.2.5. Preparation and culture of microcuttings for rooting

Experiments for root formation on shoots proliferated in vitro were conducted only after obtaining sufficient amount of shoot cultures. Different rooting experiments were conducted to find out the culture conditions, suitable medium composition, and optimum requirements of plant growth regulator. After 6-8 weeks, the proliferated multiple shoots were separated from each other, and they were rescued aseptically from the culture vessels. Then each of the microcuttings of shoots was cultured on freshly prepared rooting medium containing various concentrations of hormonal supplements or without hormone. Roots were induced on these excised shoots by placing them on different media added with various concentrations of IBA, NAA, and IAA. Gellan gum was used as gelling/supporting material.

2.2.3. Setting and designing of different experiments

For evaluation of optimum growth requirements of the cultures (both proliferating and rooting), experiments
were conducted with different chemical and physical factors of the medium. In general, cultures were grown on media containing 3.0% sucrose (Wako Pure Chem. Indus. Ltd., Japan), 0.8% agar (Wako Pure Chem. Indus. Ltd., Japan), or 0.2% gellan gum (Wako Pure Chem. Indus. Ltd., Japan), pH 5.7 ± 0.1, and they were maintained at 16 hrs photoperiod at 25 ± 1°C. When any one of these factors was varied, the others remained unchanged. There were 20 replicated cultures for shoot proliferation, callusing, and rooting experiments. Proliferating cultures were grown for four consecutive cultures, and were used for recording the data on different growth parameters. Cultures for rooting experiment were grown for total period of 6-8 weeks. The special purposes of an experiment and the important point regarding collections of data have been elaborated in the proper places under specific experiment.

2.2.4. Culture environment

The inoculated culture vessels were incubated in a growth chamber providing special culture environment. All cultures were grown in the growth chamber, where temperature was controlled at 25 ± 1°C with light intensity varied from 2000-3000 lux (50-70 μmol·m⁻²·s⁻¹). The photoperiod was maintained generally 16 hrs light and 8 hrs dark. The culture vessels were checked daily to note the morphogenic response of cultured explants in different experiments conducted in the present investigation.

2.2.5. Transplantation and acclimatization of plantlets under ex vitro environment

For transferring onto soil, the in vitro regenerated plantlets were taken out from the culture tubes, washed thoroughly under running tap water and kept in water for about 1 hr to remove any remains of sugar and agar. Plantlets were then transferred to plastic pot (6 × 9 cm) containing Kanuma soil (Kanuma Bonsai Potting Medium Co., Japan), covered with a transparent plastic cap to ensure high humidity during the acclimatization period of 20 days. They were maintained under culture room conditions. The potted plants were irrigated with MS basal salts solutions (¼ strength) devoid of sucrose and myo-inositol every 4 days for 3 weeks. Plastic cups were opened after three weeks in order to acclimatize plants to room conditions. Acclimatized plants were then transferred to the larger pot (18 × 24 cm) and maintained in green house for 3 months, and then placed outdoors under full sun.

2.3. Data Collection

Data were collected using the following parameters and the methods are given bellow.

2.3.1. For shoot induction

2.3.1.1. Percentage of explants induced shoots

Excised explants were used for shoot initiation. They were cultured separately for each combination of plant growth regulator. After 7-10 days of inoculation, the culture tubes that were contaminated by microorganisms were separated and rejected. Among the responded explants having showed shoot proliferation, data were recorded after required days of culture. Percentages of explants induced shoots were calculated using following formula:

\[
\text{Percentage of explants induced shoots} = \frac{\text{No. of cultures induced shoots}}{\text{Total number of explants cultured}} \times 100
\]

(Excluding contaminated and dead explants)

2.3.1.2. Number of shoots and average length of shoots per explant

Number and length of shoots per explant were counted after required days of culture. Mean number and length of adventitious shoots per explant were calculated using following formula:

\[
\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}
\]

Where,

\[
\bar{X} = \text{Mean number / length of shoot.}
\]

\[
n = \text{Number of observation.}
\]
\( X = \text{Total number / length of shoot.} \)
\( \Sigma = \text{Summation.} \)

2.3.2. For callus induction

2.3.2.1. Percentage of explants induced callus

Explants (hypocotyl, cotyledon, internode, and leaf) were cultured in petridishes containing culture media for callus induction. Percentage of explants induced callus was calculated using following formula:

\[
\text{Percentage of explants induced callus} = \frac{\text{No. of explants induced callus}}{\text{Total number of cultured explants}} \times 100
\]

2.3.3. For root induction

2.3.3.1. Percentage of shoots induced roots

Shoots were cultured separately in 25×120 mm culture tubes, and 200 ml culture bottles for each of the combination of media with rooting growth regulators were used. Percentages of root formation, mean number of roots per shoot, and average length of roots (cm) were calculated as mentioned above.

2.3.4. Analysis of data

Twenty explants were used for all experiments, and experiments were repeated four times. Data were subjected to ANOVA and were analyzed by JMP Statistical Discovery Software (SAS institute, SAS Campus Drive, Cary, NC, USA). When the ANOVA indicated statistical significance, a Tukey’s multiple comparison test was used to distinguish difference between treatments.

3. RESULTS

3.1. Establishment of Aseptic Cultures

3.1.1. Standardization of NaOCl treatment period for the surface sterilization of explants

For the primary establishment of in vitro culture from field-growing plants, surface sterilization of the explants was essential for loosing any contamination attached to the surface of the explants. Standardization for surface sterilization was carried out by trial and error experiments. Two types of explant, nodal and shoot tip explants from the field-growing plants of *Phellodendron amurense* were treated with 3.0% NaOCl for raising aseptic culture. To overcome contamination problem, surface sterilization of the explants was done with 3.0% (v/v) NaOCl for 10, 15, 20, 25, 30, 35, and 40 min. Nodal explants collected from mature plants were found to be very sensitive to NaOCl treatment. In the first attempt of surface sterilization with 3.0% NaOCl solution, treatment duration of 10 min, caused contamination of 100.0% explants. The most of the cultured explants showed fungal and bacterial contamination within 3 to 10 days of inoculation. Only 15.0-40.0% contamination-free cultures were obtained, when the explants were treated for 15-20 min with 3.0% NaOCl. No death of explants occurred in these treatments. These explants remained green and showed healthy growth and proliferation of axillary shoots. When the explants treated for 25-35 min, 50.0-90.0% contamination-free explants were obtained, while 10.0-70.0% explants died. Hundred-percent explants died, on the other hand, when the explants were treated with NaOCl for 40 min. Among all the treatments, nodal segments (NS) showed best performance than shoot tip (ST) segments for contamination-free, while the highest percentage of explant death was observed in shoot tip segments (Fig. 1.2). Considering the sensitivity of the explants, treatment with 3.0% NaOCl for 35 min

![Fig. 1.2. Effects of NaOCl treatment duration for the sterilization of nodal and shoot tip explants from mature plants. NS: nodal segment, ST: shoot tip.](image-url)
duration was found to be most suitable for shoot explants.

3.1.2. Standardization of NaOCl treatment period for the surface sterilization of seeds and for raising of aseptic seedlings

For raising aseptic seedlings, thoroughly washed seeds were treated with 3.0% NaOCl for various duration periods, viz. 15, 20, 25, 30, 35, and 40 min. Fifty seeds from each treatment were cultured on medium for testing their contamination and germination rates. The data were collected as percentages of contamination-free seeds after 8 days and germination of seeds after 25 days of culture. In this experiment, the highest percentage (100.0%) of contamination-free seeds was recorded in the 6 different sterilization periods, when the seeds were treated for 30-40 min with 3.0% NaOCl, while the highest frequency (80.0%) of seed germination was recorded in 30 min treatment. The lowest percentage (20.0%) of contamination-free cultures was observed in 15 min treatment with the same concentration of NaOCl. The survival ability of the sterilized seeds was a problem. It was found that the seeds treated with 3.0% NaOCl for 40 min did not germinate in maximum cultures probably due to intoxication, though they were 100.0% contamination free (Fig. 1.3). Hence, 3.0% NaOCl for 30 min duration of treatment was optimum for seed germination in this experiment to avoid contamination for higher survival.

3.2. Proliferation of Axillary Shoots

The explants carrying pre-formed meristems, shoot tips and node segments, were cultured for proliferating the axillary shoots. On the basis of a preliminary investigation on in vitro growth behavior of the explants, following parameters were considered as a selection criterion for the assessment of medium suitability, type and origin of explant, and type and concentration of plant growth regulator with the view of optimizing shoot multiplication and plantlet regeneration.

![Graph](graph.png)

**Fig. 1.3.** Effects of NaOCl treatment duration for the sterilization of seeds.

In all the subsequent experiments, these parameters were used for evaluating the success of an experiment. The growth parameters were as follows:

- a) Percentage of explants showing shoot proliferation.
- b) Number of total shoots per culture.
- c) Average length of shoots (cm).

3.2.1. Selection of explants for axillary shoot proliferation

In this experiment, 1.0-1.5 cm segments consisting of either node or shoot tip from the mature plants and in vitro raised seedlings were cultured on proliferation medium (MS) fortified with 0.5, 1.0, 2.0, and 4.0 μM BAP. Explants from the field-growing mature plants as well as from the in vitro grown seedlings were incorporated in this experiment for proliferating axillary shoots.

The proliferation efficiency of the explants from the in vitro grown seedlings was remarkably higher than that of the mature plants, when evaluated after 6 weeks of culture incubation. Moreover, nodal explants showed comparatively better proliferating efficiency than shoot tip explants, irrespective of their origin, while the nodal explants from the in vitro grown seedlings were found to be best for axillary shoot proliferation (Figs. 1.AA-D).

Fig. 1.4. Axillary shoot proliferation from the mature and seedling explants.

A, B: Shoots development from nodal (A) and shoot tip (B) explants obtained from field-growing mature plants cultured on MS with 2.0 μM BAP after 6 weeks of culture. C, D: Shoots developed from seedling-derived nodal (C) and shoot tip (D) explants cultured on MS with 2.0 μM BAP after 5 weeks of culture.

The results presented in Table 1.1 show that the seedlings raised nodal explants produced 100.0 ± 2.1% shoots, with 8.4 ± 0.8 number of total shoots per culture, and 5.7 ± 0.3 cm average length of shoots per culture, when they were cultured on MS medium with 2.0 μM BAP. When shoot tip explants were cultured on the same medium, they produced 91.3 ± 1.7% shoots, where number of total shoots per culture was 3.9 ± 0.4, and average length of shoots per culture was 6.8 ± 0.5 cm. On the other hand, the highest 60.0 ± 2.2% of shoot formation, 2.9 ± 0.2 total shoots, and 2.2 ± 0.3 cm average length were obtained from nodal explants of mature plants, when they were cultured on MS medium containing 2.0 μM BAP. However, the highest frequency 50.0 ± 1.6%, total number of shoots 1.9 ± 0.3, and average length 5.2 ± 0.4 cm were recorded from shoot tip explants of mature plants on the same medium.

Mean separation was conducted using Tukey's multiple comparison test at the 0.05 level of probability. It was observed that the highest percentage of shoot formation and the greatest number of total shoots per culture were given by the *in vitro* grown nodal explants, followed by nodal explants from field-growing mature plants, and the differences between them were statistically significant. On the other hand, the highest average length of shoots was found in the *in vitro* grown shoot tip explants, followed by shoot tip explants from field-growing mature plants, and also differences between them were statistically significant (Table 1.1).

### 3.2.2. Effects of different nutrient medium on axillary shoot proliferation

After selection of most suitable explants, experiment was further carried out to select the suitable nutrient medium. In this experiment nodal and shoot tip explants were collected from both sources (mature plants and seedlings) and cultured on different nutrient media viz. MS, MMS, and Woody plant (WP) medium supplemented with 2.0 μM BAP in each nutrient medium for standardizing the suitable medium for *in vitro* shoot proliferation.

The results presented in Table 1.2 show that among different nutrient media used, MS medium produced the best proliferation of shoots. As a result, the highest frequency (97.5 ± 1.5%) of shoot proliferation and the highest number (8.6 ± 0.3) of shoots per explant were obtained from seedling-raised nodal explants, while the highest length (7.5 ± 0.2 cm) of shoots was recorded from shoot tip explants, when they were cultured on MS medium containing 2.0 μM BAP. On the other

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Explant origin</th>
<th>Explant type</th>
<th>Shoot formation (%)</th>
<th>Total number of shoots/ explant</th>
<th>Average length of shoots/ explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature nods</td>
<td>Node</td>
<td>43.8 ± 3.5</td>
<td>34.6 ± 2.9</td>
<td>20 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>Mature shoot</td>
<td>Tip</td>
<td>44.8 ± 2.7</td>
<td>32.3 ± 2.1</td>
<td>49 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Seedlings nod</td>
<td>Node</td>
<td>91.3 ± 3.9</td>
<td>44.1 ± 0.2</td>
<td>43 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Seedlings shoot</td>
<td>Tip</td>
<td>63.2 ± 2.2</td>
<td>30 ± 1.5</td>
<td>63 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Matues nods</td>
<td>Node</td>
<td>51.3 ± 1.8</td>
<td>41.9 ± 0.4</td>
<td>48 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Matues shoot</td>
<td>Tip</td>
<td>45.3 ± 1.2</td>
<td>34 ± 0.3</td>
<td>43 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Seedlings nod</td>
<td>Node</td>
<td>46.2 ± 2.1</td>
<td>35 ± 0.5</td>
<td>67 ± 0.5</td>
<td></td>
</tr>
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<td>Seedlings shoot</td>
<td>Tip</td>
<td>80 ± 0.5</td>
<td>39 ± 0.3</td>
<td>77 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Mature nods</td>
<td>Node</td>
<td>40 ± 1.5</td>
<td>34 ± 0.2</td>
<td>57 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Mature shoot</td>
<td>Tip</td>
<td>50 ± 1.5</td>
<td>35 ± 0.6</td>
<td>63 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Seedlings nod</td>
<td>Node</td>
<td>51.5 ± 1.5</td>
<td>35 ± 0.6</td>
<td>68 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Seedlings shoot</td>
<td>Tip</td>
<td>62 ± 1.5</td>
<td>31 ± 0.3</td>
<td>62 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Data were recorded after 6 weeks of culture. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level.
WP medium, and the differences among them were statistically significant (Table 1.2).

### 3.2.3. Effects of different cytokinins and their concentrations on axillary shoot proliferation

Both the nodal and shoot tip segments responded to axillary shoot proliferation, when MS medium was fortified with different concentrations of cytokinin. From the initial experiment, it was evident that nodal segments of seedlings was the best explant and MS medium was the best medium for proliferating shoots in vitro of *P. amurensis*. In this experiment, therefore, nodal segments were collected from the *in vitro* grown seedlings and cultured on MS medium supplemented with BAP, Kn, and CPPU at concentrations of 0.5, 1.0, 2.0, 4.0, and 6.0 μM for selecting optimum type and concentration of cytokinin for the maximum shoot proliferation (Table 1.3).

In this experiment, among the three types of cytokinin, the better results were recorded on the medium supplemented with BAP after 6 weeks of culture. The concentration of 2.0 μM BAP gave the best responses, the shoot formation being 97.5 ± 1.5%. The cultured explants produced the highest number 8.6 ± 0.4 of shoots per explant. On the other hand, the highest average length 6.2 ± 0.1 cm of shoots per explant was obtained from the medium containing 1.0 μM BAP among all the treatments.

Like BAP, Kn also produced axillary shoots from nodal explants. In this experiment, among different
formulations of Kn, the medium fortified with 2.0 μM produced the highest frequency 73.8 ± 2.3% of shoot proliferation and the highest number 4.9 ± 0.4 of shoots per explant. However, the lowest result was observed for 6.0 μM Kn, where only 41.3 ± 1.9% explants showed proliferation with 1.0 ± 0.2 shoots per culture.

Besides, when the nodal explants were cultured on CPPU based medium, maximum frequency 82.5 ± 1.8% of explants showed shoot proliferation and the greatest number of total shoots per explant was 5.2 ± 0.2 at 1.0 μM, whereas the other two cytokinins (BAP and Kn) showed that maximum frequency and number of total shoots per explant were the highest at 2.0 μM. Only one shoot formed per explant at 6.0 μM CPPU.

The results of this experiment revealed that different cytokinins and their concentrations largely influenced shoot differentiation. BAP was more effective cytokinin in the proliferation efficiency than that of Kn and CPPU (Figs. 1.6A-C). The optimum concentration of BAP for shoot proliferation was found to be 0.5 to 2.0 μM.

The means compared by the Tukey’s multiple comparison test at the 0.05 level of probability. The highest percentage of shoot formation, the greatest total number of shoots per culture, and the greatest average length of shoots were obtained in BAP, followed by CPPU and Kn, and the differences among them were statistically significant (Table 1.3).

### 3.2.4. Effects of BAP and auxins on axillary shoot proliferation from the nodal explants

The nodal segments from the in vitro grown shoots were also cultured on MS medium supplemented with different concentrations of BAP (1.0 and 2.0 μM) in combinations with different concentrations (0.5, 1.0, and 2.5 μM) of NAA or IBA. The results of this experiment are presented in Table 1.4.

It was observed that the relative amount and ratio of BAP and NAA present in the medium influenced axillary shoot proliferation from the nodal explants. The highest percentage of shoot proliferation was 90.0 ± 1.1%, the greatest number 7.4 ± 0.5 of shoot per culture, and the greatest average length 5.2 ± 0.3 cm of shoots per culture, which were obtained on MS medium with 2.0 μM BAP and 0.5 μM NAA. On the other hand, a comparatively lower frequency of shoot proliferation was obtained in BAP + IBA than BAP + NAA combinations. In BAP + IBA combination, the highest percentage 66.3 ± 2.2% and 62.5 ± 1.5% of shoot proliferation were obtained with 2.0 μM BAP plus

![Fig. 1.6. Effects of cytokinin alone or with GA₃ on axillary shoot proliferation from the nodal explants of in vitro grown seedling.](image)

A-C: Axillary shoots developed on MS medium with 2.0 μM BAP (A), 2.0 μM Kn (B), and 1.0 μM CPPU (C) from the nodal explants of seedlings after 7 weeks of culture. D: Axillary shoots developed on MS medium with 2.0 μM BAP + 1.0 μM GA₃ from the in vitro raised nodal explants after 8 weeks of culture.

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Concentration (μM)</th>
<th>Shoot formation (%)</th>
<th>Total number of shoots (per explant)</th>
<th>Average length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>0.5</td>
<td>85.3 ± 1.4</td>
<td>32.6 ± 0.5</td>
<td>5.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>95.6 ± 1.2</td>
<td>67.5 ± 0.7</td>
<td>6.7 ± 0.3</td>
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<tr>
<td></td>
<td>2.0</td>
<td>95.9 ± 1.3</td>
<td>81.3 ± 0.8</td>
<td>7.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>95.2 ± 1.2</td>
<td>114.0 ± 0.5</td>
<td>10.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>53.3 ± 2.1</td>
<td>18.0 ± 0.5</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Kn</td>
<td>0.5</td>
<td>57.1 ± 1.2</td>
<td>19.0 ± 0.5</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>67.5 ± 1.3</td>
<td>24.0 ± 0.5</td>
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<tr>
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<td>2.0</td>
<td>78.3 ± 2.1</td>
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<tr>
<td></td>
<td>4.0</td>
<td>88.3 ± 3.0</td>
<td>26.0 ± 0.5</td>
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</tr>
<tr>
<td></td>
<td>6.0</td>
<td>93.3 ± 4.0</td>
<td>29.0 ± 0.5</td>
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<td>CPPU</td>
<td>0.5</td>
<td>67.5 ± 1.3</td>
<td>16.0 ± 0.5</td>
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<td>1.0</td>
<td>82.3 ± 1.2</td>
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<td></td>
<td>2.0</td>
<td>93.4 ± 1.7</td>
<td>46.0 ± 0.5</td>
<td>3.3 ± 0.5</td>
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<td>4.0</td>
<td>93.2 ± 2.0</td>
<td>43.0 ± 0.5</td>
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</tr>
<tr>
<td></td>
<td>6.0</td>
<td>96.0 ± 1.2</td>
<td>104.0 ± 0.5</td>
<td>2.3 ± 0.7</td>
</tr>
</tbody>
</table>

Values represent mean ± standard error of 10 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey’s multiple comparison test at 0.05 probability level.
0.5 μM IBA and 2.0 μM BAP plus 1.0 μM IBA, respectively. Among the BAP plus IBA combinations, the highest number 5.1 ± 0.3 of shoot and the greatest average length 4.4 ± 0.5 cm of shoot per explant were obtained on MS medium with 2.0 μM BAP and 0.5 μM IBA.

Among different combinations and concentrations of BAP with auxins, 2.0 μM BAP plus 0.5 μM NAA showed best performance for axillary shoot proliferation. BAP was more suitable for axillary shoot proliferation than BAP with auxin-supplemented medium.

Mean separation was conducted using Tukey’s multiple comparison test at the 0.05 level of probability. It was observed that the highest percentage of shoot formation, the greatest total number of shoots per culture, and the greatest average length of shoots were obtained in BAP plus NAA, followed by BAP plus IBA, and the differences between them were statistically significant (Table 1.4).

### 3.2.5. Effects of BAP and CPPU in combination with different concentrations of GA₃ on axillary shoot proliferation from nodal explants

Nodal segments excised from the in vitro grown shoots were cultured on MS medium supplemented with different concentrations (1.0 and 2.0 μM) of BAP and CPPU in combinations with different concentrations (0.5, 1.0, and 2.5 μM) of GA₃. Results of this experiment are presented in Table 1.5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot formation (%)</th>
<th>Total number of shoots (per explant)</th>
<th>Average length of shoots (per explant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP + NAA</td>
<td>1.0 μM</td>
<td>63.5 ± 1.5</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2.0 μM</td>
<td>75.2 ± 1.2</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>25 μM</td>
<td>75.2 ± 1.2</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>75.2 ± 1.2</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>75.2 ± 1.2</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>250 μM</td>
<td>75.2 ± 1.2</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>500 μM</td>
<td>75.2 ± 1.2</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

In this experiment, data were recorded after 5 weeks of culture. Data on percentage of shoot formation, number of total shoots per culture, and average length of shoots per culture revealed that the certain combinations of cytokinin (BAP or CPPU) with GA₃ promoted the formation of axillary shoots from the nodal explants. Maximum shoot proliferation was observed on the medium supplemented with 2.0 μM BAP plus 1.0 μM GA₃. On this growth regulator combination, 82.5 ± 1.5 % explants produced 5.2 ± 0.2 total shoots and 8.1 ± 0.6 cm average length of shoot per culture. On the other hand, 63.8 ± 2.4% shoot proliferation, 4.1 ± 0.3 total number of shoots, and 5.2 ± 0.7 cm average length of shoot per culture were observed on the medium supplemented with 2.0 μM CPPU and 1.0 μM GA₃. Proliferation efficiency of the nodal explants was influenced variously by combinations and concentrations of BAP and CPPU with GA₃ present in the culture media. Although the results obtained here revealed that the addition of GA₃ with cytokinin either of BAP or CPPU considerably increased the shoot length, it decreased multiple shoot formation over the control treatment (compare to Table 3) (Fig. 1.6D).

The means compared by the Tukey’s multiple comparison test at the 0.05 level of probability revealed that the highest percentage of shoot formation, the greatest total number of shoots per culture, and the greatest average length of shoots were obtained in BAP plus GA₃, followed by CPPU plus GA₃, and the differences between them were statistically significant (Table 1.5).

### 3.3. Proliferation of Adventitious Shoots

The explants without any pre-formed meristems viz. hypocotyl, cotyledon, leaf, and internode explants were used for regenerating adventitious shoots, directly or indirectly, i.e. without or with intermediate callus phase. In the following sections, results on adventitious shoot formation are presented on the basis of morphological nature of the cultured explants. The hypocotyl,
Table 1.5. Effects of BAP and CPPU in combinations with different concentrations of GA3 on axillary shoot proliferation from the nodal explants of in vitro grown shoots on MS medium after 8 weeks of culture.

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Concentration (µM)</th>
<th>Shoot formation (%)</th>
<th>Total number of shoots / explants</th>
<th>Average length of shoot (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP + GA3</td>
<td>0.05</td>
<td>41.2 ± 1.2</td>
<td>23.9 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>63.2 ± 1.5</td>
<td>34.6 ± 0.7</td>
<td>6.6 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>30.9 ± 1.2</td>
<td>14.3 ± 0.2</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2.6 + 0.5</td>
<td>11.3 ± 2.3</td>
<td>4.2 ± 0.3</td>
<td>6.6 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>83.3 ± 1.5</td>
<td>52.2 ± 0.2</td>
<td>8.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>53.3 ± 1.6</td>
<td>2.1 ± 0.3</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>CPPU + GA3</td>
<td>1.0 + 0.5</td>
<td>28.8 ± 1.9</td>
<td>1.6 ± 0.8</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>43.3 ± 1.2</td>
<td>3.1 ± 0.6</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>75.0 ± 2.3</td>
<td>3.1 ± 0.5</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4.0 + 0.5</td>
<td>53.3 ± 1.6</td>
<td>3.3 ± 0.6</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>63.3 ± 1.9</td>
<td>4.1 ± 0.3</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>75.0 ± 2.3</td>
<td>1.4 ± 0.5</td>
<td>4.3 ± 0.2</td>
</tr>
</tbody>
</table>

Values represent means ± standard error of 10 replicates per treatment in 3 repeated experiments. Means followed by the same letter are not significantly different by Tukey’s multiple comparison test at 0.05 probability level.

cotyledon, leaf, and internode explants from the aseptically raised in vitro grown seedlings were cultured on MS medium supplemented with different combinations and concentrations of BAP with either NAA or IBA for inducing and developing the adventitious shoots.

(A) Direct Adventitious Shoot Regeneration

In order to find out the different responses of different explants, viz. hypocotyl, cotyledon, leaf, and internode explants, were tested for direct regeneration of adventitious shoots through no callus phase. All the four types of explant were cultured on MS medium containing different combinations and concentrations of plant growth regulators. Results on adventitious shoot formation are presented in the following sections on the basis of different explants.

3.3.1. Effects of BAP with auxins on shoot regeneration from hypocotyl explants

Hypocotyl explants (1.0 - 1.5 cm) were cultured on MS medium supplemented with different concentrations of BAP in combinations with two auxins, NAA and IBA. Almost all of the media induced adventitious shoots without forming callus after 7 weeks of culture. Results obtained on morphogenic responses of the cultured explants are presented in Table 1.6. Among various combinations of cytokinin and auxin, the cultured explants showed the best proliferation result in BAP with NAA combination than BAP with IBA combination. Out of the nine BAP-NAA combination, the medium containing 2.2 µM BAP plus 0.5 µM NAA induced the highest frequency of 97.5 ± 1.2% adventitious shoots, maximum number 45.0 ± 0.3 of shoots, and the greatest average length 5.0 ± 0.2 cm of the elongated shoot per explant with small callusing (Figs. 1.7A,B). Relatively high shoot formation (92.5 ± 1.2%), total number of shoot (40.4 ± 0.3), and average length of shoot (3.5 ± 0.4 cm) were obtained from the medium supplemented with 4.4 µM BAP with 0.5 µM NAA.

Among the BAP-IBA formulations, maximum frequency (75.0 ± 2.1%) of shoot proliferation was found in the medium containing 2.2 µM BAP plus 0.5 µM IBA. This combination also produced the maximum 32.4 ± 0.3 total shoot, the greatest average length 3.9 ± 0.1 cm of the elongated shoot per explant. In this experiment, it was found that 2.2 µM BAP plus 0.5 µM NAA was the best medium supplementation for
proliferating adventitious shoots from hypocotyl explants.

The means compared with the help of Tukey’s multiple comparison test showed that the highest percentage of shoot formation, the greatest total number of shoots per culture, and the greatest average length of shoots were obtained with BAP plus NAA, followed by BAP plus IBA, and the differences between them were statistically significant at the 0.05 level of probability (Table 1.6).

### 3.3.2. Effects of BAP with auxins on shoot regeneration from cotyledon explants

Cotyledon explants were cultured on MS media supplemented with different concentrations of BAP in combinations with two auxins, NAA or IBA for regenerating adventitious shoots. Results on morphogenic responses of the cultured explants are presented in Table 1.7. After 4 weeks of culture, all the cultured explants showed development of adventitious shoot buds from the cut ends without callusing (Figs. 1.7C,D). Among the media containing different concentrations of cytokinin and auxin tested here, BAP-NAA formulation showed a better performance than the other formulations of BAP-IBA. Among 18 formulations of plant growth regulator used here, the cultured explants produced adventitious shoots in 16 formulations. A combination of 2.2 μM BAP and 2.5 μM NAA failed to produce any adventitious shoots, but produced large amount of callus (Data not shown). Percentage of shoot formation, total number of shoots per explant, and average length of shoots from different treatments were recorded after 4-week interval of culture. Among the BAP with NAA combinations, maximum percentage of 77.5 ± 1.6% explants showed shoot bud differentiation and the greatest number of 36.0 ± 0.3 of shoots at 6.6 μM BAP plus 0.5 μM NAA, whereas the greatest average length of 7.2 ± 0.7 cm per explant was obtained with 4.1 μM BAP plus 0.5 μM NAA.

Among BAP-IBA formulations, maximum number of shoots per culture was 25.4 ± 0.8 and maximum average length of the elongated shoots was 4.8 ± 0.8 cm that were observed in 71.3 ± 2.2% cultures at 0.6 μM BAP plus 0.5 μM IBA and in 42.5 ± 2.4% cultures at 4.4 μM BAP plus 0.5 μM IBA, respectively. Between the two auxins tested, NAA was more effective than IBA for inducing adventitious shoots. In this experiment, it was found that 0.6 μM BAP plus 0.5 μM NAA was the best medium supplementation for proliferating adventitious shoots from cotyledon explants.

The means were compared using the Tukey’s multiple comparison test, the results indicating that the highest percentage of shoot formation, the greatest total number of shoots per culture, and the greatest average length of shoots were obtained in BAP plus NAA, followed by BAP plus IBA, and the differences between them were statistically significant at the 0.05 level of probability (Table 1.7).

### 3.3.3. Effects of BAP with auxins on shoot regeneration from the leaf explant of in vitro grown shoots

In this experiment, the leaf explants were collected from in vitro grown seedlings and cultured on MS medium supplemented with various concentrations of BAP (2.2, 4.4, and 6.6 μM) and in combinations with either of NAA or IBA for proliferating adventitious shoots from the leaf explants. Under appropriate combinations, the cultured explants formed a number of adventitious shoot

---

**Table 1.6. Effects of different combinations and concentrations of BAP with NAA or IBA on adventitious shoot regeneration from hypocotyl explants.**

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Concentration (μM)</th>
<th>Shoot formation (%)</th>
<th>Total number of shoots (mean)</th>
<th>Average length of shoots (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP + NAA</td>
<td>0.0 - 0.5</td>
<td>20.6 ± 1.5</td>
<td>45.6 ± 2.7</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>50.3 ± 2.1</td>
<td>57.7 ± 2.8</td>
<td>1.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>97.5 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>97.3 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>4.4 - 0.5</td>
<td>95.1 ± 1.5</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>6.0 - 0.5</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>8.0 - 0.5</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>13.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>77.1 ± 1.0</td>
<td>204.6 ± 2.7</td>
<td>20.4 ± 0.7</td>
</tr>
</tbody>
</table>

Data were recorded after 10 weeks of culture. Values represent mean ± standard error of 20 explants per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey’s multiple comparison test at 0.05 probability level. (Symbol indicates no response.)
buds within 7 weeks of culture. Results obtained on morphogenic responses of the cultured explants are presented in Table 1.8.

Among various combinations of BAP-NAAs, the highest regeneration response achieved with each explant was recorded after 6 weeks of incubation, and the maximum frequency of shoot bud differentiation was 72.5 ± 1.7% explants, this fact being observed with medium containing 4.4 μM BAP plus 0.5 μM NAA. Besides, another combination of 6.6 μM BAP plus 0.5 μM NAA also produced considerable percentage of shoots, where 62.5 ± 2.3% shoot proliferation was recorded. The maximum number of 24.4 ± 0.2 shoots and the greatest average length 6.5 ± 0.4 cm of shoot per explant were observed on the medium containing 4.4 μM BAP plus 0.5 μM NAA (Figs. 1.7E,F). Among BAP-IBA formulations, the maximum frequency 51.3 ± 2.1% of shoot formation was obtained, when they were cultured on the medium containing 4.4 μM BAP plus 0.5 μM IBA. Maximum number and average length of shoots were 18.1 ± 0.3 and 5.9 ± 0.5 cm, respectively, which were also recorded on the medium containing 4.4 μM BAP plus 0.5 μM IBA.

The means compared by the Tukey’s multiple comparison test showed that the highest percentage of shoot formation, the greatest total number of shoots per culture, and the greatest average length of shoots were obtained in BAP plus NAA, followed by BAP plus IBA, and the differences between them were statistically significant at the 0.05 level of probability (Table 1.8).

### 3.3.4. Effects of BAP with auxins on shoot regeneration from the internode explants of in vitro grown shoots

Internode explants were cultured on MS media with different concentrations of BAP in combinations with two auxins, NAA or IBA for inducing direct adventitious shoot regeneration. The data on percentage of shoot growth, number of total shoots per explant, and average length of shoots from different treatments were recorded at 8 weeks of culture initiation. Results obtained on morphogenic response of the cultured explants are presented in Table 1.9.

In this experiment, among nine concentration of BAP with NAA, the cultured explants showed the best results on the medium with 2.2 μM BAP plus 0.5 μM NAA that induced direct adventitious shoot development without intervening unorganized callusing stage. Frequency of shoot proliferation was maximum 62.5 ± 1.3%, the greatest number of total shoots per culture was 19.3 ± 0.3, and the greatest average length of the shoot was 4.8 ± 0.2 cm, when they were cultured on the medium containing 2.2 μM BAP plus 0.5 μM NAA (Figs. 1.7G,H). On the other hand, among BAP-IBA formulations, maximum frequency of 48.8 ± 1.8% adventitious shoots formation, the greatest number of 15.3 ± 0.1 shoots, and the greatest average length 3.2 ± 0.3 cm of shoot per culture were observed, when

### Table 1.7. Effects of different combinations and concentrations of BAP with NAA or IBA on adventitious shoot regeneration from cotyledon explants.

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Concentration (μM)</th>
<th>Shoot formation (%)</th>
<th>Total number of shoots / explant</th>
<th>Average length of shoots / explant (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAP + NAA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 + 0.5</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td><strong>BAP + IBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 + 0.5</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Data were recorded after 8 weeks of culture. Values represent mean ± standard error of 20 repetitions per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey’s multiple comparison test at 0.05 probability level. Hyphen indicates no response.

### Table 1.8. Effects of different combinations and concentrations of BAP with NAA or IBA on adventitious shoot regeneration from leaf explants.

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Concentration (μM)</th>
<th>Shoot formation (%)</th>
<th>Total number of shoots / explant</th>
<th>Average length of shoots / explant (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAP + NAA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 + 0.5</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td><strong>BAP + IBA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 + 0.5</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>72.5 ± 1.7</td>
<td>18.1 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Data were recorded after 30 weeks of culture. Values represent mean ± standard error of 20 repetitions per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey’s multiple comparison test at 0.05 probability level. Hyphen indicates no response.
they were cultured on the medium containing 2.2 μM BAP plus 0.5 μM IBA. As the results, this experiment revealed that 2.2 μM BAP plus 0.5 μM NAA was the best supplementation for induction and proliferation the adventitious shoots from internode explants of experimental species of *P. amurense*.

Mean separation was conducted using Tukey's multiple comparison test at the 0.05 level of probability. The results showed that the highest percentage of shoot formation, the greatest total number of shoots per culture, and the greatest average length of shoots were obtained in BAP plus NAA, followed by BAP plus IBA, and the differences between them were statistically significant (Table 1.9).

### 3.3.5. Effects of plant growth regulators on regeneration efficiency of hypocotyl, cotyledon, leaf, and internode explants

The effects of different combinations and concentrations of cytokinin-auxin on adventitious shoot regeneration were studied using hypocotyl, cotyledon, leaf, and internode explants.

The pooled mean results presented in Table 1.10 show that between two types of plant growth regulators, BAP plus NAA and BAP plus IBA, BAP plus NAA produced the highest percentage (35.0 ± 2.4%) of shoot formation, the greatest number (12.7 ± 0.5) of shoots and the maximum average length (2.7 ± 0.3 cm) of shoots for all explants. However, BAP plus IBA combination produced 26.3 ± 9.9% shoot formation, 9.5 ± 0.3 total shoots, and 1.9 ± 0.1 cm average length of shoot. The highest frequency 39.3 ± 2.5% of shoot formation and the greatest total number of 17.7 ± 0.4 shoots were obtained from hypocotyl explants, while cotyledon, leaf, and internode explants produced 31.5 ± 1.9%, 27.7 ± 2.7%, and 24.1 ± 2.4% shoot formation, 12.1 ± 0.6, 8.1 ± 0.9, and 6.4 ± 0.7 total shoots per explant respectively, when they were cultured on BAP with NAA supplemented medium. The highest average length of shoot was 3.0 ± 0.5 cm, which was obtained from cotyledon explant. In this experiment, it was revealed that BAP plus NAA was the best growth regulator formulation and hypocotyl explant was the best explant for direct adventitious shoot proliferation.

The differences between the pooled mean of different plant growth regulators and explants were compared with the help of Tukey's multiple comparison test at the 0.05 level of probability. BAP plus NAA combination was significantly different from BAP plus IBA combination for all the parameters. The regeneration efficiency significantly depended on explant types. High percentage of shoot formation, and maximum total number of shoots per culture were obtained in hypocotyl, followed by cotyledon, leaf, and internode explants, and the differences among them were statistically significant. On the other hand, the highest average length of shoots was obtained in cotyledon, followed by leaf, hypocotyl, and internode explants, and also differences among them were statistically significant (Table 1.10).

### (B) Indirect Adventitious Shoot Regeneration

Experiments were also conducted to find out the optimum condition for induction and growth of the adventitious shoots indirectly (through callus formation) from the *in vitro* grown seedling explants. In the present study, the cultured explants like hypocotyl, cotyledon, leaf, and internode explants responded to callus...
formation and subsequently regenerated shoots under the influence of plant growth regulators. These morphogenic activities were mostly dependent on different combinations and concentrations of cytokinin and auxin applied to the culture medium. Results on indirect regeneration of the adventitious shoots are presented in the following sections.

### 3.3.6. Effects of cytokinin and auxin on callus induction from different explants

The effects of different combinations and concentrations of cytokinin-auxin on callus induction were studied with using hypocotyl, cotyledon, leaf, and internode explants. These explants were cultured on MS medium supplemented with different concentrations of BAP and TDZ in combinations with two auxins, 2,4-D or NAA for callus induction. Morphology and the amount of callus formed varied with different levels of auxin in combination with cytokinin. After 4 weeks of culture, all the cultured explants showed growth of callus from the cut ends. Among the media combinations containing different concentrations of cytokinin and auxin, TDZ + 2,4-D formulation showed the best performance compared to all the other combinations used. Percentage of callus and callus fresh weight (g) from different treatments were recorded after 4-week interval of culture (Table 1.11). The lower concentration (2.0 μM) of auxin with 2.0 μM BAP produced low frequency and less amount of callus. Hypocotyl, cotyledon, leaf, and internode explants produced highly proliferating friable callus showing green in the medium containing 2.0 μM TDZ with 4.0 μM 2,4-D, and the highest percentage 100.0 ± 2.1% for hypocotyl, 88.5 ± 1.9% for cotyledon, 85.0 ± 1.2% for leaf, and 95.0 ± 1.3% for internode explants. The highest amount 4.3 ± 0.3 g of callus for hypocotyl, 3.6 ± 0.4 g for cotyledon, 3.2 ± 0.2 g for leaf, and 4.0 ± 0.7 g for internode were also obtained on the same medium. The second highest frequency were 92.5 ± 1.1%, 80.0 ± 1.2%, 72.5 ± 1.0%, and 87.5 ± 1.1%, for hypocotyl, cotyledon, leaf, and internode explants, respectively, which were recorded in MS medium containing 2.0 μM TDZ with 4.0 μM NAA, and their callus weights were 3.7 ± 0.2 g, 3.0 ± 0.1 g, 2.6 ± 0.4 g, and 3.3 ± 0.4 g, respectively. BAP with 2,4-D or NAA also produced considerable callus (Table 1.11). In the present study, it was revealed that 2.0 μM TDZ with 4.0 μM 2,4-D or NAA was the best formulation, and hypocotyl was the best explant for callus production in P. amurense.

Mean separation was conducted using Tukey’s multiple comparison test at the 0.05 level of probability. It was observed that the highest percentage of callus formation and callus fresh weight were obtained in TDZ plus 2,4-D, followed by TDZ plus NAA, BAP plus NAA, and BAP plus 2,4-D, and the differences among them were significant. The callusing efficiency significantly was dependent on explant types. High percentage of callus formation, and high callus weight were obtained in hypocotyl, followed by internode, cotyledon, and leaf explants, and also differences among them were statistically significant (Table 1.11).
3.3.7. Effects of cytokinin and auxin on shoot induction from hypocotyl-derived callus

After proper establishment of the callus culture, the next step of the experiment was to induce shoot differentiation from callus. For this, hypocotyl-derived callus induced on MS medium containing 2.0 μM TDZ plus 4.0 μM NAA was cultured on MS medium supplemented with different concentrations of BAP and CPPU combined with either NAA or IBA. The results are shown in Table 1.12. It was noticed that adventitious shoot buds were formed from the hypocotyl-derived callus within 4 weeks of culture, and they elongated accompanied with leaf formation within 8 weeks of culture (Figs. 1.8A, B). Among the different cytokinin-auxin combinations tested, 1.5 μM BAP with 1.0 μM NAA showed the highest shoot regeneration frequency (90.0 ± 2.2%), the greatest number (45.0 ± 0.1) and the greatest shoot length (6.8 ± 0.3 cm) of shoot per callus clump. Shoots regenerated on CPPU with NAA or IBA showed slow growth with poor internodal elongation, whereas medium supplemented with BAP and IBA produced considerable shoot regeneration. The present study showed that low and high concentrations of cytokinin and auxin suppressed the rate of shoot regeneration.

The means compared with the help of Tukey’s multiple comparison test at the 0.05 level of probability showed that the highest percentage of shoot formation, mean number of total shoots and mean shoot length were obtained in BAP plus NAA, followed by BAP plus IBA, CPPU plus NAA, and CPPU plus IBA, and the differences among them were statistically significant (Table 1.12).

3.3.8. Effects of cytokinin and auxin on shoot induction from cotyledon-derived callus

The effects of different combinations and concentrations of cytokinin-auxin on multiple shoot induction were studied using cotyledon-derived calli induced on MS medium containing 2.0 μM TDZ plus 4.0 μM NAA. These calli were cultured on MS medium supplemented with different concentrations of BAP and CPPU in combinations with two auxins, NAA or IBA for regenerating adventitious shoots. After 4 weeks of culture, cultured explants showed growth of callus with adventitious shoot buds from the callus (Fig. 1.8C).

The results are shown in Table 1.13. Among the different combinations and concentrations of cytokinin and auxin, BAP-NAA formulation showed a better performance than the other combinations. The combination of 1.5μM BAP and 1.0 μM NAA showed

![Fig. 1.8. Indirect adventitious shoot regeneration from callus of different explants.](image)

Table 1.12. Effects of plant growth regulators on adventitious shoot regeneration from hypocotyl-derived callus.

<table>
<thead>
<tr>
<th>Growth regulator(s)</th>
<th>Shoot formation (%)</th>
<th>Number of total shoots/callus</th>
<th>Average length of shoot/callus (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP + NAA 1.0-4.0 μM</td>
<td>68.5 ± 3.0*</td>
<td>362 ± 12.0*</td>
<td>5.2 ± 0.7*</td>
</tr>
<tr>
<td>3.5-1.0</td>
<td>75.5 ± 1.0*</td>
<td>450 ± 13.0*</td>
<td>6.8 ± 0.7*</td>
</tr>
<tr>
<td>4.0-1.0</td>
<td>75.5 ± 1.0*</td>
<td>353 ± 13.0*</td>
<td>5.5 ± 0.7*</td>
</tr>
<tr>
<td>BAP + IBA 1.0-4.0 μM</td>
<td>46.5 ± 2.0*</td>
<td>278 ± 10.0*</td>
<td>4.0 ± 0.7*</td>
</tr>
<tr>
<td>3.5-1.0</td>
<td>60.5 ± 2.0*</td>
<td>360 ± 13.0*</td>
<td>6.5 ± 0.7*</td>
</tr>
<tr>
<td>4.0-1.0</td>
<td>60.5 ± 2.0*</td>
<td>260 ± 14.0*</td>
<td>5.2 ± 0.7*</td>
</tr>
<tr>
<td>CPPU + NAA 1.0-4.0 μM</td>
<td>42.0 ± 1.0*</td>
<td>202 ± 8.0*</td>
<td>3.2 ± 0.7*</td>
</tr>
<tr>
<td>3.5-1.0</td>
<td>55.0 ± 1.0*</td>
<td>320 ± 6.0*</td>
<td>4.7 ± 0.7*</td>
</tr>
<tr>
<td>4.0-1.0</td>
<td>55.0 ± 1.0*</td>
<td>205 ± 10.0*</td>
<td>4.1 ± 0.7*</td>
</tr>
<tr>
<td>CPPU + IBA 1.0-4.0 μM</td>
<td>38.5 ± 1.0*</td>
<td>312 ± 6.0*</td>
<td>3.5 ± 0.7*</td>
</tr>
<tr>
<td>3.5-1.0</td>
<td>42.0 ± 1.0*</td>
<td>245 ± 8.0*</td>
<td>3.5 ± 0.7*</td>
</tr>
<tr>
<td>4.0-1.0</td>
<td>42.0 ± 1.0*</td>
<td>213 ± 10.0*</td>
<td>3.0 ± 0.7*</td>
</tr>
</tbody>
</table>

Data were recorded after 8 weeks of culture. Values represent means ± standard error of 30 replicates per treatment in 3 replicated experiments. Means followed by the same letter are not significantly different by Tukey’s multiple comparison test at 0.05 probability level.
the highest shoot regeneration frequency (75.0 ± 1.4%) , the greatest number (36.5 ± 1.2) and the greatest length (5.0 ± 0.5 cm) of shoots per callus clump (Fig. 1.8D). The second highest frequency (67.5 ± 2.3%) was recorded in MS medium containing 1.5 μM BAP plus 1.0 μM IBA. Shoots regenerated on CPPU with NAA or IBA showed lower frequency than BAP with NAA or IBA combinations. The present study showed that low and high concentrations of a cytokinin (1.0 and 3.0 μM of BAP or CPPU) and an auxin (0.5 and 2.0 μM of NAA or IBA) suppressed the rate of shoot regeneration.

The means were compared with the help of Tukey’s multiple comparison test at the 0.05 level of probability. It was observed that the highest percentage of shoot formation, mean number of total shoots, and mean shoot length were obtained in BAP plus NAA, followed by BAP plus IBA, CPPU plus NAA, and CPPU plus IBA, and the differences among them were statistically significant (Table 1.13).

### 3.3.9. Effects of cytokinin and auxin on shoot induction from leaf-derivered callus

The light green-friable callus from leaf induced on MS medium containing 2.0 μM TDZ plus 4.0 μM NAA was cultured in MS medium supplemented with different combinations and concentrations of cytokinins (1.0, 1.5, 3.0 μM BAP or CPPU) and auxins (0.5, 1.0, 2.0 μM NAA or IBA) to find out the shoot differentiation efficiency from leaf-derivered callus. Data on percentage of calluses forming shoots, and number and length of differentiated shoots were recorded after 8 weeks of inoculation. The results are shown in Table 1.14.

It was noticed that adventitious shoot buds were formed from the leaf-derived callus within 4 weeks of culture, and they elongated accompanied with leaf formation within 8 weeks of culture. Among the different cytokinin-auxin combinations tested, 1.5 μM BAP with 1.0 μM NAA showed the highest shoot regeneration frequency (80.0 ± 1.5%), the greatest number (42.0 ± 1.2), and the greatest shoot length (6.0 ± 0.5 cm) of shoot per callus clump (Figs. 1.8E,F). Shoots regenerated on CPPU with NAA or IBA showed slow growth with poor internodal elongation, whereas the media supplemented with BAP and IBA showed considerable shoot regeneration. The present study showed that low and high concentrations of cytokinin (1.0 and 3.0 μM of BAP or CPPU) and auxin (0.5 and 2.0 μM of NAA or IBA) suppressed the rate of shoot regeneration.

The means compared with the help of Tukey’s multiple comparison test indicated that the highest percentage of shoot formation, the greatest mean number of total shoots, and the greatest mean shoot length were obtained in BAP plus NAA, followed by BAP plus IBA, CPPU plus NAA, and CPPU plus IBA, and the differences among them were statistically significant at the 0.05 level of probability (Table 1.14).

### 3.3.10. Effects of cytokinin and auxin on shoot induction from internode-derived callus

The regeneration efficiency significantly depended on explant types. In previous studies, the shoot regeneration efficiency of hypocotyl- , cotyledon- and leaf-derivered calli was tested. In this experiment, the internode-derived calli were used for testing their shoot forming efficiency. Internode-derived calli which were induced on MS medium containing 2.0 μM TDZ with 4.0 μM NAA were cultured on MS medium supplemented with 1.0, 1.5, and 3.0 μM of BAP or CPPU in combination
with 0.5, 1.0, and 2.0 μM of NAA or IBA. The results are shown in Table 1.15. The maximum frequency of shoot formation from internode-derived callus was 78.7 ± 1.2%, which were recorded on 1.5 μM BAP plus 1.0 μM NAA-containing MS medium. The highest number 35.2 ± 0.1 of shoots and maximum length 5.7 ± 0.3 cm of shoot per callus clump were also obtained on the same medium (Figs. 1.8G,H). Among the BAP-IBA formulations, on the other hand, maximum of 70.0 ± 1.4% callus clump produced adventitious shoots, with the greatest number 33.2 ± 0.1 of shoots and the greatest length 5.1 ± 0.2 cm, when they were cultured on the medium containing 1.5 μM BAP plus 1.0 μM IBA. Shoots regenerated on CPPU with NAA or IBA showed lower frequency than BAP with NAA or IBA combinations. The present study also revealed that low and high concentrations of cytokinin (1.0 and 3.0 μM of BAP or CPPU) and auxin (0.5 and 2.0 μM of NAA or IBA) suppressed the rate of shoot regeneration from internode-derived callus.

The means compared with the help of Tukey's multiple comparison test at the 0.05 level of probability showed that the highest percentage of shoot formation, mean number of total shoots, and mean shoot length were obtained in BAP plus NAA, followed by BAP plus IBA, CPPU plus NAA, and CPPU plus IBA, and the differences among them were statistically significant (Table 1.15).

### Table 1.14. Effects of plant growth regulators on adventitious shoot regeneration from leaf-derived callus.

<table>
<thead>
<tr>
<th>Growth regulator (μM)</th>
<th>Shoot formation (%)</th>
<th>Number of total shoots (cm)</th>
<th>Average length of shoots (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP + NAA</td>
<td>1.0 - 0.5</td>
<td>55.2 ± 1.5a</td>
<td>27.9 ± 0.9b</td>
</tr>
<tr>
<td></td>
<td>1.0 - 1.0</td>
<td>58.6 ± 3.1a</td>
<td>24.2 ± 1.5c</td>
</tr>
<tr>
<td></td>
<td>1.0 - 2.0</td>
<td>65.1 ± 1.8a</td>
<td>35.7 ± 1.6c</td>
</tr>
<tr>
<td>BAP + IBA</td>
<td>1.0 - 0.5</td>
<td>56.7 ± 3.1a</td>
<td>26.9 ± 0.9b</td>
</tr>
<tr>
<td></td>
<td>1.0 - 1.0</td>
<td>56.7 ± 1.7a</td>
<td>33.0 ± 0.9b</td>
</tr>
<tr>
<td></td>
<td>1.0 - 2.0</td>
<td>58.7 ± 1.2a</td>
<td>27.0 ± 0.9b</td>
</tr>
<tr>
<td>CPPU + NAA</td>
<td>1.0 - 0.5</td>
<td>52.2 ± 2.1a</td>
<td>52.2 ± 0.9b</td>
</tr>
<tr>
<td></td>
<td>1.0 - 1.0</td>
<td>68.6 ± 1.7a</td>
<td>26.2 ± 0.9b</td>
</tr>
<tr>
<td></td>
<td>1.0 - 2.0</td>
<td>67.2 ± 2.7a</td>
<td>19.7 ± 0.8b</td>
</tr>
<tr>
<td>CPPU + IBA</td>
<td>1.0 - 0.5</td>
<td>52.2 ± 1.3a</td>
<td>27.1 ± 0.8b</td>
</tr>
<tr>
<td></td>
<td>1.0 - 1.0</td>
<td>47.2 ± 1.4a</td>
<td>24.9 ± 0.9b</td>
</tr>
<tr>
<td></td>
<td>1.0 - 2.0</td>
<td>38.6 ± 1.1a</td>
<td>16.7 ± 0.8b</td>
</tr>
</tbody>
</table>

Data were recorded after 8 weeks of culture. Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level.

### Table 3.3.11. Effects of subculture on shoot regeneration efficiency of leaf-derived callus

Another experiment was designed to test the effects of repeated subculturing on shoot regeneration. After excising the elongated shoots, the callus pieces (approximately 1.0 g fresh weight of each callus piece) were subcultured at 4-week intervals onto fresh MS medium supplemented with 2.2 μM BAP. Up to 6 passages, the effects of subculture were tested for its regeneration ability. Data on percentage of callus forming shoots, number and length of differentiated shoots were recorded after 8 weeks of culture. The formation of fresh shoot buds from callus was noticed at every second week of subculture. These shoot buds attained an appropriate height by the third week of subculture. Elongated shoots were excised and transferred for rooting in every subculture. The frequency of shoot regeneration reached its maximum (91.3 ± 1.6%) after the 3rd subculture. The number of regenerated shoots per callus increased drastically from the first subculture (21.5 ± 0.3) to 3rd subculture (65.0 ± 0.9) and then gradually decreased (Fig. 1.9). Nevertheless, there was no significant difference in the frequency of shoot regeneration among the 3rd and 4th subculture, whereas they showed significant differences in the number and length of shoots.
3.3.12. Effects of sucrose concentrations on growth and multiplication of shoots

Sucrose is a main factor for in vitro shoot proliferation. In this experiment, nodal explants from the in vitro regenerated shoots were cultured on MS medium supplemented with 2.0 μM BAP and six different concentrations of sucrose viz. 10, 20, 30, 40, 50, and 60 g/l. Absence of sucrose in the medium (gelled with 8.0 g/l of agar) resulted in complete inhibition of growth and sprouting of axillary shoots and shoot formation rate was only 5.0 ± 2.1%. The whole explants became chlorotic and eventually necrotic within 3-4 weeks of incubation. However, presence of different concentrations of sucrose (10-60 g/l) in the medium largely changed the degree of shoot growth.

After 6 weeks of culture on the media containing different concentrations of sucrose, percentage of shoot formation, total number of shoots, and average length of shoots (cm) per culture were shown in Fig. 1.10. Among the different concentrations of sucrose in MS medium, the media containing 30 g/l sucrose showed the optimum result for percentage of shoot formation, number of total shoot per explant, and length of the longest shoot, and they were 97.5 ± 1.7%, 8.6 ± 0.6 shoots per explant, and 6.0 ± 0.2 cm in length, respectively. Among the 60 g/l sucrose containing medium showed the lowest frequency of shoot formation, number of total shoot and average length of shoot per culture, and they were 20.0 ± 2.5%, 1.2 ± 0.2, and 1.6 ± 0.1 cm, respectively. The present investigation showed that different concentrations of sucrose affected in vitro growth of _P. amurense_ shoots variously. Complete inhibition of sprouting and development of shoots cultured on sucrose-free medium indicated the essentiality of an easily accessible energy source in the proliferation medium.

3.3.13. Effects of pH levels in the medium on growth and multiplication of shoots

The pH of the culture medium is an other important factor for proliferating shoots in vitro. Excised nodal segments were taken from the in vitro grown shoots and used as explants in this experiment for optimizing pH level of the medium. Nodal segments were cultured on MS medium containing 2.0 μM BAP at six different pH levels viz. 4.0, 4.7, 5.0, 5.7, 6.0, and 6.7. The results are shown in Fig. 1.11.

Among these pH levels, the highest percentage 98.8 ± 1.5% of explants showing proliferation, the maximum number 8.2 ± 0.2 of shoot and the greatest length 6.1 ± 0.3 cm of shoot were observed on the medium adjusted to pH 5.7. The lowest frequency 20.0 ± 1.7% of explants showing proliferation was observed on the medium, the pH of which was adjusted to 4.0. The lowest number 1.9 ± 0.1 and length 2.0 ± 0.5 cm
3.4. Induction of Roots in Regenerated Shoots

3.4.1. Effects of different auxins and various strength of MS medium on root induction in microcuttings

Leafy shoots (2-4 cm in length) obtained from in vitro differentiated leaf-derived shoots were transferred to MS, MMS$_1$, and MMS$_2$ media for rooting, and one week after inoculation, root formation was noticed from basal cut portion of the shoots. The percentage of shoot forming root, number of root per shoot, and length of root were influenced by the medium type, the concentrations, and kind of auxins (Fig. 1.12). A satisfactory improvement was obtained in rooting experiment, where about 98.8 ± 1.1% shoots induced roots on MS medium containing 2.0 µM IBA with a fairly good length (5.5 ± 0.4 cm) and number (7.2 ± 0.8) of root per shoot (Fig. 1.13A). In contrast, the media containing 1.0 µM NAA produced a maximum frequency of 91.3 ± 1.8% root formation and the
greatest number 5.4 ± 0.7 of root, and root length was about 3.4 ± 0.2 to 4.0 ± 0.8 cm (Fig. 1.13B). Medium supplemented with 1.0 and 2.0 μM IAA produced a maximum frequency of 58.8 ± 2.3% and 61.3 ± 1.6% root formation (Fig. 1.13C). The greatest length of a root per shoot was 2.9 ± 0.2 cm and 3.6 ± 0.8 cm, respectively, while no significant difference was observed among root numbers in IAA-containing media (Fig. 1.12). On the other hand, high concentrations (4.0 μM) of IBA and IAA caused intensive callusing and produced relatively few roots, while NAA (4.0 μM) failed to produce any roots in all strengths of MS medium. Malformation and slow growth of roots were also observed on the MS medium containing 2.0 μM NAA (Fig. 1.13E). On the other hand, all kind of media supplemented with 4.0 μM NAA failed to produce any roots but produced only callus at the base of microcuttings (Fig. 1.13F). In this study, no rooting was found from the base of any microcuttings prior to the first week of culture. Rooting frequency increased gradually with the increase in culture period, and reached the maximum percentage after 20 days of culture on full strength of MS medium. However, maximum percentage of roots was obtained from MMS₁ and MMS₂ media after 30 and 40 days of culture, respectively. Moreover, a drastic inhibitory effect on both formation and elongation of root was noted at MMS₁ and MMS₂ media.

As shown in Table 1.16, the differences between the pooled mean of different medium and auxins were compared with the help of Tukey’s multiple comparison test at the 0.05 level of probability. MS medium was significantly different from MMS₁ and MMS₂ media for all the parameters. The rooting efficiency was significantly dependent on auxin types. The highest percentage of root formation and the greatest total number of root per culture were obtained in IBA, followed by NAA and IAA, and the differences among them were statistically significant. Although the highest average length of root was also found in IBA, followed by IAA and NAA, the differences between IAA and NAA were not statistically significant (Table 1.16).

### 3.4.2. Effects of dark treatment on adventitious root formation in auxin-free medium

In the preliminary rooting experiments, it was noticed that although every auxin treatment produced 98.8% rooting of *P. amurense* microcuttings, there were difficulties like excessive callus formation and malformation of roots in certain treatments. However, some plants produced roots in vitro even in the absence of auxin. To prove whether or not *P. amurense* microcuttings can root without auxin, an experiment was also conducted. The results are shown in Fig. 1.14. For this study, microcuttings were prepared from the elongated shoots, and were cultured on MS medium without any auxin supplement. This experiment was conducted using two sets of microcuttings (20 cuttings in each set) cultured on auxin-free rooting medium. One set was cultured in incubators maintained at 26°C under dark condition for initial one week, and thereafter

### Table 1.16. Effects of different strengths of MS media and auxins on adventitious root formation in vitro.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root Formation (%)</th>
<th>Number of root/cm</th>
<th>Length of root/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effect of Medium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>64.4 ±0.8</td>
<td>3.3 ±0.8</td>
<td>3.1 ±0.8</td>
</tr>
<tr>
<td>MMS₁</td>
<td>49.6 ±1.8</td>
<td>2.5 ±0.8</td>
<td>2.8 ±0.8</td>
</tr>
<tr>
<td>MMS₂</td>
<td>35.7 ±1.2</td>
<td>1.9 ±0.8</td>
<td>1.9 ±0.8</td>
</tr>
<tr>
<td><strong>Effect of Auxin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBA</td>
<td>35.1 ±1.8</td>
<td>1.8 ±0.8</td>
<td>2.1 ±0.8</td>
</tr>
<tr>
<td>IAA</td>
<td>40.2 ±1.5</td>
<td>2.2 ±0.8</td>
<td>2.0 ±0.8</td>
</tr>
<tr>
<td>NAA</td>
<td>42.2 ±3.4</td>
<td>1.9 ±0.8</td>
<td>2.3 ±0.8</td>
</tr>
</tbody>
</table>

Data were recorded after 5 weeks of culture. Values represent the pooled mean ± standard error of four replicates. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level.

![Fig. 1.14. Effects of dark treatment on adventitious root formation efficiency on auxin-free MS medium.](image-url)
they were transferred to normal growth room condition (16 hrs photoperiod and 25°C temperature). Another set of microcuttings was maintained under normal growth room light and temperature conditions, and rooting was observed every day. In this experiment, no rooting was found from the base of any microcuttings before 10 days of culture. Observations after 15 days of culture on auxin-free medium revealed that the microcuttings incubated under 26°C in dark showed 70.0 ± 1.9% root formation, whereas only 40.0 ± 1.2% root formation was obtained from the microcuttings which were maintained directly under normal growth room conditions. After 30 days of culture incubation, the maximum 85.0 ± 2.1% root formation was recorded from microcuttings treated under dark condition (Fig. 1.13F). On the other hand, microcuttings treated under normal light conditions showed 60.0 ± 2.0% root formation. The emergence of roots was 7-10 days earlier in the microcuttings incubated with dark condition, and the average length of root was also higher in those microcuttings. The over all rooting performance showing the higher frequency, and early and uniform emergence was found to be the best in microcuttings incubated under 26°C in dark for initial one week. The maximum number and average length of root per microcutting was 4.6 ± 1.6 and 7.0 ± 0.4 cm, respectively. There was also no basal callusing from any microcutting base even after five weeks of incubation. When rooting behavior of *P. amurense* microcuttings by dark treatment was compared with that by light treatment in any auxin-free medium, it was noticed that incubation at 26°C under dark condition for initial one week was proved to be the best treatment for root induction.

3.5. Acclimatization and Establishment of In Vitro Regenerated Plantlets

Fifty rooted plantlets (5-6 cm long) with 4-5 fully expanded leaves and well developed roots were transferred to pot containing Kanuma soil for hardening under diffuse light (16hrs photoperiod) condition.

![Fig. 1.15. Ex vitro establishment of in vitro regenerated plantlets.](image)

A-C: Plantlets growing on the Kanuma soil under culture room conditions after 2 weeks (A) and 4 weeks (B) of transfer. Plantlets grown under greenhouse conditions on garden soil after 12 weeks of transfer.

Normal growth of the potted plants was observed after 10-15 days of transfer (Fig. 1.15A). After one month, they (Fig. 1.15B) were transferred to larger pot containing the same soil and moved to green house. As the results, 90.5% of rooted plantlets survived after transplantation to soil and grew to maturity under greenhouse conditions. The regenerated plants did not show detectable variation in morphological or growth characteristics, when compared with the donor plants (Fig. 1.15C).

The information obtained by this study on the micropropagation of *P. amurense* could be exploited for micropropagating a selected tree of *P. amurense*, although the conventional propagation method of this tree is very difficult and the percentage of success was very low. An efficient micropropagation protocol for large scale clonal propagation via axillary and adventitious shoot regeneration are shown in micropropagation flow chart (Figs. 1.16A-C).

4. DISCUSSION

The present investigation was designed to establish a protocol of micropropagation of *Phellodendron*

Fig. 1.16A. Flow chart for micropropagation of *P. amurense* through axillary shoot proliferation.

*amurense* Rupr. using the explants both from the field-growing mature plants and from the *in vitro* grown seedlings. The results obtained are as follows:

For the primary establishment of *in vitro* culture, NaOCl was used as the surface sterilant. Surface sterilization with 3.0% NaOCl solution for a treatment duration of 10 min resulted in 0.0% contamination-free of explants. Only 15.0-60.0% contamination-free cultures were obtained, when the explants were treated for 20-25 min with 3.0% NaOCl. In these treatments, no explant death occurred. These explants remained green and showed healthy growth and proliferation of axillary shoots. Increasing the treatment duration, percentage of contamination-free explants and percentage of explant death also increased gradually. When the explants were treated for 40 min, 100.0% explants were contamination-free, but all of them died. Similar results were found in *Angelica sinensis* (Shi-Yu ad Kuo-Chang, 1989), *Magnolia ovovata* (Nakamura et al., 1995), and *Fraxinus pennsylvanica* (Kim et al., 1997). Surface sterilization of the seeds with 3.0% NaOCl for 30 min also produced 100.0% contamination-free seed cultures and subsequently yielded 80.0% aseptic seedlings. Camper et al. (1997) and Turker et al. (2001) reported the same results in *Ginkgo biloba* and *Verbascum thapsus*, respectively. The treatment removing the nodal and shoot tip explants from surface micro-flora completely was found to be lethal for the explant itself.

*In vitro* methods of plant propagation include shoot culture with proliferation of axillary and/or adventitious shoots and callus culture with organogenesis or embryogenesis. Though all plant cells are theoretically totipotent, attempts using many tissues to get whole plants frequently lead to failure due to lack of proper techniques and insufficient knowledge about nutrient media and other physical and chemical conditions which are essential for proper growth of cells, tissues, and organs of the plant concerned (Johni, 1982). Although variation in the indigenous hormonal levels of the buds is present in juvenile regions of the stem, they play an important role in their sprouting (Lane, 1978). In the present investigation, significant difference regarding axillary shoot proliferation from the nodal and shoot tip explants was also observed.

For axillary shoot proliferation, seedlings raised nodal explants were found to be better than that of field-growing mature plants. In the present investigation, nodal explants of seedling produced significantly higher number of shoots in 100.0% culture. Ariyoshi (1988) reported axillary shoot proliferation from intrapetiolar bud culture of *P. amurense* mature plant, where shoot proliferation rate and shoot number was very low. Many authors reported that explants from *in vitro* grown seedlings were better than mature plant explants. This fact was found also in case of *Citrus halimii* (Normah et al., 1997), *Jojoba* (Roussos et al., 1999), *Eucalyptus grandis* × *E. urophylla* (Cid et al., 1999),
and Bauhinia vahlii (Bhatt and Dhar, 2000).

Among the different types of medium used here, the full strength MS medium produced significantly better results regarding proliferation of shoots and callus than those produced by other two media (MMS; and WPM), irrespective of growth regulator supplements. Although most plants exhibit various degrees of responses to MS medium, the herbaceous and semi-woody species respond better than the woody ones (Bhojwani and Razdan, 1983). Many authors reported that many medicinal plant species highly responded on MS medium, e.g. Eucalyptus species (Sita, 1981), Cinnamomum zeylanicum (Rai and Chandra, 1987), Azadirachta indica (Rao et al., 1988), Rauvolfia serpentina (Roja et al., 1990), Magnolia obovata (Nakamura et al., 1995), Ginkgo biloba (Camper et al., 1997), Cinnamomum camphora (Huang et al., 1998), and Gymnema sylvestre (Komalavalli and Rao, 2000). Results of the above investigations are in agreement with those of the present study, and showed that full strength of MS medium was also better for in vitro propagation of woody species, P. amurense.

Some cytokinins, BAP, Kn, and CPPU, were tested in a concentration range 0.5-6.0 μM to assess the optimum concentration of the cytokinin for obtaining early sprouting and maximum proliferation of axillary shoots.
BAP was found to be more effective than Kn and CPPU on the proliferation and development of *P. amurense* shoots, this fact being similar to those observed by Ariyoshi (1986). Superiority of BA over other cytokinins in producing *in vitro* shoots has also been confirmed in other plants like *Camellia sinensis* (Aralpragasam and Latiff, 1988), *Atropa belladonna* (Benjamin et al., 1987), *Citrus halepini* (Normah et al., 1997), blue honeysuckle (Karhu, 1997), Jojoba (Roussos et al., 1999), and *Ceratonia siliqua* (Romano et al., 2002). Wareing and Phillips (1981) showed that synthetic cytokinin, such as BA, was more active for shoot proliferation than naturally occurring cytokinin.

For regeneration of shoots from nodal explants, BAP-NAA combination was also found to give better results than other combinations. However, the explants produced only axillary shoots, but no roots. On the shoot proliferation medium, 1.0-2.0 μM of BAP along with 0.5-1.0 μM of NAA showed the best result. This is in agreement with the results of *Camellia sinensis* (Phukan and Mitra, 1984), *Capsicum annuum* (Agrawal et al., 1988), and *Eucalyptus globosus* (Islam et al., 1994). It was also reported that *Coffea arabica* (Raghuramula et al., 1989) produced multiple shoots from nodal explants on MS medium supplemented with Kn and NAA or IAA. Komalavalli and Rao (2000) has
reported that BAP with Kn and NAA was suitable for axillary shoot proliferation of a multipurpose medicinal plant *Gynemona sylvestre*.

*In vitro* response of buds to nodal elongation was largely influenced by the presence of gibberellin in the medium. Combinations and concentrations of BAP with GA$_3$ present in the culture media also influenced proliferation efficiency of the buds variably. The addition of GA$_3$ to the BAP-containing media considerably increased shoot length, whereas drastically decreased shoot multiplication. This is contrary to the report by Wilna De Winnar (1988) who found a fact that GA$_3$ with BAP formulation stimulated both proliferation and elongation of shoots. Xie and Hogn (2001) also reported that TDZ with GA$_3$ increased the shoot length of *Acacia mangium*. In the present study, GA$_3$ stimulated shoot elongation, but not shoot bud proliferation. This result is in consistent with the findings obtained in papaya (Conover and Litz, 1978).

Concentration plant growth regulator also gave a marked effect on shoot regeneration from different explants of *P. amurense*. In general, percentage of shoot formation, number of total shoots, and average length of shoot per explant increased up to a certain concentration depending on the kind of plant growth regulator and the explants. A higher concentration of cytokinin supports profuse callus forming and reduction of shoot bud induction (Tiwari *et al.*, 2001). In the present experiment, hypocotyl appeared to be the best explant for direct adventitious shoot bud regeneration, which is in agreement with the findings in *Psidium guajava* (Sigh *et al.*, 2002), *Limum* sp. (Mundhara and Rashid, 2001), and *Anonna squamosa* (Nagori and Purohit, 2004). Furthermore, hypocotyl explants have the advantage of manipulating easily and large number of explants could be obtained successfully from a single plant.

Shoot regeneration from cotyledon has also been achieved in a wide range of tree species with using a BAP plus NAA combination (Niedz *et al.*, 1989; Dong and Jia, 1991; Jahan and Hadiuzzaman, 1996). Superior effect of the BAP plus NAA combination on direct adventitious bud proliferation from cotyledon was also reported by Singh *et al.* (2002) for *Dalbergia sissoo*. Results obtained here showed a consistency with other studies, where the addition of NAA with BAP promoted the proliferation and elongation of shoots in *Eucalyptus grandis* (Luís *et al.*, 1999) and *Hybanthus enneaspermas* (Prakash, 1999). In the present study, it was found that BAP with NAA or IBA successfully produced adventitious shoots from cotyledon with the high percentage of 71.5% and 71.3%, respectively, although cotyledon explants showed less shoot proliferation rate than hypocotyl explants. Similar result was also reported in *Panax ginseng* (Choi *et al.*, 1998).

Plant regeneration was also achieved from leaf explants of *P. amurense* on MS medium containing BAP with NAA or IBA. The frequency of shoot regeneration was the highest (72.5%) at 4.41 μM BAP with 0.5 μM NAA, whereas BAP plus IBA was less effective than BAP plus NAA. Similar observations were made on leaf segment culture of *Adhatoda vasica* (Amin *et al.*, 1997), *Echinacea purpurea* (Koroch *et al.*, 2002), and *Platanus acerifolia* (Liu and Bao, 2003). In this experiment, leaf explants produced the greatest length of shoot than the other explants. This result was similar to that of *Morus alba* (Bhau and Wakhlu, 2001).

Among various combinations and concentrations of BAP and NAA, internode explants of *P. amurense* showed the best result on the medium containing 2.22 μM BAP and 0.5 μM NAA. Similar observations were reported on internode segment cultures of *Piper longum* (Bhat *et al.*, 1992) and *Adhatoda vasica* (Azad and Amin, 1990). It was also reported that BAP alone produced adventitious shoots from internode segments of *Piper cubridnum* (Kelkar and Krishnamurthy, 1998) and cumin (Tawfik and Noga, 2001). Kn-IBA combination produced shoot buds from internode segments of *Coccinia grandis* (Gulati, 1988). These results indicate that suitability of auxin-cytokinin combination for regeneration of adventitious shoots *in vitro* depends on the genotypic variation of the experimental explants.
Morphology and the amount of callus formed varied with different levels of auxin in combination of cytokinin. Within 3 weeks of incubation, callus was proliferated from cut margin of all the treated explants. Hypocotyl explants produced highly proliferating friable callus showing green in the medium containing 2.0 μM TDZ with 4.0 μM 2,4-D, while another three explants showed less performance than hypocotyl. Schween and Schwenkel (2003) reported the similar result for callus production in Primula ssp. The second highest frequency and callus fresh weight were also recorded in MS medium containing 2.0 μM TDZ with 4.0 μM NAA from hypocotyl explants. Ikuta et al. (1998) reported that 0.1 μM Kn with 1.0 μM 2,4-D was better for callus induction from the stem segment in P. amurensce. In the present study, it was revealed that 2.0 μM TDZ with 4.0 μM 2,4-D or NAA was the best formulation, and hypocotyl was the suitable explant for callus production in P. amurensce. This is in agreement with callus induction of Astragalus adsurgens by Luo and Jia (1998).

Indirect induction of adventitious shoot buds was observed from the hypocotyl-, cotyledon-, leaf-, and internode-derived callus surface within 4-6 weeks of culture, and they elongated accompanied with leaf formation within 8 weeks of culture. Among the different cytokinin-auxin combinations tested, BAP with NAA showed the highest shoot regeneration frequency of shoot per callus clump, while CPPU with NAA or IBA showed slow growth with poor internodal elongation. The medium supplemented with BAP and IBA showed considerable shoot regeneration. Results obtained here showed a consistency with other studies, where the BAP and NAA promoted the proliferation and elongation of shoots in Adhatoda vasica (Azad and Amin, 1998), Astragalus adsurgens (Luo and Jia, 1998), and Eucalyptus grandis (Luis et al., 1999). Yang et al. (2001) reported that adventitious shoots regenerated from immature cotyledon-derived callus of Swainsona salsula in the presence of TDZ alone or in combination with IBA. Phillip et al. (2001) also described the same results in cotyledon-derived callus of almond. It was reported that the BA-IAA combination successfully produced shoot buds from leaf-derived callus in Flaveria trinervia (Sudarshana and Shanthamma, 1991) and Ladebouria hyacinthiana (Turakhia and Kulkarni, 1988). Kelkar and Krishnamurthy (1998) also reported that the combination of 0.5 mg/l BA with 1.0 mg/l 2,4-D or NAA (0.5 and 1.0 mg/l) induced organogenesis in leaf-derived callus of Piper colubrinum. Kulkarni et al. (2000) noticed that BAP alone produced shoot buds from the internode-derived callus of Withania somnifera. It was also reported that Zeatin-IAA combination produced adventitious shoot buds from hypocotyl-derived callus in beech (Cuenca et al., 2000). The present study revealed that low and high concentrations of a cytokinin (1.0 and 3.0 μM of BAP or CPPU) and an auxin (0.5 and 2.0 μM of NAA or IBA) suppressed the rate of shoot regeneration. It is considered that the optimal plant growth regulator combination is needed for the shoot regeneration from callus of P. amurensce.

The regenerating callus was subcultured continuously onto the fresh regenerating medium every four weeks for further amplification and continuous inductions of shoot buds. These shoot buds attained an appropriate height by the third week of subculture. The number of regenerated shoot per callus increased drastically from the first to the 3rd subculture and then gradually decreased. From the 6th subculture, it was obstructed to regenerate shoots for another subculture. Rout et al. (1999) reported that shoot bud regeneration rate per callus in Plumbago zeylanica was stable up to the eighth subculture. Reddy et al. (2001) also reported that frequency of shoot regeneration per callus was stable up to the 6th subculture in Coleus forskohlii.

Inhibitions of chlorophyll synthesis and shoot growth on sucrose-deficient medium have also been reported by Amin and Jaiswal (1989). At 50 g/l and 60 g/l sucrose concentrations, although the shoot size was bigger, its number decreased and root growth was inhibited. The findings obtained in the present study also indicate that
the sucrose not only acts as a carbon cum energy source in the medium, but also acts as an osmotic, which is in agreement with the results by Skirvin (1981). They also indicate that different concentrations of sucrose act as one of the controlling factors for the induction and growth of shoots.

Since it is well known that medium pH affects nutrient uptake and shoot proliferation, the effects of medium pH (4.0, 4.7, 5.0, 5.7, 6.0 and 6.7) were tested with using an MS medium supplemented with 2.22 μM BAP. Although the optimum pH for shoot proliferation and elongation was 5.7, in more acidic media they were severely inhibited. These data were similar to those of Huang et al. (1988), Onay (2000), Castillo et al. (2001), and many others that the number of shoots was the greatest on the medium at pH 5.7. On the other hand, Parlman et al. (1982b) reported that the lower level of pH (4.9) on the medium was suitable for the rhizome explant culture of Dianoeac muscipula Ellis.

The percentage of root formation, number of root per shoot, and length of root were largely influenced by the medium type, and also by the concentrations and kinds of auxin. There was a satisfactory improvement in rooting, as about 98.8% shoots could induced roots on MS medium containing 2.0 μM IBA with a fairly good length and number of root per shoot. Reddy et al. (2001) reported the similar results in Coleus forskohii. Ariyoshi (1986) reported that WP medium was effective for root induction in P. amurense. Faisal and Anis (2003) also reported that half strength of MS medium was better for root induction in Tylaphora indica. The present study showed the best result of root formation in MS medium, whereas MMS1 and MMS2 medium showed less performance than MS medium. Among the three types of auxin used here, IBA was found to be the best for root induction, followed by NAA. The auxin IAA at the concentrations tested produced smaller numbers of roots compared to IBA and NAA. These findings are in agreement with those observed in other medicinal plants, such as Adhatoda vasica (Azad et al., 1999), Malus domestica (Tantos et al., 2001), and Holostemma ada-kodiien (Martin, 2002).

Another experiment was conducted for finding out the influences of dark effect on root induction with using auxin-free medium. In this experiment, a progressive result was obtained. As described in previous study, root initiation in shoot started within 10 days on auxin-containing medium. In some cases, culture of regenerated shoots on medium with auxin delayed root formation, and decreased both the rooting percentage and number of roots, when high concentrations of auxin were used. When microcuttings were treated under dark condition for one-week at 26 °C on auxin-free medium, they produced maximum 85.0% of rooting within 30 days of culture incubation. In addition, no malformation and profuse roots were observed in auxin-free medium. These findings are in agreement with those observed in jackfruit (Amin, 1990) and carambola (Amin et al., 1992). Hammerschlag (1982) stated that a 2-week dark period was essential for obtaining the maximum rooting in vitro of Calita plum. Apple microcuttings also showed increased rooting in darkness (Welander, 1983). When comparing the both root initiation duration and rooting efficiency, IBA-containing MS medium was more effective. Considering the duration of root induction and cost of auxin, auxin-free medium was better for root induction. However, no survival effects were observed between plantlets originated from both auxin-containing and auxin-free media. Similar results were reported in Foeniculum vulgare (Hunault, 1984), cumin (Tawfik and Noga, 2001), and Bupleurum fruticosum (Fraternale et al., 2002).

In in vitro regenerated plantlets that had been transferred onto the soil, 90.5% of them could tolerate transplantation shock and survived under ex vitro environment. Rest of the transplants could not survive either due to desiccation or microbial infection. Damping off and necrosis of the transplants were also observed during acclimatization under ex vitro conditions of Eucalyptus tereticornis (Gill et al., 1993), Solanum sisymbriifolium (Ara et al., 1993), Rauwolfia
serpentina (Ihli, 1993), and Rosa damascena (Kumar et al., 1995). Being the delicate nature of in vitro regenerated plantlets, special arrangement such as controlled green house conditions, use of soil free potting mixture like perlite, vermiculite, peat plugs, and application of fungicides are needed for easy and successful acclimatization of the plantlets (Degerh and Read, 1990).

In conclusion, an efficient protocol for axillary and adventitious shoot morphogenesis and for in vitro hardening of P. amurense regenerants has been established, the flow chart of which are presented in results chapter. The protocol was optimized by manipulations of different explants from axenic plants and explant placement on the induction medium containing various concentrations of cytokinin and auxin. This protocol may be useful for large-scale clonal propagation and studies on producing P. amurense transgenic plants via an Agrobacterium-mediated system.

5. SUMMARY
The present investigation was based on axillary shoot proliferation, direct and indirect adventitious shoot regeneration, and rooting of the in vitro proliferated shoots. Regeneration of complete plantlets that could be transplanted and established under ex vitro conditions has been achieved by this study.

Nodal and shoot tip explants, and seeds collected from the field-growing mature plants were surface-sterilized with 3.0% NaOCl for 25 and 30 min. Aseptic seedlings were raised in vitro on MS medium with 2.0 μM BAP. Nodal and shoot tip segments of the seedlings and mature plants were used for axillary shoot proliferation, while hypocotyl, cotyledon, leaf, and internode explants from seeds were used for direct and indirect adventitious shoot proliferation.

The sterilized explants were cultured on MS, MMS, and WP media supplemented with different cytokinin alone or in combination with auxins. The plant growth regulators acted differently on different explants for axillary and adventitious shoot regeneration, and their multiplication. Among the nodal and shoot tip explants of mature plants and seedlings, nodal segments of seedlings showed the best result for axillary shoot proliferation on MS medium containing 2.0 μM BAP. The highest percentage of shoot proliferation and the greatest number of total shoots per culture were 100.0% and 8.5 ± 0.8, respectively, whereas the greatest length 7.5 ± 0.2 cm of shoots was obtained from seedling-derived shoot tip explants on MS medium containing 1.0 μM BAP.

With proper manipulation of cytokinin and auxin combination and concentration, it was possible to induce callus and subsequent plantlet regeneration from hypocotyl, cotyledon, leaf, and internode explants. Among different explants, morphogenic potentiality of hypocotyl and cotyledon explants was higher than that of leaf and internode explants. Among different combinations and concentrations of cytokinin and auxin in MS medium, 2.2 μM BAP with 0.5 μM NAA combination was found to be the best formulation for direct proliferating adventitious shoots from hypocotyl and internode explants, whereas 6.6 μM BAP plus 0.5 μM NAA, and 4.4 μM BAP plus 0.5 μM NAA combinations were better for adventitious shoot regeneration from cotyledon and leaf explants, respectively. For the induction of callus from hypocotyl, cotyledon, leaf, and internode explants, the suitable medium combination was 2.0 μM TDZ with 4.0 μM 2,4-D. This combination produced only fast-growing callus, while they could not produce any shoot buds. When this callus was subcultured on MS medium supplemented with 1.5 μM BAP + 1.0 μM NAA, it produced shoots successfully.

Microcuttings were prepared from the in vitro proliferated shoots (2–4 cm long) and cultured for adventitious rooting in MS medium with 0.5-2.0 μM of IBA, NAA, and IAA. Root-forming performance of IBA was proved to be the best among the three auxins tested. Maximum rooting (98.8%) with 7.2 ± 0.8 roots per cutting was recorded on the medium containing 2.0 μM of IBA. The high concentrations (4.0 μM) of all
the auxins gave either callus formation at the microcutting base or malformation of roots. These abnormalities were particularly remarkable, when NAA was used for rooting. Eighty five percent root formations also occurred in hormone-free medium within 30 days of incubation. Rooted shoots were acclimatized successfully and established onto the soil under greenhouse conditions with survival rate of 90.5%.

Chapter 2: Plant Regeneration from Protoplast of *Phellodendron amurense* Rupr.

1. INTRODUCTION

Protoplast technology has been considered as an important and valuable approach to produce novel genotypes with desired traits through parasexual hybridization, genetic engineering, and somaclonal variation. Protoplasts also provide an unique opportunity for genetic improvement of plant species. The transfer of bulk DNA by protoplast fusion may not only produce interesting novel germplasm for fundamental studies, but also lead to the development of plants with synergistic combinations of secondary metabolites. It would be advantageous to combine medicinal plants containing different metabolites to understand the biosynthesis of secondary metabolites related to the production of specific medicinal contents (Murphy and Saxena, 2001). Although initial work on protoplasts employed medicinal species such as *Nicotiana* and *Atropa*, the studies on the use of protoplast system in medicinal plant biotechnology are very limited (Nagata and Bajaj, 2001).

In order to fully explore the application of protoplast biotechnology in plant-based medicines, an efficient system of protoplast isolation followed by induction of cell divisions and thereafter plant regeneration is a prerequisite. The successful culture of protoplasts and their subsequent regeneration into complete plants depend on a number of factors, such as genotype, tissues for protoplast isolation, the physiological conditions under which the material plants cultures have been raised, purity of the enzymes, the plasmolyticum, period of incubation, culture media, plant growth regulators, milieu of protoplasts / plating density, viability of protoplasts, method of culture (liquid / solid), incubation conditions etc. (Bajaj, 1989). In the present study, based on these views, successful plant regeneration has been achieved from leaf- and stem segment-derived callus, and cell suspension culture-derived protoplasts of *P. amurense*.

2. MATERIALS AND METHODS

2.1. Preparation of Protoplasts

2.1.1. Plant materials and source of explants

Plant materials for starting *in vitro* culture were seedlings (Fig. 2.1A). The seed collection, sterilization, and culture procedures were the same as those described in Materials and Methods in Chapter 1. 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. Culture conditions were 25 ± 1°C, 16 hrs illumination per day at 50 μmol·m⁻²·s⁻¹, and optimum humidity. Generally, each cultured node cutting gave numerous shoots bearing three to four fully expanded

![Fig. 2.1. Plant materials for protoplast culture.](image)

A: A seedling of *P. amurense*. B: Plantlets of *P. amurense* from which young leaves and internode were collected for protoplast isolation.
leaves, and microcuttings gave single shoots with roots bearing also three to four expanded leaves. The sources of protoplasts were leaves and stem segments of *P. amurense*. The leaves and stem segments were taken from 4-5 week-old *in vitro* grown shoots (Fig. 2.1B). These materials were used for induction of callus and cell suspension cultures.

### 2.1.2. Callus induction

The young leaves (1 cm²) and stem segments (1-1.5 cm long) were incubated in MS medium containing different concentrations (viz. 0.5, 1.0, 2.0, 4.0, and 6.0 μM) of NAA, IBA or 2,4-D in combination with either of 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 ± 0.1. The explants were cultured on medium surface in glass petri dishes (9 × 1.5 cm, Asahi Techno Glass, Japan). Each petri dish contained 10 explants and sealed with Parafilm (Parafilm M, American National Can., USA), and the cultures were grown at 25 ± 1 °C under the illumination of warm-white fluorescence tubular lamp with a light intensity of 50 μmol·m⁻²·s⁻¹ for 16 hrs photoperiod. All cultures were transferred to the new medium containing the same composition every 4 weeks for callus proliferation. The experiments were repeated 4 times.

### 2.1.3. Cell suspension culture

Cell suspension cultures were initiated by placing about 3-4 g fresh weight of stem segment-derived friable callus into 25 ml of liquid medium in a 100 ml Erlenmeyer flask. For induction of cell suspension cultures from friable calli, three types of liquid media, MS, MMS₁, and BS (Gamborg et al., 1988; see Appendix 5), all of which contained 3.0% sucrose (w/v) and were adjusted to pH 5.7 ± 0.1, were examined to select the best medium by adding various combinations of plant growth regulators as follows: NAA, IBA, or 2,4-D (viz. 1.0, 2.0, and 4.0 μM) as auxin, and BAP, TDZ, or CPPU (viz. 1.0, 2.0, and 4.0 μM) as cytokinin. The cultures were incubated under the light condition on a rotary shaker at 100 rpm to allow the callus to be disaggregated. They were subcultured every 2 weeks by adding 5 ml of them to 25 ml of fresh medium in a 100 ml Erlenmeyer flask. The culture was performed at 25 ± 1°C under the illumination of warm-white fluorescence tubular lamp with a light intensity of 50 μmol·m⁻²·s⁻¹ for 16 hrs photoperiod.

### 2.1.4. Protoplast isolation

Protoplasts were isolated by enzymatic digestion of the cell wall of different materials of *P. amurense*. Three materials were used as the primary protoplast sources, leaves, calli, and suspension cultures.

#### 2.1.4.1. Protoplast isolation from leaf and callus

The young leaves were collected aseptically from the plantlets aseptically grown from the seeds in the laboratory. About 0.2 g fresh weight of leaves were excised and then used for protoplast isolation. 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 isolating protoplasts.

Twenty-four enzyme combinations were examined to select the best one for protoplast isolation. The following enzymes were used here: 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 used at 1.0% concentration, except for Pectolyase Y-23 at 0.5%. In the case of Driselase, aqueous suspension of the enzyme was subjected to centrifugation at 100 × g for 3 min, and the obtained supernatant was used for the experiments.

About 0.2 g fresh weight of the leaves or friable calli were incubated with 4 ml of enzyme solution in 30 ml sample bottle for different duration (6 to 20 hrs) to
find out the optimum treatment duration for protoplast isolation. After digestion, the enzyme mixtures were passed through a 40 μm nylon mesh, and resulting protoplasts were washed three times with 0.6 M mannitol (Wako Pure Chem. Indus. Ltd., Japan) solution by centrifugation at 100×g for 3 min.

2.1.4.2. Protoplast isolation from cell suspension cultures

Twenty-four enzyme combinations were examined to select the best one for protoplast isolation from cell suspension cultures. Isolation of protoplasts was performed 3 weeks after subculture. The protoplasts from cell suspension cultures induced from stem segment-derived friable calli were isolated by the enzyme combinations as follows; Cellulase Onozuka RS, Cellulase Onozuka R-10, Driselase, Hemicellulase, Macerozyme R-10, and Pectolyase Y-23. All enzymes were used at 1.0% concentration except for Pectolyase Y-23 at 0.5%. When 24 enzyme combinations were tested to isolate protoplasts, cell suspension cultures of about 50 mg fresh weight were immersed in 400 μl enzyme solution in a 24-well plastic culture plate (FALCON, Becton Dickinson and Co., USA) and then incubated at culture room temperature for 24 hours. The experiment of protoplast isolation was repeated 3 times.

After selection of the best combination for protoplast isolation, the cell suspension cultures of about 0.3 g fresh weight were separated from the culture medium by filtration through a 40 μm nylon mesh, washed with 0.6 M mannitol solution, and then immersed in 10 ml of the enzyme solution in 50 ml sample bottle. After 8-hr incubation 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, then followed by 3-time washing with 0.6 M mannitol solution, the obtained protoplasts were used for protoplast culture.

2.1.4.3. Purification of protoplasts and determination of the yield and viability of protoplasts

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-time washing with 0.6 M mannitol solution. Protoplast yield was measured with a haemocytometer (Nitrin, Tokyo, Japan) under an inverted microscope (OLYMPUS IX70-S8F, Olympus Optical Co. Ltd., Japan), and calculated by the following equation:

\[
\text{Protoplast yield (protoplast / ml) = } \frac{5 \times \text{number of protoplast} \times 10^3}{\text{final volume}}
\]

After measuring the protoplast yield, protoplasts were stained with 0.01% (w / v) fluorescein diacetate (FDA) in 0.6 M mannitol to examine their viability (Larkin, 1976). FDA was mixed with 0.6 M mannitol solution in a ratio of 1: 25 (v / v). An equal volume of this FDA solution was added to the protoplast suspension. After 3 min, the protoplasts were observed under a fluorescence microscope (OLYMPUS IX70-S8F) with using excitation with blue light (WBV). The excitation filter (BA 475) was served as a barrier filter. Protoplast viability (%) was calculated as the percentage of the number of viable protoplasts to the number of total protoplasts. To find out the effects of the osmotic potential of enzyme solution on the yield and viability of protoplasts, different concentrations (viz. 0.5, 0.6, and 0.7 M) of mannitol were also tested.

2.2. Culture of Protoplast

2.2.1. Protoplast culture media

2.2.1.1. Culture of protoplasts from leaf

Protoplasts isolated from leaf were cultured in liquid or solid MS medium solidified with 0.2% gellan gum (w/v). The media contained 2.0% sucrose (w/v), 0.6 M mannitol, and various concentrations (viz. 0.1, 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).

2.2.1.2. Culture of protoplast from stem-segment-derived callus
Protoplasts obtained from stem segment-derived callus were cultured in liquid or solid MS medium solidified with 0.2% gellan gum (w/v) containing different concentrations (viz. 0.1, 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.

2.2.1.3. Culture of protoplast from cell suspension cultures
Protoplasts isolated from stem-segment-derived friable callus were cultured in liquid or solid B₅ medium solidified with 0.2% gellan gum (w/v) containing 3.0% sucrose (w/v) and different concentrations (viz. 0.1, 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). In this case, mannitol was not used. All the protoplast culture media were adjusted to pH 5.7 ± 0.1.

To examine the effects of pH of the medium on protoplast responses, B₅ medium with 2.0 μM BAP and 4.0 μM NAA was used as a standard medium. The pH of medium was varied to 4.7, 5.7, and 6.0 with 0.1M KOH or 0.2M HCl before autoclaving.

2.2.2. Plating efficiency
In all the experiments, cell density was adjusted to 2 × 10⁵, 4 × 10⁵, and 6 × 10⁵ protoplast / ml by adding 2.5 μl of the concentrated protoplast suspension to 50 μl of the medium in a well of a 96-well plastic culture plate (FALCON, Becton Dickinson and Co., USA). About 10 μl of the sterilized distilled water was dispensed between the wells to maintain the humidity, and the plate was tightly sealed with Parafilm. Protoplast suspension (1.5-2.0 ml) was also cultured on the surface of MS solid medium in a petridish (6 × 1.5 cm), where the protoplast density was 4 × 10⁵ protoplast / ml. Petridishes were sealed with Parafilm. The protoplasts were maintained at 25 ± 1°C under the illumination of cool-white fluorescence tubular lamp with a light intensity of 50 μmol·m⁻²·s⁻¹ for 16-hr photoperiod. Protoplasts were observed at 24-hr intervals under an inverted microscope (OLYMPUS IX70-S8F) during 3 months of culture. After 2 months of culture, the number of colonies larger than 200 μm in diameter in each well was counted twice under an inverted microscope. Frequency of colony formation was calculated as the percentage of the number of colonies per the number of originally plated protoplasts as follows:

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

2.3. Plant Regeneration from Protoplast-Derived Callus

2.3.1. Proliferation of protoplast-derived callus
After 2 months of culture, the microcalli (about 2-3 mm diameter) obtained from leaf- and stem segment-derived protoplasts were used for callus proliferation. These microcalli were transferred to petridishes (9 × 1.5 cm, Asahi Techno Glass, Japan) or 200 ml flasks (Asahi Techno Glass, Japan) 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.

To proliferation callus from protoplasts isolated from cell suspension cultures, microcalli were collected after 2 months and cultured on MS solid medium. MS medium contained 3.0% sucrose (w/v), 0.2% gellan gum, and different combinations and concentrations of BAP or TDZ (viz. 1.0, 2.0, and 4.0 μM) with NAA, IBA, or 2,4-D (viz. 2.0 and 4.0 μM). In each case, petridishes (9 × 1.5 cm) or 200 ml flasks were used as culture vessels. All culture media were adjusted to pH 5.7 ± 0.1, and the cultures were incubated at 25 ± 1°C under the illumination of cool-white fluorescence
tubular lamp (50 μmol · m⁻² · s⁻¹) for 16-hr photoperiod. Protoplast-derived microcalli were subcultured every 4 weeks. The data were obtained from triplicate cultures of callus.

2.3.2. Plant regeneration from protoplast-derived callus

For plant regeneration, protoplast-derived calli were transferred to a sequence of regeneration media supplemented with various cytokinins and auxins. The calli from all sources were transferred to 200 ml conical flask containing 50 ml of MS medium supplemented with different concentrations (viz. 0.5, 1.0, 2.0, and 4.0 μM) of BAP or CPPU in combination with of NAA or IBA (0.5, 1.0, 2.5, and 5.0 μM) for shoot regeneration.

For the elongation of shoots regenerated from protoplast-derived calli, small shoots with calli were transferred to MS medium containing different concentrations (viz. 0.5, 1.0, 1.5, and 2.0 μM) of BAP. All of the shoot regeneration and elongation media were adjusted to pH 5.7 ± 0.1, and they were fortified with 3.0% sucrose (w/v) and gelled with 0.8% agar (w/v). The cultures were grown at 25 ± 1°C under illumination of cool-white florescence tubular lamp with a light intensity of 50 μmol · m⁻² · s⁻¹ for 16-hr photoperiod.

2.3.3. Rooting of in vitro regenerated shoots

Microcuttings (2-4 cm long) obtained from protoplast callus-derived elongated shoots were cultured on MS basal medium fortified with either of 0.5, 1.0, 2.0, and 4.0 μM IBA, NAA, or IAA for adventitious rooting to improve the overall growth of roots and to reduce the time duration of root induction. Data were recorded on percentage of rooting and the number and length of root after 4 weeks of transfer onto the rooting media.

2.4. Data Analysis

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

3. RESULTS

3.1. Maintenance of Experimental Materials

In this study, seedling explants were used as initial plant materials for protoplast culture. Seeds were germinated within 2-3 weeks of culture, and gave rise to shoots which developed 2-3 nodes 1 month later (Fig. 2.1A). Nodes and shoot tips obtained from seedlings were cultured on MS medium supplemented with 2.0 μM BAP or IBA separately. The shoot cultures gave better expanded and more normal-looking leaves, when shoots were grown on MS medium with 2.0 μM IBA (Fig. 2.1B). The inclusion of BAP led to extensive adventitious shoot formation and developed small leaf blades. However, BAP was essential for the multiplication of shoots.

3.2. Induction of Callus from Leaf and Stem Segment Explants

Morphology and the amount of formed callus varied with different levels of an auxin in combination of a cytokinin. In this study, different concentrations (viz. 0.5, 1.0, 2.0, and 6.0 μM) of NAA, IBA, or 2,4-D in combination with either of BAP or TDZ (1.0, 2.0, and 4.0 μM) were tested for their effects on callus formation from leaf and stem segment explants. Within 3 weeks of incubation of leaf explants, callus was proliferated from cut margin (Fig. 2.2A). Lower
Table 2.1. Effects of plant growth regulators on callus formation from leaf explants on MS medium.

<table>
<thead>
<tr>
<th>NAA (μM)</th>
<th>IBA (μM)</th>
<th>2,4-D (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>2.0</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td>10.0</td>
<td>20.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

CULTIVATION TEMPERATURE: 25°C ± 2°C.

* + = 0.0-20.0% callus formation, ++ = 20.0-40.0% callus formation, +++ = 40.0-60.0% callus formation.

Data were recorded after 8 weeks of culture. Twenty explants were used in each treatment.

Concentration (1.0 μM) of NAA or IBA with 2.0 μM BAP or 4.0 μM TDZ produced low frequency (20.0%) and less amount of callus, while 0.5 μM NAA or IBA, and 0.5-1.0 μM 2,4-D in combination with all cytokinins used here failed to produce any callus (Table 2.1). As increasing the concentration of auxin from 2.0 to 4.0 μM, gradual increase in percentage of callus formation was recorded. Further increase in the concentration of auxin (4.0 to 6.0 μM) gave the gradual decrease of callus formation rate (Table 2.1). Leaf explants produced the highest percentage (80.0%) of callus showing green colour in the MS medium containing 2.0 μM TDZ with 4.0 μM NAA after 8 weeks of incubation. The considerable callus formation was recorded in the MS medium containing 2.0 μM BAP with 4.0 μM of NAA, IBA or 2,4-D. Formation of friable callus was observed in MS medium supplemented with 2.0 μM TDZ with 4.0 μM 2,4-D.

Callus was induced successfully from stem segment explants (Fig. 2.2B). The media containing 2.0 μM BAP, or TDZ in combination with 4.0 μM NAA, IBA or 2,4-D were more effective for callus induction. This combination produced maximum frequency (80.0%) of callus formation (Table 2.2). 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 should browning within 6 weeks of culture incubation. 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 production in P. amurense.

3.3. Induction of Cell Suspension Cultures from Friable Callus Derived from Stem Segment.

Proliferating friable at from the third subculture phase were used for induction of cell suspension cultures. Newly initiated cell suspension cultures were composed of isodiametric, thin-walled, and richly cytoplasmic cells (Figs. 2.3A,B). By sieving suspensions, it was possible to obtain fine cell aggregates for plating.

Three types of medium, MS, MMS1, and B5, and different concentrations (1.0, 2.0, and 4.0 μM) of BAP, TDZ, or CPPU in combination with either of NAA, IBA or 2,4-D (1.0, 2.0, and 4.0 μM) were used for

![Fig. 2.3. Cell suspension cultures induced in internode-derived friable callus in B5 liquid medium with 2.0 μM BAP and 4.0 μM NAA. A: Cell suspension cultures subcultured at intervals for two weeks showing green colour. B: Cell suspension cultures after 6 weeks of culture.](image-url)
induction of cell suspension cultures. When placed in liquid medium, friable calli were easily broken apart and dispersed into clumps of 0.5-5.0 mm. Further agitation fragmented these clumps into suspension cultures consisting of small cell aggregates. The results are presented in Table 2.3. Among different types of medium and plant growth regulator used here, B₃ medium containing 2.0 μM BAP with 4.0 μM NAA, IBA or 2,4-D showed the best performance for induction of cell suspension cultures (Table 2.3; Fig. 2.3A). When the suspension cultures were subcultured in the same medium and combination of plant growth regulators, they showed active growth without browning. The friable callus cultured on either MS or MMS₁ medium started cell suspension later than that on B₃ medium. The lowest level for induction of cell suspension cultures was observed in MMS₁ medium, while moderate level of cell suspension cultures was obtained in MS medium. These media also produced green-coloured suspension cultures, whereas after one month of subculture they turned to brown colour. This event revealed that B₃ medium is suitable for suspension culture. Therefore, in the subsequent experiments only B₃ medium was used for induction of cell suspension cultures of *P. amurense*.

### 3.4. Isolation of Protoplasts

#### 3.4.1. Protoplast isolation from leaf- and stem-segment-derived 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 both Macerozyme R-10 and Hemicellulase were not effective in combination with Cellulase Onozuka R-10 or RS for isolation of leaf protoplasts (Table 2.4). Among the 24 enzyme combinations examined here, 4 enzyme mixtures (Cellulase Onozuka R-10 plus Driselase, Cellulase Onozuka R-10 plus Pectolyase Y-23, Cellulase Onozuka RS plus Driselase, and Cellulase Onozuka RS plus Pectolyase Y-23) were suitable for protoplast isolation from leaf (Table 2.4). Protoplasts obtained from leaves are shown in Fig. 2.4A. They contained many chloroplasts and ranged from 20 to 40 μm in diameter. Vacuolated protoplasts were also obtained in this experiment (Fig. 2.4A).

Among the 24 enzyme combinations, on the other hand, Cellulase Onozuka R-10 plus Driselase, Cellulase Onozuka RS plus Driselase, Cellulase Onozuka RS plus Hemicellulase plus Pectolyase Y-23, and Cellulase Onozuka RS plus Pectolyase Y-23 were suitable for protoplast isolation from stem-segment-derived callus (Table 2.5). Protoplasts obtained from stem-segment-derived callus are shown in Fig. 2.4B.

Generally, vigorous protoplasts emit yellow-green fluorescence under a fluorescence microscope, when protoplasts were stained with FDA, whereas damaged protoplasts do not (Larkin, 1970). In the present study, protoplasts with yellow-green fluorescence were judged to be vigorous, and the viability of protoplast was measured from the number of fluoresced leaf protoplasts (Figs. 2.5A,D), stem-segment-derived callus protoplasts.
Table 2.5. Effects of enzyme combination on the protoplast isolation from stem-segment-derived friable callus by treatments for 24 hrs with using 24 enzyme combinations.

<table>
<thead>
<tr>
<th>R-10</th>
<th>H + M</th>
<th>H + D + P</th>
<th>H + M</th>
<th>H + D + P</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>D + P</td>
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<table>
<thead>
<tr>
<th>RS</th>
<th>P</th>
<th>P</th>
<th>M</th>
<th>M</th>
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<td>H</td>
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<td>H + P</td>
<td>++</td>
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<td>++</td>
</tr>
<tr>
<td>D + P</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>


1: Yield more than $1.5 \times 10^6$ protoplasts per g fresh weight of callus.
++: Yield more than $5.3 \times 10^5$ protoplasts per g fresh weight of callus.
+++: Yield more than $7.5 \times 10^2$ protoplasts per g fresh weight of callus.

(Figs. 2.5B,E), and cell-suspension culture protoplasts (Figs. 2.5C,F), respectively. Figures 2.6 and 2.7 show the influence of enzymatic treatment time from 6 to 18 hrs on the yield and viability of protoplasts from the leaf and stem segment-derived callus, respectively. The yield gradually increased as increasing the enzymatic treatment time. On the other hand, the viability considerably decreased with the increase in enzymatic treatment time. In this study, the highest 86.0% and 92.0% viability with the yields of $7.1 \times 10^2$ and $2.4 \times 10^5$ protoplast / g fresh weight were obtained from leaf and callus, respectively, when the materials were treated for 6 hrs with Cellulase Onozuka R-10 plus Driselase. The highest yield of leaf protoplasts ($13.6 \times 10^5$ protoplast / g fresh weight) and callus protoplasts ($10.0 \times 10^5$ protoplast / g fresh weight) were obtained by the treatment for 18 hrs with Cellulase Onozuka RS plus Driselase, whereas the protoplast viability was 0%. In this experiment, it was revealed that among the different enzyme combination and treatment time, the treatment with Cellulase Onozuka R-10 plus Driselase for 8 hrs was most suitable for protoplast isolation from *P. amurensis* leaf and stem-segment-derived callus, where

Fig. 2.6. Effects of enzymatic treatment time on the yield and the viability of protoplasts isolated from leaf.
the yield of protoplasts were $10.5 \times 10^6$ and $5.5 \times 10^5$ protoplast / g fresh weight and the viability were 80.0% and 90.0%, respectively.

3.4.2. Protoplast isolation from cell suspension cultures

Twenty four enzyme combinations were preliminary tested for 24 hrs to isolate protoplasts from cell suspension cultures induced from friable callus derived from stem segments. The results obtained are shown in Table 2.6. The single use of Cellulase Onozuka R-10 or RS failed to isolate protoplasts. However, large number of protoplasts was isolated, when they were used in combination with other enzymes. Driselase and Pectolyase Y-23 in combination with Cellulase Onozuka R-10 or RS were effective for protoplast isolation whereas, Macerozyme R-10 and Hemicellulase were not.

Among 24 enzymatic combinations examined, the following four enzyme mixtures gave higher protoplast yields: Cellulase Onozuka R-10 plus Driselase, Cellulase Onozuka R-10 plus Pectolyase Y-23, Cellulase Onozuka RS plus Driselase, and Cellulase Onozuka RS plus Pectolyase Y-23.

In order to choose the best one from the four combinations described above, next experiment was carried out with the enzymatic treatment time for 6, 8, 10, 12, and 18 hrs, respectively. The results obtained are shown in Fig. 2.8. When the enzyme combination of Cellulase RS and Pectolyase Y-23 was used, all of the three enzymatic treatment times of more than 10 hrs gave higher values of the protoplast yield and lower protoplast viability. The combination of Cellulase Onozuka R-10 plus Driselase was most effective for the isolation of protoplasts. The highest protoplast viability (90.0%) (Fig. 2.5C,F) with yield of $4.5 \times 10^5$ protoplast / g fresh weight was obtained by the treatment for was 8 hrs. The protoplasts isolated from the cell suspension cultures ranged from 20-40 μm in diameter, and were different in colour from leaf protoplast (Fig. 2.4C)

3.4.3. Effects of osmotic potential on the yield and viability of protoplasts

Mannitol is generally used as the osmoticum. The osmotic potential in isolation mixtures also influenced the yield and viability of protoplasts from leaf, callus,

Table 2.6. Effects of enzyme combination on the protoplast isolation from cell suspension cultures induced from stem-segment-derived friable callus by treatments for 24 hrs with using 24 enzyme combinations.

<table>
<thead>
<tr>
<th>R-10</th>
<th>RS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H + M = ++</td>
</tr>
<tr>
<td></td>
<td>H + M = ++</td>
</tr>
<tr>
<td></td>
<td>H + D = ++</td>
</tr>
<tr>
<td></td>
<td>D + M = ++</td>
</tr>
</tbody>
</table>


+: Yield more than $1.5 \times 10^9$ protoplasts per 1 g fresh weight of suspension cultures.

++: Yield more than $5.2 \times 10^5$ protoplasts per 1 g fresh weight of suspension cultures.

+++ : Yield more than $5.5 \times 10^5$ protoplasts per 1 g fresh weight of suspension cultures.

Fig. 2.8. Effects of enzymatic treatment time on the yield and the viability of protoplasts isolated from cell suspension cultures induced from stem-segment-derived friable callus.
and suspension cultures. 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 effects of osmotic potential on protoplast isolation. The yield of protoplasts from leaf increased with increase in mannitol concentration from 0.4 to 0.6 M, and the maximum viability (86.0%) and yield (10.5 × 10^3 protoplast / g fresh weight) of protoplasts were obtained with 0.6 M mannitol (Fig. 2.9). The callus and suspension cultures also showed the highest values with 0.6 M mannitol. It was also noted that higher osmolality induced lower protoplast yield and lower osmolality resulted in more cell burst.

3.5. Culture of Protoplasts

Successful protoplast isolation has been achieved primarily in a medicinal tree species, *P. amurense*. However, the formation of callus from the protoplasts has only occasionally been successful. In this experiment, protoplasts from the leaf, callus, and cell suspension cultures were used. The various combinations and concentrations of plant growth regulators, and different types of medium for successful culture of protoplasts were examined.

3.5.1. Culture of protoplasts from leaf

Protoplasts were successfully isolated from leaf by using the enzyme combination of 1.0% Cellulase Onozuka R-

![Graph](image)

**Fig. 2.9.** Effect of the osmotic potential of enzyme solution on the yield and viability of protoplasts.

10 plus 1.0% Driselase, as described before. The obtained protoplasts were cultured in liquid MS medium supplemented with the various combinations of cytokinins (BAP and TDZ) and auxins (NAA, IBA, and 2,4-D) at different concentrations. The protoplasts were cultured at three different cell densities, 2 × 10^5, 4 × 10^5, and 6 × 10^5 protoplast / ml. After 48 hrs of culture, several masses composed of two cells each showing the first cell division were observed, and these masses then developed into many microcolonies after 15 days of culture (Figs. 2.10A, B). The distinct colony formation was observed within one month (Fig. 2.10C). The microcolony formation was observed in the media with various combinations of cytokinins and auxins at every cell density, in which the cells composed of more than 10 cells to about 200 cells are defined as a microcolony, as shown in Fig. 2.10B. After 6 weeks of culture, the number of colonies larger than 200 µm in diameter (Fig. 2.10C) in each well was counted, and then the frequency of colony formation was calculated as the percentage of the number of colonies per the number of originally plated protoplasts. The results obtained are shown in Fig. 2.11. The plating densities of 4 × 10^5 protoplast / ml induced the active growth and cell division of protoplasts, while 2 × 10^5 protoplast / ml showed less performance. Among the different combinations and concentrations of cytokinins and
auxins, 2.0 μM BAP with 4.0 μM NAA showed the best performance of colony formation. A comparison of microcolony formation among the media showed that BAP gave higher colony efficiency than TDZ.

After 8 weeks of culture, the colonies larger than about 500 μm in diameter were transferred to a 24-well culture plate containing the fresh liquid medium of the same components as those of the first culture, except for no addition of mannitol. The subcultured colonies developed into the microcalli larger than 1.0 mm in diameter within two weeks of culture. Table 2.7 shows the effects of plant growth regulator and plating density on the microcallus formation after 4 weeks of new subculture. After 12 weeks of subculture, the microcalli developed into 5 mm in diameter were observed in the MS medium containing the combinations of 2.0 μM BAP with 4.0 μM NAA, IBA, or 2,4-D. The leaf protoplasts were also cultured on MS solid medium containing 2.0 μM BAP and 4.0 μM NAA. In this medium, the mesophyll protoplasts grew into microcalli within 10 weeks of culture incubation (Fig. 2.10D).

### 3.5.2. Culture of protoplasts from stem segment-derived callus

Protoplasts were released from the friable callus derived from stem segment of *in vitro* grown *P. amurense* shoots, and were cultured on MS liquid medium supplemented with different combinations and concentrations of a cytokinin and an auxin. In this experiment, protoplasts were cultured at three different cell densities, $2 \times 10^3$, $4 \times 10^3$, and $6 \times 10^3$ protoplast / ml. After one week of culture, protoplast growth with the same nature was observed in the media. It seemed that the cell wall regeneration had already commenced. After 2 weeks of culture, the first cell division occurred in the media containing high concentration (4.0 μM) of NAA, IBA, or 2,4-D with 2.0 μM BAP (Fig. 2.12A), suggesting that the time required for the cell wall regeneration is 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 (Fig. 2.12B).

### Table 2.7. Effects of plant growth regulators and plating density on microcallus formation and proliferation from mesophyll protoplasts.

<table>
<thead>
<tr>
<th>Plating density</th>
<th>4 × 10^2 protoplast / ml</th>
<th>6 × 10^2 protoplast / ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA (μM)</td>
<td>IBA (μM)</td>
<td>BAP (μM)</td>
</tr>
<tr>
<td>2.0</td>
<td>4.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

| BAP (μM) | |
| 2.0 | ++ |
| 1.0 | + |
| 0.5 | + |

+: Microcallus formation.
++: Considerable microcallus proliferation.

### Fig. 2.12. Culture of protoplasts isolated from callus.

A: Dividing cells after two weeks of culture (arrowheads) in MS medium containing 2.0 μM BAP and 4.0 μM IBA. B: 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. C: Colony formation after 5 weeks of culture in MS medium containing 2.0 μM BAP and 4.0 μM IBA. D: Callus formation after 4 months of culture in MS medium containing 2.0 μM BAP and 4.0 μM IBA.
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, and 4.0 μM) and NAA, IBA, or 2,4-D (2.0 and 4.0 μM) after 5 weeks of culture (Fig. 2.12C). The colonies actively proliferated from one month of culture, and they grew up to 1 mm in diameter. Among the different densities of protoplasts examined here, the plating density of $4 \times 10^5$ protoplast / ml induced the active growth and cell division of protoplasts (Fig. 2.13).

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 as those of the first culture without mannitol. After 4 months of culture in this medium, the microcalli of about 4 mm in diameter and their proliferation were observed (Fig. 2.12D). The results shown in Table 2.8 indicate that MS media containing 2.0 μM BAP with 4.0 μM NAA or IBA are suitable for the proliferation of stem-segment-derived callus, when protoplasts were cultured at the density of $4 \times 10^5$ protoplast / ml.

### 3.5.3. Culture of protoplasts isolated from cell suspension cultures

Protoplasts were successfully isolated from the cell suspension cultures induced from friable callus derived from stem segment by using the enzyme combination of 1.0% Cellulase Onozuka R-10 plus 1.0% Driselase. The obtained protoplasts were cultured in liquid B5 medium supplemented with the various combinations of a cytokinin (BAP or TDZ) and an auxin (NAA, IBA, or 2,4-D). In this experiment, the protoplasts were also cultured at three different cell densities of $2 \times 10^5$, $4 \times 10^5$, and $6 \times 10^5$ protoplast / ml. After one week of culture, protoplasts showed spherical and non-spherical cell enlargement in the B5 medium containing 2.0 μM BAP and 4.0 μM IBA (Fig. 2.14A). The first cell division occurred after 10 days of culture in the B5 liquid media containing high concentration (4.0 μM) of NAA, IBA, or 2,4-D with 2.0 μM of BAP (Fig. 2.14B). After one month of culture, microcolony was formed in the media with various combinations of NAA, IBA, or 2,4-D and BAP or TDZ at all cell densities.

![Fig. 2.13. Effects of different combinations and concentrations of a cytokinin and an auxin, and plating density on colony formation from protoplasts isolated from stem-segment-derived callus.](image)

![Table 2.8. Effects of plant growth regulators and plating density on microcallus formation and proliferation of protoplasts from stem segment-derived callus.](image)

![Fig. 2.14. Culture of protoplasts isolated from cell suspension culture in B5 medium containing 2.0 μM BAP and 4.0 μM IBA.](image)
densities, in which the cluster composed of more than 10 cells are defined as a microcolony (Fig. 2.14C). After 7 weeks of culture, the number of colonies larger than 3 mm in diameter in each well was counted (Fig. 2.14D). The results obtained are shown in Fig. 2.15. In the culture of protoplasts from cell suspension cultures induced from stem-segment-derived callus, cell density of $4 \times 10^5$ protoplast / ml was effective for microcolony formation, while that of $2 \times 10^5$ protoplast / ml was less effective. A comparison of the microcolony formation among the media showed that BAP gave higher colony efficiency than TDZ. Further more, many irregular-shaped cells, such as enlarging cells (Fig. 2.16A) and pearl-chain-like cells (Fig. 2.16B), were observed in some media supplemented with BAP and NAA or IBA. Many colourless, fiber-like cells (Fig. 2.16C), crescent-like cells (Fig. 2.16D), and enlarging cells were observed in BAP- and 2,4-D-supplemented media. At a certain time, some enlarging cells showed ring-like shape (Figs. 2.16 F-H). Cell division was also occurred in enlarging cells (Figs. 2.16D,E). As the results, the most active colony formation occurred under the following conditions: B5 liquid medium containing 2.0 $\mu$M BAP and 4.0 $\mu$M NAA at cell density of $4 \times 10^5$ protoplast / ml, where the colony frequency 1.5% was obtained.

After 2 months of culture, the obtained colonies were transferred to a 24-well plastic culture plate containing the fresh liquid B5 medium or MS solid medium of the same components as those of the first culture, except for no addition of mannitol. The subcultured colonies developed into the microcalli larger than 1 mm in diameter after 1 month of subculture (Fig. 2.14E). Table 2.9 shows the effects of plant growth regulator and cell density on the formation and proliferation of microcallus. In this experiment, it was revealed that 2.0 $\mu$M BAP and 4.0 $\mu$M NAA or IBA, and plating density of $4 \times 10^5$ protoplast / ml were the best conditions for callus induction and proliferation from the protoplasts of cell suspension cultures which were induced from friable callus derived from stem segment.

![Fig. 2.15. Effects of different combinations and concentrations of a cytokinin and an auxin, and plating density on colony formation from protoplasts isolated from cell suspension cultures.](image1)

![Fig. 2.16. Irregular types of cells in cell suspension cultures. A,B: Irregular-shaped (A) and pearl-chain-like cells (B) after two weeks of culture in B5 medium containing 2.0 $\mu$M BAP and 4.0 $\mu$M NAA. C,D: Fiber (C) and crescent-like cells (D) after two weeks of culture in B5 medium containing 2.0 $\mu$M BAP and 4.0 $\mu$M 2,4-D. E,F: Dividing and enlarging cells after 2 weeks of culture in B5 medium containing 2.0 $\mu$M BAP and 4.0 $\mu$M 2,4-D. Arrowheads indicate cell division. F-H: Continuous-ring-like cell formation in B5 medium containing 2.0 $\mu$M BAP and 4.0 $\mu$M 2,4-D.](image2)

**Table 2.9. Effects of plant growth regulator and cell density on microcallus formation and proliferation from protoplasts induced from the suspension cultures of stem-segment-derived callus.**

<table>
<thead>
<tr>
<th>Plating density</th>
<th>4 $\times 10^5$ protoplast / ml</th>
<th>6 $\times 10^5$ protoplast / ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA ($\mu$M)</td>
<td>BAP ($\mu$M)</td>
<td>2,4-D ($\mu$M)</td>
</tr>
<tr>
<td>2.0</td>
<td>4.0</td>
<td>+</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>++</td>
</tr>
<tr>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+: Microcallus formation.
++: Considerable microcallus proliferation.
+++: Massive microcallus proliferation.
3.5.4. Effects of culture media on colony formation from the different sources of protoplasts

The effects of components and conditions of medium, and source of protoplasts on colony formation from cultured protoplasts were examined. Protoplasts isolated from different sources were cultured in liquid or solidified (0.2% gellan gum) medium with each of three different media, MS, MMS₁, 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 protoplast / ml. After one month of culture, data were recorded and are presented in Table 2.10. This experiment was repeated three times. Both media and types of culture, and their interaction had a significant effect on colony formation. Colony formation of protoplasts induced from suspension cultures was significantly higher (0.62%) with MS solid medium than other media (Table 2.10). This fact revealed that the culture of suspension-culture-derived protoplasts on MS solid medium was the best for protoplast culture of *P. amurense*.

3.5.5. Effects of pH on colony formation

To find out the effects of pH on colony formation in protoplast culture, other experiments were conducted. The protoplasts isolated from stem-segment-derived suspension cultures were cultured in B₅ liquid medium containing 3.0% sucrose, 2.0 µM BAP, and 4.0 µM NAA. The medium adjusted to pH 4.7, 5.7, and 6.0. Percentage of visually intact protoplasts was recorded after one month of culture. As shown in Fig. 2.17, there were no great differences in the percentage of visually intact protoplasts over the pH ranges tested in this experiment. However, it was noted that pH 5.7 was slightly better than 4.7 and 6.0.

3.6. Plant Regeneration from Protoplast-Derived Callus

3.6.1. Proliferation of protoplast-derived callus

The microcalli (about 2-3 mm in diameter) obtained from protoplasts of leaf, stem-segment-derived callus, and suspension cultures were used for callus proliferation. The protoplast-derived microcalli were cultured on MS medium supplemented with different combinations and concentrations of a cytokinin and an auxin. The previous study (Table 2.10) revealed that liquid media were less effective for colony formation or proliferation of callus. In contrast, solid medium more 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 medium 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.

![Table 2.10. Effects of culture media on colony formation.](image)

All media had 2.0 µM BAP and 4.0 µM NAA, and plating density was 4×10⁵ protoplast / 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.

![Fig. 2.17. Effects of medium pH on the percentage of visually intact protoplasts isolated from suspension cultures.](image)
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 used here, considering that in general green callus is in physiologically active and suitable for differentiation of shoots.

Among the three sources of protoplast-derived microcalli, stem-segment-derived callus and suspension cultures showed best results than that from leaf. The maximum 80.0% of callus proliferation was obtained from the protoplast-derived microcalli derived form both stem segments and suspension cultures on MS medium containing 2.0 μM BAP and 4.0 μM NAA or IBA, while leaf protoplast-derived microcalli showed maximum 60.0% of callus proliferation (Fig. 2.18). The cytokinin 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 (Figs. 2.19A,B). In the present study, successful proliferation of callus through the protoplast culture was obtained.

3.6.2. 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.

Leaf-protoplast-derived calli successfully regenerated small shoot buds after 6 weeks on the regeneration media (Fig. 2.20A). The number of calli which regenerated shoots varied with types and concentrations of plant growth regulators. Among the different combinations and concentrations, only two combinations 2.0 μM BAP plus 1.0 μM NAA and 4.0 μM BAP plus 2.5 μM IBA, showed 40.0% shoot differentiation with green calli, whereas 0.5 μM BAP with 0.5 μM NAA or 1.0 μM IBA only produced white-greenish callus. Other combinations failed to produce any callus or shoots (Table 2.11).

The adventitious shoots were successfully induced from the protoplast-derived green calli, produced from stem-segment-derived callus (Fig. 2.20B), whereas root differentiation occurred from the white-greenish calli on MS medium containing 0.5 μM BAP and 1.0 μM IBA (Fig. 2.21). The green calli were soft compact, while white-greenish calli 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.
Fig. 2.20. Shoot differentiation from protoplast-derived callus.
A-C: Shoot differentiation of calli derived from leaf (A), callus (B), and cell-suspension-culture protoplasts on MS medium containing 2.0 μM BAP and 1.0 μM NAA. D-F: Multiplication and elongation of shoots differentiated from calli derived from leaf (D), callus (E), and suspension-culture-protoplast on MS medium containing 1.0 μM BAP.

Table 2.11. Effects of plant growth regulators on adventitious shoot regeneration from leaf-protoplast-derived callus.

<table>
<thead>
<tr>
<th>NAA (μM)</th>
<th>BAP (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>WG</td>
<td>WG</td>
</tr>
</tbody>
</table>

+ : Indicates 40.0% shoot differentiation.
++ : Indicates 60.0% shoot differentiation.
+++ : Indicates 80.0% shoot differentiation.
WG : Indicates white-greenish callus formation.
G : Indicates green callus formation.

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 (Table 2.12). In this study, 1.0 μM CPPU and 1.0-2.5 μM NAA developed only green callus, which failed to produce any shoots.

Active shoot differentiation also occurred after 6 weeks of culture on protoplast-derived calli from suspension cultures (Fig. 2.20C). The callus proliferation and shoot development frequency were recorded after 10 weeks of culture. This study showed the same results as those of stem-segment protoplast-derived callus culture. In this study, the maximum 60.0% of shoot proliferation was obtained on MS medium after 6 weeks of culture, when 4.0 μM BAP plus 1.0 μM NAA and 4.0 μ BAP plus 2.5 μM IBA were used as plant growth regulators (Table 2.13). In all the experiments, two combinations, 0.5 μM BAP plus 0.5 μM NAA and 0.5 μM BAP plus 1.0 μM IBA proliferated only white-greenish calli derived from leaf, stem-segment-derived callus, and suspension-culture protoplasts.

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 (Figs. 2.20D,E,F, 2.22). The results of protoplast culture in P. amurense that have been investigated in this study were summarized in Table 2.14.
Table 2.13. Effects of plant growth regulators on adventitious shoot regeneration from protoplast-derived callus induced from suspension cultures from stem-segment-derived callus.

<table>
<thead>
<tr>
<th>NAA (μM)</th>
<th>IBA (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>WG</td>
</tr>
<tr>
<td>1.0</td>
<td>G</td>
</tr>
<tr>
<td>1.0</td>
<td>G ++</td>
</tr>
<tr>
<td>2.0</td>
<td>G ++</td>
</tr>
</tbody>
</table>

CRR (%) (μM), BAP (μM)

WG: Indicates white-greenish callus formation.
G: Indicates green callus formation.

Table 2.14. Summary of protoplast culture in P. amurense.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean fresh weight (g)</th>
<th>Total number of shoots</th>
<th>Total number of shoots with root formation</th>
<th>Protoplast number</th>
<th>Protoplast number of shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>10% Calliobiochimia,</td>
<td>1.0</td>
<td>5</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>80% Calliobiochimia,</td>
<td>1.0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>80% Calliobiochimia</td>
<td>2.0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
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</table>

Fig. 2.23. Rooting of the protoplast-derived shoots. A: Root formation at the base of microcuttings obtained from calli derived from leaf protoplast on MS medium containing 2.0 μM IBA. B: Root formation at the base of microcuttings obtained from calli derived from leaf protoplast on MS medium containing 1.0 μM NAA.

3.6.3. Rooting of the in vitro proliferated shoots

The microcuttings (2-4 cm long) were prepared from elongated shoots induced from leaf-protoplast-derived 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 basal cut portion of the shoots. There were satisfactory results in rooting: 95.0% shoots, 4.0 total number of root, and 5.0 cm average length could be obtained on MS medium containing 2.0 μM IBA (Fig. 2.23A), while 85.0% root formation, 2.0 maximum number of root, and 3.9 cm average length were obtained with 1.0 μM NAA (Fig. 2.23B).

4. DISCUSSION

The most significant finding of this study was the development of protocols for plant regeneration from protoplasts of a medicinal plant P. amurense. The initial plant material used for protoplast isolation proved to be an important factor for successful isolation and regeneration of P. amurense. Reasonable yield and viability of protoplasts were obtained from in vitro grown shoot cultures. The leaves obtained from in vitro germinated seedlings only produced a few protoplasts that were unable to form cell colonies (data not shown). Pan et al. (2003) reported the same results in two Egyptian medicinal plants, Artemisia judaica and Echinops spinosissimus, and they also mentioned that micropropagated shoots were the suitable materials for protoplast isolation. Binding (1975) demonstrated that shoot-culture-derived leaves of tobacco produced higher yields of viable protoplasts which showed cell division at a relatively higher frequency. The use of shoot cultures further proved to be highly efficient for producing protoplasts of other plant species, Solanum medongena (Saxena et al., 1987) and Pithecellobium dulce (Saxena and Gill, 1987). Sihachakr et al. (1995)
also reported that the lamina of 3-4 weeks old cultured shoot of *Solanum khasianum* was the best source of protoplasts. The uniformity of growth conditions and the conditioning of the tissues to in vitro growth are the potential reasons for a greater viability and regeneration ability of the protoplasts isolated from shoot cultures.

Before starting protoplasts culture, it should be needed to find out essential culture conditions and culture environment of callus induction from the experimental explants. In this study, leaf and stem segment explants were used as the source of protoplasts. To find out their callus induction efficiency, leaf and stem segment explants were cultured on MS medium containing different combinations and concentrations of a cytokinin and an auxin. The leaf explants produced considerable callusing on the MS media containing 2.0 \( \mu \text{M BAP} \) with 4.0 \( \mu \text{M NAA} \), IBA, or 2,4-D. Friable callus was formed on MS medium supplemented with 2.0 \( \mu \text{M TDZ} \) and 4.0 \( \mu \text{M 2,4-D} \). Li-Chun et al. (1998) reported the same results for callus production from leaf and stem in *Cinnamomum camphora*. Stem segment explants produced highly proliferating friable callus showing green on the medium containing 2.0 \( \mu \text{M BAP} \) with 4.0 \( \mu \text{M NAA} \), IBA, or 2,4-D. Schween and Schwenkel (2003) reported the similar results for callus production in *Primula* spp. Ikuta et al. (1998) reported that 0.1 \( \mu \text{M KI} \) with 1.0 \( \mu \text{M 2,4-D} \) was better for callus induction from the stem segment in *P. amurense*. In the present study, it was revealed that 2.0 \( \mu \text{M BAP} \) or TDZ with 4.0 \( \mu \text{M NAA} \), IBA, or 2,4-D were the best formulations, and stem segment was the suitable explant for callus production in *P. amurense*. The results are similar to these of callus induction in *Astragalus adsurgens* (Luo and Jia, 1998). In the present study, callus was induced successfully from the leaf and stem segment explants. No adventitious embryos were induced from the subcultured callus during the prolonged culture periods in this study. Further research is needed to establish the culture conditions for inducing adventitious embryos from callus.

The growth characteristics have been measured with using several growth parameters in cell suspension cultures of many plants, such as *Daucus*, *Convulvus*, and *Haplopappus* (Torrey et al., 1962), *Nicotina* (Flinear, 1965), *Rubus*, *Lims*, *Parthenocissus*, and *Acer* (Hanshaw et al., 1963). Uchimiya and Murashige (1974) reported that growth stages of cell suspension cultures derived from *Nicotina tabacum* largely affected the yield of protoplasts. In the present study, cell suspension cultures were prepared from stem segment-derived friable callus. The selection of callus lines is most essential for the establishment of suspension cultures. Wang et al. (2001) reported that embryogenic callus was better for suspension cultures. Straub et al. (1988) also reported that the embryogenic calli were not friable and grew as lumps in liquid culture. In the present study, friable, and generative callus was selected to initiate suspension culture of *P. amurense*.

In general, although the medium containing high concentrations of inorganic salts as MS medium seems optimal for callus growth, the medium with low contents of inorganic salts seems effective in the differentiation from callus rather than that with high contents (Uddin et al., 1988). B5 medium used in the present study also has lower contents of inorganic salts than does MS medium. Furthermore, the amount of the total inorganic nitrogen in the B5 medium is about one half of MS medium, 27 mM. Park and Choi (1999) demonstrated that MS liquid medium and dark condition at 25°C were suitable for cell suspension culture from callus in *P. amurense*. In this study, B5 medium with 2.0 \( \mu \text{M BAP} \) and 4.0 \( \mu \text{M NAA} \), IBA, or 2,4-D under light condition at 25°C was more effective than other media and plant growth regulators. This is in agreement with *Colesus* spp. (Petersen, 1994), *Anchusa officinales* (De-Eknamkul and Ellis, 1988), and *Astragalus* sp. (Ionkova, 1995).

Cocking (1960) has first used enzymes to isolate protoplasts from higher plants. He applied culture filtrates of a cellulolytic fungus *Myrothecium verrucaria* to release protoplasts from tomato root tips. Later,
Takebe and co-workers succeeded in applying commercially available cell-wall-degrading enzymes, Macerozyme and Cellulase Onozuka, to isolate large quantities of protoplasts from a wide variety of plant tissues (Takebe et al., 1968; Otsuki and Takebe, 1969). Thereafter, many enzyme preparations have been produced commercially and used successfully for the isolation of plant protoplasts. These enzymes are divided into three categories, cellulase, hemicellulase, and pectinase by their degrading actions toward substrates. Combined use of these enzymes and development of isolation procedures have made it possible to isolate protoplasts from almost all kinds of plant tissues. However, certain tissues, such as pollen cells or pollen tetrad, require specific enzyme combinations or special treatment to release protoplasts (Bajaj, 1983).

It is very important to select enzymes to digest cell wall components for obtaining a high yield of protoplasts with a high viability. In the present study, 24 enzyme combinations with different treatment duration were tested to select the best one for the protoplast isolation from leaf, stem-segment-derived callus, and cell suspension cultures. Among the 24 combinations, Cellulase Onozuka R-10 with Driselase for 6-8 hrs enzymatic treatment was relatively suitable for protoplast isolation from all of the experimental explants. Comparing protoplast viability obtained by using Cellulase Onozuka RS system with those by R-10, the latter gave better results. Wakita (1997) demonstrated the same results on the protoplast isolation in Betula platyphylla leaf. Wakita (1997) also reported that Cellulase Onozuka RS with Driselase was better for highest yield and viability of leaf protoplasts in Alnus firma. So far, there is no report on protoplast isolation of P. amurenses. This study, therefore, is the first experiment of protoplast isolation in this plant species.

Most of the scientists used either mannitol or sorbitol as an osmotic stabilizer for both green plant materials and cell suspension cultures. The osmotic potential in isolation mixtures also influenced protoplast isolation of P. amurenses. In this study, three concentrations (0.4, 0.6, and 0.8 M) of mannitol were used to isolate protoplasts from leaf, stem-segment-derived callus, and cell suspension cultures. The yield of protoplasts from all the experimental explants increased, when mannitol concentration was increased from 0.4 to 0.6, and then with 0.8 M decreased. Similar results were reported in Prunus amygdalus (Wu and Kuniyuki, 1985), Ginkgo biloba (Trémouillaux-Guiller et al., 1996), Alnus firma (Wakita, 1997), Artemisia judaica, and Echinops spinossissimus (Pan et al., 2003).

According to several reports, the use of young explants from micropropagated plants as well as nature of the protoplast source is an important factor (Lense and Chupeau, 1986; Dupuis et al., 1990; Dorion et al., 1994, 1999). In this study, it was shown the first time with P. amurenses that the leaves were better than all other materials (callus and suspension culture) tested for protoplast isolation, whereas callus and cell suspension cultures gave better results than leaves for protoplast viability and colony formation. This may be a reflection of the unique physiology and growth requirement of this species. Similar results were reported in Artemisia judaica and Echinops spinossissimus (Pan et al., 2003). A minimal number of protoplasts per ml is always required to ensure cell divisions (Yang and Laliberté, 1996). It was found in this study that protoplasts could divide from a plating density as low as $2 \times 10^2$ protoplast / ml, and an optimum of $4.6 \times 10^4$ protoplast / ml. In order to optimize colony formation, the incubation period has to be limited to 6 hrs despite a maximal protoplast yield recorded after 12-18 hrs. Similar observation was found in Platamnus orientalis (Wei and Xu, 1995). In this study, the highest percentage of colony formation was 0.8%, 1.2%, and 1.5% for leaves, callus, and suspension cultures, respectively. 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 leaf and callus protoplasts. This agrees with reports for other
woody species, such as *Gentiana scabra* (Takahata and Jomori, 1989) and *Ginkgo biloba* (Tremouillaux-Guiller et al., 1996). In this study, it was revealed that cell-suspension-derived protoplasts gave better results in B; liquid medium. Similar results were found in *Mentha piperita* (Sato et al., 1996).

Apparently, the enzymes frequently show some toxicity. Light is necessary during enzyme incubation, as darkness was shown to increase protoplast mortality. It has been reported that leaf protoplasts retain the same photosynthetic and respiratory activities of complete leaves (Nishimura et al., 1984; Smith et al., 1989). Therefore, light may contribute to protoplast defence against enzyme toxicity and osmotic stress (Hahne and Lorz, 1988). 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 (Yang and Laliberté, 1996), in which the first week of protoplast culture was performed in the dark. In *P. amurense* leaf protoplast, photosynthesis also seems to be needed at early stages of culture.

More colonies are produced in MS solid medium without mannitol than in liquid medium with mannitol, whereas MMS; 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 is probably lower. The beneficial effects of solid medium on protoplast culture have been previously reported (Lorz et al., 1983). However, the beneficial effects varied with the nature of the gelling agent (Saito and Suzuki, 1999). 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 (Nairn et al., 1995) or by the influence on nutrients or growth regulator availability (Bornman and Vogelmann, 1984; Brand, 1993).

Individual groups of 20-25 protoplast-derived microcalli (approx. 2-3 mm in diameter) were transferred to petridishes 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, and incubated at 25 ± 1°C under the illumination of cool-white fluorescence tubular lamp (50 μmol·m⁻²·s⁻¹) for 16 hrs photoperiod. Microcalli obtained from stem-segment-derived callus and suspension cultures showed better performance than leaf protoplast-derived ones. Similar observation was reported in *Picea excelsa* (Strmen and Cierna, 1981). In this study, BAP and NAA or IBA were the most effective plant growth regulators for callus proliferation of protoplast-derived callus. Same results were found in many other woody species, such as: *Gentiana scabra* (Takahata and Jomori, 1989), *Ginkgo biloba* (Tremouillaux-Guiller et al., 1996), and *Rosa hybrida* (Marchant et al., 1997). It was noted that in case of cell suspension cultures, medium suitable for suspension culture or colony culture was B; whereas MS medium was optimum for callus proliferation or shoot induction. This is in agreement with protoplast culture of *Duboisia* (Kitamura, 1993).

Many regenerated plants were obtained from protoplasts of leaf-derived calli, stem-segment-derived calli, and cell-suspension culture-derived calli in this study. 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; or WPM medium. Similar results were reported in *Actinidia delicosa* (Cai et al., 1993), *Rosa species* (Matthews et al., 1994), and *Diospyros kaki* (Tamura et al., 1995).
Hormonal conditions influencing the callus growth apparently differed depending on the origin of tissue sources from which the protoplasts had been isolated. Organogenesis from the callus also depended on the culture conditions. It is very important to note that organogenesis from the callus apparently differed depending on the starting materials from which protoplasts were isolated and the callus induced. Therefore, physical and chemical conditions for organogenesis from the callus must be surveyed in more detail in this species.

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 observations were found in Solanum khasianum (Serraf et al., 1988; Sihachak et al., 1995).

*In vitro* rooting was achieved, 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 on the species and clone used, on the media combination, and on the concentration of the auxin used (Chalupa, 1992). In general, low salt media stimulated root number and length in Phellodendron (Maene and Derbergh, 1985). Kim et al. (1992) 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.

Several regenerants were multiplied and used for protoplast isolation under the same condition as seedlings. However, yield, planting efficiency, and regeneration capacities of these true genotypes were not compared to the original heterogeneous seedling-derived protoplasts. Despite this, further studies may lead to the development of shoot regeneration strategies based on protoplasts of this plant species. Moreover, successful protoplast culture and plant regeneration in *P. amurense* now makes it possible to manipulate this species by genetic engineering.

5. SUMMARY

In the present investigation, an important medicinal plant species *Phellodendron amurense* was chosen as the material for plant tissue culture. Expanded leaves and stem segments from shoot culture or sterilized seedlings were excellent materials for protoplast isolation and culture. It was examined here that callus induction efficiency of leaf and stem segments, suitable medium and growth regulators for suspension cultures, enzymatic conditions for the isolation of protoplasts, and culture conditions of protoplasts (e.g. basal medium, plant growth regulator, and planting density) for the colony formation and subsequent plantlet regeneration from the protoplast-derived callus.

Morphology and the amount of callus formed varied with different levels of auxin and cytokinin. Callus was induced most effectively from the leaves and stem segments with using MS solid media containing 2.0 μM BAP and 4.0 μM NAA or IBA. The growth of callus was largely promoted in those media without browning throughout the subculture of callus.

Successful suspension cultures were induced from friable callus derived from stem segment in B5 liquid media supplemented with 2.0 μM BAP and 4.0 μM NAA, IBA, or 2,4-D with the highest growth rate.

The protoplasts were isolated successfully from the leaves, stem-segment-derived callus, and cell suspension cultures. The optimal enzyme solution for protoplast isolation was 1.0% Cellulase Onozuka R-10 plus 1.0% Driselase, and optimal osmoticum was 0.6 M mannitol. Leaf tissues produced a large protoplast yield of 10.5 × 10^3 protoplast / g fresh weight in this mixture, and the protoplasts obtained showed the highest viability of 86.0% by 6 hrs enzymatic treatment. Protoplasts from both friable callus derived from stem segments and cell suspension cultures were also isolated successfully by using the same enzyme mixtures. In this case, the satisfactory yield and viability of protoplasts were
obtained at 8 hrs enzymatic treatment and they were 5.5 × 10^5 protoplast / g fresh weight and 90.0% from callus, and 4.5 × 10^4 protoplast / g fresh weight and 95.0% from suspension cultures, respectively.

Protoplasts obtained from leaves and calli were cultured on MS liquid medium containing different combinations and concentrations of a cytokinin and an auxin with 2.0% sucrose and 0.6 M mannitol. The highest frequency 0.8% and 1.2% of colony formation were observed from leaf and callus-derived protoplasts, respectively, on MS medium containing 2.0 μM BAP and 4.0 μM NAA, where the effective plating density was 4 × 10^5 protoplast / ml. On the other hand, the highest frequency 1.5% of colony formation was obtained from cell-suspension-culture-derived protoplasts, when they were cultured in B_5 liquid medium containing 2.0 μM BAP with 4.0 μM NAA and 3.0% sucrose at the culture density of 4 × 10^5 protoplast / ml. After 8 weeks of culture, microcolonies were transferred to the same medium for their development into microcalli.

Microcalli obtained from leaf, callus, and suspension-culture-derived protoplasts were cultured on MS medium supplemented with 1.0-1.0 μM BAP or TDZ and 2.0-4.0 μM NAA or IBA for callus proliferation. The maximum 80.0% of callus proliferation was observed from the callus- and suspension-culture-protoplast-derived microcalli, when they were cultured on MS media containing 2.0 μM BAP and 4.0 μM NAA or IBA, while leaf-protoplast-derived microcalli showed 60.0% of callus proliferation on the same medium.

For plant regeneration from protoplast-derived calli, they were transferred to regenerating medium. About 40.0% of shoot regeneration was obtained from leaf-protoplast-derived calli on the MS media supplemented with 2.0 μM BAP plus 1.0 μM NAA or 4.0 μM BAP plus 2.5 μM IBA. On the other hand, about 80.0% of shoot regeneration were observed on MS media containing 2.0 μM BAP plus 1.0 μM NAA or 4.0 μM BAP plus 2.5 μM IBA for protoplast-derived callus induced from stem-segment-derived callus, and 4.0 μM BAP plus 1.0 μM NAA or 2.5 μM IBA produced 60.0% shoot regeneration from protoplast-derived callus induced from cell suspension cultures. For multiplication and elongation of protoplast-callus-derived shoot buds, they were transferred to 1.0 μM BAP-supplemented MS medium. Elongated shoots were rooted on 2.0 μM IBA-supplemented MS medium.

The present study is the first to have established successful plant regeneration system through the culture of protoplasts derived from the leaf, stem segment-derived callus, and cell suspension cultures of *P. amurenses*.

Chapter 3: Morpho-Histological Studies of Shoot Regeneration System from Callus of *Phellodendron amurenses* Rupr.

1. INTRODUCTION

Plant regeneration protocols for *Phellodendron amurenses* has been established, which were described in chapter 1 and chapter 2. Axillary shoots proliferation was achieved from nodal and shoot tip explants on MS medium containing 2.0 μM BAP from both of mature and seedling explants. In this study, nodal explants of *in vitro* grown seedling showed better performance than mature plant explant. Direct and indirect adventitious shoots were also regenerated successfully from hypocotyl, cotyledon, leaf, and internode explants. Regenerated shoots were rooted on MS medium containing 2.0 μM IBA, and they were acclimatized successfully and established onto the soil under greenhouse conditions.

Successful plant regeneration was also achieved from protoplast culture of *P. amurenses*. Protoplasts were isolated from the leaves, stem segment-derived callus, and cell suspension cultures by using 1.0% Cellulase Onozuka R-10 plus 1.0% Driedase in 0.6 M mannitol solution. Isolated protoplasts were cultured on MS or B_5 liquid medium containing 2.0 μM BAP with 4.0 μM
NAA for producing callus. Plant regeneration was obtained from proplast-derived callus on MS medium containing 2.0 μM BAP plus 1.0 μM NAA. Regenerated shoots were rooted on MS medium containing 2.0 μM IBA, and then they were established under ex vitro conditions.

Proper selection of the combination and concentration of cytokinin and auxin is the most important factor for callus induction and subsequent plant regeneration from different explants. However, the response to regeneration treatments is generally dependent on the genotype (Chevreau et al., 1997). Cytokinins have been defined as substances that stimulate cell divisions in plants and interact with auxins in determining the direction of cell differentiation (Wareing and Phillips, 1970). In the recent years, activation of cell division by cytokinins has been related to their action on the cell cycle at mitosis (Riou-Khamlichi et al., 1999). It is well documented that exogenous cytokinins play a major role in adventitious shoot regeneration. Nevertheless, studies on their distribution in tissue and site of their actions are very limited (Centeno et al., 1996).

Somatic embryogenesis is a process by which somatic cells undergo a developmental sequence similar to that seen in zygotic embryos (Williams and Maheswaran, 1980). Knowledge of the morphogenic and histological events that occur during somatic embryogenesis may make it possible to improve the efficiency of this process and to apply biotechnological methods to produce artificial seeds. Many biochemical and physiological studies have shown that abscisic acid promotes normal development of somatic embryos in vitro by stimulating the reserved substances and inhibiting precocious germination (Roberts et al., 1990; Gutmann et al., 1996).

The process of somatic embryogenesis and organogenesis from in vitro produced cultures has been examined histologically in order to determine the single or multicellular origin of somatic embryos. The foremost factors which affect somatic embryo or shoot bud formation are genotype, age, and origin of the initial tissues (Wann, 1988), which affect endogenous hormonal condition. Furthermore, the importance of the culture medium, particularly the exogenous application of plant growth regulators (PGRs) has been much discussed (Terzi and LoSchiavo, 1990). Thus, the embryogenic potential of explants is closely associated with their contents of both endogenous and exogenously applied PGRs. Unfortunately, little is known about this topic mainly due to the complexity and sophistication of the techniques required for PGRs analyses. Although a few research teams have found links between the embryogenic capacity of plant tissues and specific endogenous hormonal contents (Ivanova et al., 1994), in most cases, these results have been obtained in herbaceous plants and not in woody species.

In the present investigation, a morpho-histological study was undertaken to get a better understanding of cellular changes which take place during the plant regeneration process from callus tissues. This study led to the identification of different types of callus, cellular origin, specific cell characterization, and developmental pattern of regenerative callus. Further information regarding developmental stages of embryogenesis and organogenesis are needed to elucidate the developmental pattern of in vitro morphogenesis in Phellodendron amurense. Such work may lead to a better understanding of in vitro development in P. amurense, and consequently result in higher regeneration rates which would benefit clonal propagation and transformation work. This study describes, at the cellular level, the developmental pattern of organogenesis in P. amurense induced from the same explant source.

2. MATERIALS AND METHODS

2.1. Collection and Sterilization of Source Materials

Fruits of Phellodendron amurense Rupr. were collected from a 50-year-old tree growing at the Medicinal Plant Garden of Kumamoto University, Japan. Flesh was removed from fruits and seeds were recovered. They
were washed for 15 min in neutral detergent solution (1.0 ml/l tap water) and then rinsed thoroughly with running tap water for 20 min. After these seeds were surface-sterilized with 70.0% ethanol for 3 min, they were moved to a sterilized conical flask in a laminar-air-flow cabinet. Subsequently, surface disinfection was done with 3.0% (v/v) sodium hypochlorite solution (Wako Pure Chem. Indus. Ltd., Japan) for 20 min. To remove any trace of the sterilant, the seeds were then washed with at least three changes of sterile distilled water. Sterilized seeds were germinated on 10 ml of MS medium supplemented with 2.0 μM BAP in culture tubes (120×25 mm, Asahi Techno Glass, Japan).

2.2. Induction and Culture of Callus

Hypocotyl explants were excised from in vitro grown seedlings after 4 weeks of culture and placed in petridishes (9×1.5 cm, Asahi Techno Glass, Japan) with 30 ml MS semi-solid medium supplemented with different combinations and concentrations of BAP and NAA, IBA, or 2,4-D for inducing callus. Media containing different levels (0.44, 0.89, 2.22, and 4.44 μM) of BAP in combination with NAA (1.07, 2.89, 5.37, and 10.74 μM), IBA (0.98, 2.46, 4.92, and 9.84 μM) or 2,4-D (0.90, 2.26, 4.52, and 9.05 μM) were used for inducing callus. Continuous propagation of callus was done by subculture on callus medium every 8 weeks. Initial callus (6-8 mm) was then subdivided into three to four small calli (3 mm), which were separately cultured on the callus medium for propagation. These calli were used for morphological and histological studies for understanding the differentiation mechanism of adventitious buds at cellular level. All media were adjusted to pH 5.7 ± 0.1, fortified with 3.0% sucrose, and gelled with 0.2% gellan gum. The media were autoclaved under 1.2 kg/cm² pressure at 120°C. The cultures were grown at 25 ± 1°C under 16 hr photoperiod with a light intensity of 50 μmol·m⁻²·s⁻¹.

2.3. Data Analysis

Twenty replicates were used for all experiments. Experiments were repeated four times. The effects of different treatments were quantified, and the data were evaluated by analysis of variance (ANOVA). In addition, Tukey's multiple comparison was used to distinguish differences among treatments.

2.4. Morphological and Histological Studies

2.4.1. Morphological and histological observation

Hypocotyl-derived calli, which were produced on the media containing 0.89-4.44 μM BAP plus 2.69-10.74 μM NAA, or 2.46-9.84 μM IBA, and 0.44-0.89 μM BAP plus 2.26-9.05 μM 2,4-D, were used for morphological and histological studies. Morphological evaluation of callus samples after 3-6 weeks period in culture was performed using a stereomicroscope (Olympus SZX 12), and morphological features were recorded with an Olympus camera. For histological studies, calli were sampled at weekly intervals from culture initiation and were fixed with FAA (5 ml formalin, 130 ml ethanol, and 5 ml acetic acid) at room temperature for 2 days. They were rinsed thoroughly with running tap water for 1 day and dehydrated with graded ethanol series (ethanol concentration from 50.0% to 100.0% (v/v)), 5 min in each concentration). After replaced ethanol with xylene, they were embedded in paraffin and cut into sections of 10-15 μm thick by a rotary microtome (1512, Leitz). The sections were fixed with 3.0% gelatin (v/v) on a slide glass, deparaffined with xylene, and then stained consecutively with safranin and fast green. Finally, stained sections were observed by a light microscope (BH-2, Olympus).

2.4.2. Scanning electron microscope (SEM) observation

Small pieces of callus fixed with 3.0% glutaraldehyde in phosphate buffer solution were cut into about 4×4×3 mm, dehydrated in graded ethanol series (50.0% to 100.0% (v/v)), and then dried thoroughly by critical point drying. They were mounted on specimen stubs by
using Bond gum (Konishi Co., Ltd., Japan) and coated with gold for 7 min by using an ion-sputter apparatus (JFC-1100, JEOL). Surface layer was observed by a scanning electron microscope (JSM-5200, JEOL) at 15 kV.

3. RESULTS

3.1. Induction and Culture of Callus
The morphogenic difference of the callus depended upon the supplemental PGRs. Among different combinations and concentrations of the PGRs in the MS medium, 0.89 µM BAP plus 4.52 µM 2,4-D and 4.41 µM BAP plus 5.37 µM NAA showed better performance to produce of callus with the frequencies of 90.0% and 80.0%, respectively, whereas, 2.22 µM BAP plus 4.92 µM IBA gave only 60.0% frequency of callus formation (Table 3.1). Low concentrations of auxins (1.07 µM NAA, 0.98 µM IBA, and 0.90 µM 2,4-D) in combination with all concentrations of BAP failed to produce any callus. On the other hand, 0.44 and 0.89 µM BAP with 2.69-10.74 µM NAA or 2.46-9.84 µM IBA produced slightly small amount of callus. These combinations produced only green coloured-hard-compact callus, whereas 0.44 µM BAP with 2.26-9.05 µM 2,4-D produced white coloured-hard-compact callus. The combinations of 2.22 µM BAP with 2.69-10.74 µM NAA or 2.46-9.84 µM IBA produced tuber-like callus, and the callus production rate were 25.0%, 70.0%, and 50.0%, and 30.0%, 60.0%, and 50.0%, respectively.

Besides, 4.44 µM BAP in combination with 2.69-10.74 µM NAA or 2.46-9.84 µM IBA produced friable soft callus. On the other hand, 0.89 µM BAP in combination with 2.26-9.05 µM 2,4-D produced callus with ELSs, and the frequencies were 50.0%, 90.0%, and 70.0%, whereas 0.44, 2.22, and 4.44 µM BAP in combination with 2.26-9.05 µM 2,4-D produced only few percentage of callus. Some ELSs showed a tendency to undergo dedifferentiation. Callusing started at different points on the surface of the explants after 2-3 weeks of culture irrespective of the kinds of PGRs used here, and then almost the entire surface of the explants was covered with callus within 8 weeks.

3.2. Morphological Study
Within a few days of culture, a progressive swelling of the callus occurred. At 30th day from culture initiation, four types of response, explants with amorphous callus (non regenerating callus), with regenerating callus, callus with ELSs, and with tuber-like structure could be distinguished as shown in Fig. 3.1. The amorphous callus was hard compact, friable, and also soft (Figs. 3.1A, B). It did not develop any shoots or roots and turned into yellowish or brown colour after 10 weeks of culture. The regenrating callus, which was developed on MS medium containing 2.22 µM BAP plus 5.37 µM NAA, was less friable, yellowish to green, and it formed green shoots after transferred onto the MS medium supplemented with 1.5 µM BAP plus 1.0 µM NAA (Figs. 3.1C, D). The callus with ELSs was not compact, and showed green to yellowish on MS medium supplemented with 0.89 µM BAP plus 4.52 µM 2,4-D (Fig. 3.1E). At 35th day of culture, ELSs became whitish and globular (Fig. 3.1F). Heart-like shaped globular structure was also found in this medium (Fig. 3.2A). After 50 days of culture, some ELSs were underwent to dedifferentiation. Tuber-like callus was also observed, when hypocotyl explants were cultured on MS medium with 2.22 µM BAP and 9.84 µM IBA. This was green to yellowish and hard-compact callus, and a few roots were found on the surface layer of

Table 3.1. Effects of various plant growth regulators on callus formation from the hypocotyl explants on MS medium.

<table>
<thead>
<tr>
<th></th>
<th>BAP (µM)</th>
<th>0.44</th>
<th>0.89</th>
<th>2.22</th>
<th>4.41</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>10 ± 1.1F</td>
<td>20 ± 1.8F</td>
<td>25 ± 1.5F</td>
<td>40 ± 1.5F</td>
<td></td>
</tr>
<tr>
<td>0.37</td>
<td>25 ± 1.5F</td>
<td>40 ± 1.9F</td>
<td>50 ± 1.5F</td>
<td>80 ± 1.9F*</td>
<td></td>
</tr>
<tr>
<td>0.14</td>
<td>20 ± 0.4F</td>
<td>30 ± 1.8F</td>
<td>50 ± 1.5F</td>
<td>85 ± 1.9F</td>
<td></td>
</tr>
<tr>
<td>0.08</td>
<td>2.46</td>
<td>5 ± 1.2F</td>
<td>10 ± 1.7F</td>
<td>30 ± 2.1F</td>
<td>15 ± 1.6</td>
</tr>
<tr>
<td>0.04</td>
<td>4.92</td>
<td>15 ± 1.6F</td>
<td>20 ± 0.9F</td>
<td>60 ± 1.74*</td>
<td>30 ± 2.1F</td>
</tr>
<tr>
<td>0.06</td>
<td>9.86</td>
<td>30 ± 1.7F</td>
<td>50 ± 1.5F</td>
<td>50 ± 1.2F</td>
<td>40 ± 2.3F</td>
</tr>
<tr>
<td>0.01</td>
<td>2.26</td>
<td>15 ± 1.9F</td>
<td>50 ± 1.4F</td>
<td>11 ± 1.7F</td>
<td>5 ± 1.6F</td>
</tr>
<tr>
<td>0.04</td>
<td>4.52</td>
<td>32 ± 1.8F</td>
<td>90 ± 1.9F*</td>
<td>25 ± 2.01F</td>
<td>15 ± 1.8F</td>
</tr>
<tr>
<td>0.08</td>
<td>8.01</td>
<td>25 ± 1.4F</td>
<td>70 ± 2.12</td>
<td>20 ± 1.6F</td>
<td>30 ± 1.1F</td>
</tr>
</tbody>
</table>

Data were recorded after 8 weeks of culture. Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level. * indicates no callusing. Asterisked calli were used for histological observation.
Fig. 3.1. Morphological appearance of different types of callus of Phellodendron amurense.
A: Hard-compact amorphous callus developed on MS + 2.22 µM BAP + 4.92 µM IBA after 7 weeks of culture. B: Soft-friable amorphous callus developed on MS + 4.44 µMBAP + 5.57 µM NAA after 7 weeks of culture. C: Less friable yellowish to green regenerating callus developed on MS + 2.22 µM BAP + 5.57 µM NAA after 6 weeks of culture, leading to organogenesis later. D: Torpedo-like structure developed on the surface of callus on MS + 1.5 µM BAP + 1.0 µM NAA after 9 weeks of culture. E: Callus with ELSs developed on MS + 0.9 µM BAP + 4.52 µM 2,4-D after 4 weeks of culture. F: Callus with ELSs developed on MS + 0.9 µM BAP + 4.52 µM 2,4-D after 5 weeks of culture.

callus (Fig. 3.2B). Some ovoid structures were found in the calli on MS medium containing 2.22 µM BAP plus 10.74 µM NAA (Figs. 3.2C,D).

3.3. Development of ELSs

The histological sections of callus with ELSs which were developed on MS medium containing BAP with 2,4-D after 4 weeks of culture, showed that below a layer of large and partly surface superficial cells, there was found a compact continuous layer of long or small, ovoid meristematic cells which were rich in organelles and cytoplasm (Fig. 3.3A). After 4 to 6 days of culture, vascular cells had dense cytoplasm and were stained well with safranin-fast green. After 10 days of culture, the planes of the first cell division in vascular tissues were observed, showing periclinal or anticlinal cell division (Fig. 3.3B). After 10 days of culture, the second division of the two-celled proembryo-like-

structure (PLS) took place in one of the two cells to form a three-celled PLS or in both cells simultaneously to form a four-celled PLS (Figs. 3.3C-E). After 14 days of culture, a five-celled PLS formed. In most other cases, cell divisions occurred after 10 to 15 days of culture within groups of adjacent cells of vascular tissues which thereafter developed into cell masses and globular structure of ELS (Fig. 3.3F). Finally globular- or ovoid-shaped ELSs occurred in hypocotyl-derived callus tissues.

3.4. Development of Shoots from Callus

The growth characteristics of adventitious shoots from callus were examined histologically. Two types of callus were used in this experiment: calli with ELSs which were developed on MS medium supplemented with BAP plus 2,4-D and regenerative calli which were developed on BAP plus NAA or IBA-containing MS medium.

3.4.1. Shoot development from callus with ELSs

Calli were composed of large cells with wavy walls forming vast and disorganized masses. Internally, two types of organization were observed: ELSs organization, and vascular and meristematic organization. From this callus, ovoid and globular with rugged-spherical appearance were found on MS medium containing 0.89 µM BAP plus 4.52 µM 2,4-D (Figs. 3.4A,B) after 7
weeks of culture. When these calli transferred to MS medium supplemented with 1.5 μM BAP plus 1.0 μM NAA or IBA, cell differentiation took place progressively. After 4 weeks of transfer some ELSs turned to heart-shaped, and developed meristematic dome with leaf primordium-like-structures which were easily distinguished. The heart-like-shaped structures showed a polarity, not only through different staining but also through the organisation. There was also found no connection with the vascular tissues of the initial ELSs. Shoot-tip differentiation (apical dome) was also found in ELSs (Figs. 3.1C,D). Within 7 weeks of transfer of callus with ELSs to BAP plus NAA or IBA-containing MS medium adventitious shoot development were observed.

3.4.2. Shoot development from regenerative callus

Hypocotyl-derived regenerative calli obtained from MS medium supplemented with 4.44 μM BAP plus 5.37 μM NAA, or 2.22 μM BAP plus 4.92 μM IBA were sampled at weekly intervals from culture initiation. The histological sections representing different developmental stages are presented in Figs. 3.5 & 3.6. In the presence of NAA or IBA, swollen external tissues without signs of differentiation were observed. The results gave some interesting findings showing that the shoot differentiation was derived from some structures in the lateral portion of the calli. This indicates a possible response to the PGRs applied.

Figures 3.5 & 3.6 show an organogenic process with the formation of initial shoot structures on the terminal portion of callus, which further developed to shoot. After one week of culture initiation, abundant callus formation and development were observed in the explant.
tissues. After 10 days of culture, the meristematic cells or tissues were distinguishable. Observation of regenerative callus sections showed that some cells had dense cytoplasm with large nucleus and nucleolus, a prominent feature of meristematic cells, and that a well-defined crescent-like cell region formed (Fig. 3.5A). Within 4-5 weeks, the meristematic tissues formed a meristematic zone and developed into a meristematic dome with procambium cells (Figs. 3.5B-D). It was recognized that the procambium cells were differentiated at the periphery of the central cylinder below the meristematic dome.

In the meristematic dome, three different cell divisions were observed. The first new division was anticlinal, where the new cell wall was not yet discernable; secondly, both anticlinal and periclinal, and thirdly, random direction divisions formed a continuous compact meristematic tissues consisting of small densely stained isodiametric cells with centrally located nuclei. The development of these activity growing sectors up to the early stage of shoot formation was followed day after day. This histological observation showed that the development of meristematic region was followed by the tunica-corpus theory. According to this theory, the initiating cells in the apex can be grouped into a central core (the corpus), which is enveloped by two outer layer cells organizing the tunica (Fig. 3.6F).

Histological studies showed that various development stage of meristematic dome was observed depending on the culture period of callus. After 5 weeks of culture, the active meristematic dome gave rise to leaf initial. Each meristematic dome was surrounded by leaf initials, leaf primordia, and preliminary leaves (Figs. 3.6A-D). The histological observations of tissues obtained one week after culturing in the callusing medium revealed that the division of prevascular cells of hypocotyl gave new meristematic cells. The meristematic cells formed by continuous division of prevascular cells became peripheral meristematic masses. This peripheral
meristematic zone ensured callus growth. At 40th day of culture, although the apical meristem was still distinguishable, the callus completely altered the morphology of the explant tissues. Meristematic activity was evident in the callus, and the first vegetative bud primordia were observed on the surfaces. After 60 days of culture, numerous vegetative buds originated from the callus surface, which later developed into shoots, were evident (Fig. 3.6E).

Meristematic centers consisted of small cells which occasionally coexisted with prevascular elements located in the central zone, which later on develop into the radicular primordium (Fig. 3.7A). This event was observed histologically when the callus developed on MS medium containing 2.22 μM BAP plus 9.84 μM IBA after 6 weeks of culture initiation. Distinctive cell differentiation in cortical and medullar parenchyma, primary vascularization, and initial tracheary elements were found under the leaf primordium (Fig. 3.7B). These elements were found only in the BAP plus NAA or IBA-containing MS medium after 6 weeks of culture initiation, whereas it was absent in BAP plus 2,4-D-containing medium. In this area, a spherical structure with its own vascular bundle developed.

Histological analysis revealed that shoot regeneration from callus treated with BAP plus NAA, or BAP plus IBA occurred through organogenesis.

3.5. Development of Cell Wall in Callus Tissues

At the onset of the differentiation of the woody cells, the living protoplasm produces a primary wall which can be extensively increased in surface area to keep pace with the enlarging volume of the cell as the cell develops.

To observe the cell and cell wall development in callus tissues, calli were obtained from MS medium containing 2.22 μM BAP plus 5.37 μM NAA after 5 weeks of culture initiation. Three different layers (surface, middle, and inner) of callus were examined by SEM. Detectable various developmental stages were observed in different layers. The cell wall of the cells in callus surface layer was thin and in irregular shape. Many granules were found in the surface layer's cells (Figs. 3.8A,B). A continuous cell wall development was observed, when the callus sections were examined step by step from the surface layer to inner layer, and the granules were also reduced gradually from upper layer to inner layer (Figs. 3.8A,C-E).

There were found many pit fields on the primary cell wall (Fig. 3.8D), which marks the location for later development of cell wall pits (Fig. 3.8H). Bordered pits were present in the cell walls of vessels, showing the characters of hardwood (Fig. 3.8F) of P. amurense. Figure 3.8E shows that normal development of secondary wall (S₁ layer) in wood fiber just started. When cell wall thickening was completed, the secondary wall consisted of three layers, a thin outer layer (S₁), a thick middle layer (S₂), and an inner thin layer (S₃). The similar structure of vessel and fiber was found in P. amurense callus tissues, when the inner layers of callus were observed (Fig. 3.8G).

Spiral thickenings are relatively common in hardwoods, in which they may occur in the vessel elements, fibers, and some ray cells. In the present study of P. amurense callus, spiral thickenings were also observed on the S₁ layer of vessels (Figs. 3.6F-I). This study revealed that cell wall development of callus tissues occurred continuously from surface layer to inner layer of callus.

Fig. 3.7. Development of radicular primordium and tracheary elements in callus tissues.
A: Development of radicular primordium after 6 weeks of culture when the calli were cultured on MS + 2.22 μM BAP + 9.84 μM IBA. B: Development of tracheary elements under the leaf primordium on MS + 2.22 μM BAP + 5.37 μM NAA after 6 weeks of culture.
(Sections were cut longitudinally to top of callus).
4. DISCUSSION

In this study, BAP with 2,4-D, and BAP with NAA were suitable as cytokinin-auxin combination for production of calli from hypocotyl explants of *Phellodendron amurense*. The intensity of callus proliferation was better in the medium with 0.89 μM BAP plus 4.52 μM 2,4-D for hypocotyl explants. The initial primary calli were yellowish and nodular. Choi et al. (1996) reported that LS liquid medium containing 0.44 μM BAP and 2.22 μM 2,4-D was more effective for callus induction of *Phellodendron amurense*. Ikuta et al. (1998) also reported that 4.5 μM 2,4-D and 0.44 μM Kn was the best formulation for callus induction of *Phellodendron amurense* in dark condition. In this study, it was revealed that MS media containing 0.89 μM BAP plus 4.52 μM 2,4-D, or 4.14 μM BAP plus 5.37 μM NAA were suitable formulation for callus induction and proliferation under light condition in *P. amurense*. The presents experiment showed that the acquisition ELS formation, which was reported by Pask et al. (2000) and Wassom (2001), clearly depended on the ratio between BAP and 2,4-D.

This study revealed that proliferation and morphology of callus depended upon different PGRs. Proliferation of callus was active on MS medium containing BAP plus NAA, whereas formation of ELSs was found in MS medium containing BAP plus 2,4-D. Tuber-like callus was formed, when hypocotyl explants were cultured on MS medium supplemented with BAP and IBA. Similar morphology of callus has been described in other woody species, e.g. *Mangifera indica* (Litz et al., 1984) and *Corylus avellana* (Rodriguez et al., 2000). It was observed in the present study that morphological changes of callus accompanied with its development were visible after 10 days of culture, while histological sections displayed certain cellular modifications as early as 4-5 days of culture initiation.

The histological studies of hypocotyl-derived callus showed different types of ELS and their development. Two to five-celled PLS were formed in vascular tissues within 10 to 14 days of callus culture. These events indicate a single cell origin of PLS derived from vascular tissues (Figs. 3.9A-G). Rodrigez et al. (2000) described the same observation in *Corylus avellana*. In most cases, the first cell division occurred after 10 to 12 days of culture within a group of adjacent cells of vascular tissues that developed into cell masses and globular structure of ELS. This suggests a multiple cell origin of ELS from vascular tissues (Figs. 3.10A-E). Histologically, the first stage of development of embryogenic nodules was characterized by breaking up the meristematic masses. Active cell divisions in the discontinuous zone led to the formation of protuberances composed of meristematic cells, followed by the formation of epidermis. It was considered that this
sequence of events provided a clear evidence for the multicellular origin of ELS. These findings are in agreement with Mangifera indica (Litz et al., 1984), *Pisum sativum* (Tetu et al., 1990) and *Carya illinoiensis* (Rodriguez and Wetzstein, 1998). However, the constitution of diads, tetrads, and cellular groups with densely stained cellular walls may show that a high percentage of the somatic ELS constituted is unicellularly originated. Similar results have been found in *Juglans regia* (Tucekie and McGranahan, 1985) and *Corylus avellana* (Rodriguez et al., 2000).

A distinctive cell differentiation in cortical and medullar parenchyma primary vascularization and formation of tracheary elements, was found under the leaf primordium. In this study, although formation of many ELSs was recognized in a callus, there was found no vascular connection with ELSs. Shoot-tip differentiation was also found in ELSs. These characteristics suggest that there are different capabilities of the formation and development of original tissues derived either from precocious or true type differentiation of ELS. This regeneration process has been described as organogenesis for numerous woody plants such as pomegranate (Raj Bhansali, 1990) and *Terminalia arjuna* (Nishi et al., 1997).

A sequential, developmental stage of shoot was observed in the histological studies of hypocotyl-derived callus, when the calli were cultured on MS medium containing 4.44 μM BAP plus 5.37 μM NAA, or 2.22 μM BAP plus 4.92 μM IBA, where within 4-5 weeks of culture, meristematic domes were developed. In a meristematic dome, three different cell divisions were observed; firstly anticlinal, secondly both anticlinal and periclinal, and thirdly random-directional divisions. This histological observation showed that the development of meristematic region was followed by the *tunica-corpus* theory. In the angiosperm, there is a recognizable stratification of cells in the promeristem, giving rise to the *tunica-corpus* theory of the primary cell formation (Reeve, 1948). In the dicotyledons which include all the hardwood species, the *tunica* commonly consists of two layers of cells on the surface, though *tunica* layers...
of up to five cells in thickness have been reported (Gifford, 1954, Reeve, 1948). This histological study revealed that the meristematic dome had a typical structure of a tunic-corpus arrangement consisting of the two layers as tunic cells.

There are only a few reports regarding the development of meristematic cells within callus in which different stages of somatic embryogenesis and organogenesis induced from the same explant source have been histologically examined (Shahana and Gupta, 2000; Chengalrayan et al., 2001). In sunflower, Bronner et al. (1994) showed that the morphogenetic event was multicellular in origin and occurred directly without intervening callus. The epidermis and hypocotyl cortex were the origins of organogenesis and initial cell divisions were periclinal. Histological analysis of organogenesis, induced in immature tissues of *Stylosanthes scabra* revealed that neoformed buds developed from deep-seated vascular nodule structures derived from callus tissue (Dornelas et al., 1992).

Histological studies also revealed that the active meristematic dome gave rise to leaf initial after 5 weeks of culture initiation. Similar results were reported in various dicotyledon plants such as *Aegle marmelos* (Arunugam and Rao, 1988) and *Simmondsia chinensis* (Agrawal et al., 2000). It was also found that shoot regeneration from calli occurred through organogenesis, when they were treated with BAP plus NAA or IBA, whereas ELSs were formed, when the callus was treated with BAP plus 2,4-D. Similar histological analyses were reported in apple (Caboni et al., 2000) and pear (Caboni et al., 2002). The histological observations reported here provided a novel evidence that both ELS formation and organogenesis were an indirect, transitional callus phase.

In this investigation, it was observed that meristematic centers consisted of small cells that may occasionally coexist with prevascular elements located in the central zone, which later developed into the radicular primordium. This result was confirmed by the formation of external roots, when NAA or IBA was used. Callus is a mass of unorganized dedifferentiated cells. Cell division occurs naturally in callus. Unorganized cells sometimes become organized and later developed shoots or roots under controlled conditions of PGRs. In this study cellular development of callus was studied by SEM observation. At the first step of differentiation of a woody cell, the living protoplasm produces a primary wall that can be extensively increased on its surface as the cell develops. Various developmental stages were observed in different layers of callus from the surface inward. The cell walls of cells in callus surface layer were thin, irregular shaped, and contained granules. Similar observations were found in *Carya illinoinensis* (Rodriguez and Wetzstein, 1998). The cell wall developed gradually from surface layer to inner layer of callus. The bordered pits and three-layered-secondary cell walls were observed in the inner layer of *P. amurense* callus. Harada and Côté (1967) described the same cell wall development in *Pinus palustris*. Spiral thickenings also developed gradually on the inner face of vessels from the surface inward in a callus. This feature is a characteristic of *P. amurense* species. Similar observation was found in *Tilia* sp. (Parham and Kaustinen, 1973).

Ariyoshi (1986) reported a successful plant regeneration through intrapetiolar bud in *P. amurense* using BAP. However, plant regeneration via callus or histological analysis has not been reported as yet. In this paper, the sequential development of ELS formation and organogenesis were confirmed: there are two pathways in shoot regeneration from callus (Fig. 3.11). The present study enabled to confirm that callus treated with BAP in combination of NAA or IBA resulted in successful shoot regeneration through calllogenesis. From the histological study, it was also confirmed that the ELSs, which were produced in medium containing BAP plus 2,4-D, differentiated into shoot, when they were transferred to BAP plus NAA- or BAP plus IBA-supplemented medium. These findings obtained in the present investigation are very interesting. It was found
that the emergence of organogenesis through callogensis fully depends on the physiological state of callus and the hormonal condition of the culture medium.

5. SUMMARY

A morpho-histological study was undertaken to gain a better understanding of cellular changes which take place during the shoot regeneration process from callus tissues. Hypocotyl explants were excised from in vitro grown seedlings and cultured on MS semi-solid medium supplemented with different combinations and concentrations of BAP and NAA, IBA, or 2,4-D for inducing callus. Among different combinations and concentrations of PGRs in MS media, 0.89 μM BAP plus 4.52 μM 2,4-D, and 4.44 μM BAP plus 5.37 μM NAA showed better performance to produce callus with the frequencies of 90.0% and 80.0%, respectively, whereas 2.22 μM BAP plus 4.92 μM IBA only gave 60.0% frequency of callus formation. ELSs were formed successfully in MS medium containing 0.89 μM BAP plus 4.52 μM 2,4-D. Tuber-like callus was produced from hypocotyl explants, when they were cultured on MS medium with 2.22 μM BAP and 9.84 μM IBA.

Many ovoid ELSs were found in the calli, when they were cultured on MS medium containing 2.22 μM BAP plus 10.74 μM NAA.

Anticlinal and periclinal cell divisions were observed in the histological section of regenerative callus of P. amurense. Two to five-celled PLSs were developed within 10-14 days of culture incubation. Many somatic ELSs with different shapes were found in the regenerative callus, which was produced in MS medium containing 0.89 μM BAP plus 4.52 μM 2,4-D after 6 weeks of transfer to the culture medium. Procambium and leaf primordium were also found in ELSs.

Adventitious shoot differentiation occurred in hypocotyl-derived regenerative calli, which were obtained from MS media supplemented with 4.44 μM BAP plus 5.37 μM NAA, or 2.22 μM BAP plus 4.92 μM IBA. After 10 days of culture incubation, the meristematic tissues formed crescent-like cell region in the callus. Within 3-4 weeks, the meristematic tissues formed a meristematic dome with a procambium. This histological study showed that the development of meristematic region occurred according to the tunia-corpus theory. After 5 weeks of culture initiation, the active meristematic dome successfully produced leaf initial.
Each meristematic dome was surrounded by leaf initials, leaf primordia, and preliminary leaves. After 60 days of culture, vegetative buds originated from the callus surface developed into shoots later. Histological analysis proved that in the hypocotyl-derived callus, BAP with NAA or IBA successfully induced adventitious shoots through organogenesis, whereas BAP with 2,4-D induced ELSs. These ELSs developed adventitious buds through organogenesis after transferred them to the MS medium containing BAP plus NAA.

Different layers (surface, middle, and inner) of callus from the surface inward were examined by SEM for observing the cell and cell wall development in callus tissues. A continuous cell wall development was observed, when the callus sections were observed step by step from the surface layer to inner layer, and the granules were reduced gradually accompanied with the development of cell wall from surface layer inward. Irregular shapes of cell with thin wall consisting granules were found in the surface layer of callus, whereas thick-walled cells with lacking of granules gradually appeared from the middle to inner layer of callus. Spiral thickening were also observed on the inner most layer of the three-layered secondary wall.

The results obtained in the present investigation gave the finding in that emergence of organogenesis through callogenesis depends on the physiological state of callus brought by hormonal condition.

**Conclusion**

The present investigation deals with the view of three aspects: (a) plant regeneration from mature and seedling explants, (b) plant regeneration from protoplast culture, and (c) morpho-histological studies of regeneration system of adventitious bud in in vitro produced callus. For carrying our this study, a most economically important medicinal tree, *Phellodendron amurense*, was chosen as the material for plant tissue culture.

**Chapter 1**: To establish a micropropagation protocol for *P. amurense*, node, shoot tip, and seed were collected from a field-growing mature tree. Collected materials were surface sterilized with 3.0% NaOCl for 30 min. Aseptic seedlings were raised *in vitro* on MS medium supplemented with 2.0 μM BAP. Hypocotyl, cotyledon, leaf, and internode explants were cultured for direct and indirect adventitious shoot regeneration, while node and shoot tip explants were used for axillary shoot proliferation. Three types of medium, MS, MMS, and WPM, and different kinds of cytokinin alone or in combination with different auxins were used in this investigation for proliferating axillary or adventitious shoots. Among the nodal and shoot tip explants of mature plants and seedlings, nodal segments of seedling showed the best performance (100.0%) for axillary shoot proliferation on MS medium supplemented with 2.0 μM BAP with highest 8.5 ± 0.6 shoots, whereas other media and plant growth regulators showed less effective on shoot proliferation. On the other hand, the longest average length (7.5 ± 0.2 cm) of shoot was found in seedling-derived shoot tip on MS medium with 1.0 μM BAP. However, the cytokinin and auxin combination inhibited the both shoot number and shoot length in axillary shoot proliferation.

The effects of cytokinin and auxin combination on adventitious shoot regeneration were evaluated with using MS media supplemented with different concentrations of BAP (0.9-6.6 μM) and NAA (0.5-2.5 μM) or IBA (0.5-2.5 μM). Among the different combinations and concentrations of a cytokinin and an auxins, 2.2 μM BAP plus 0.5 μM NAA combination was found to be the best formulation for proliferating direct adventitious shoots from hypocotyl and internode explants. On the other hand, 6.6 μM BAP with 0.5 μM NAA, and 4.4 μM BAP plus 0.5 μM NAA combinations were suitable for cotyledon and leaf explants, respectively. Regenerative callus was induced from hypocotyl, cotyledon, leaf, and internode explants on MS medium containing 2.0 μM TDZ and 4.0 μM 2,4-D. This combination produced only fast-growing calli, whereas they could not produce any shoots. For
producing adventitious shoots from these calli, when they were subcultured on MS medium supplemented with 1.5 \( \mu \text{M} \) BAP plus 1.0 \( \mu \text{M} \) NAA, adventitious shoots regenerated successfully.

For root induction, microshoots (2.0-4.0 cm in height with 2-3 leaflets) were prepared from \textit{in vitro} proliferated shoots, and were transferred to different strength of MS media containing 0.5-4.0 \( \mu \text{M} \) of NAA, IBA, or IAA. Maximum rooting (98.8\%) with 7.2 \( \pm \) 0.8 roots per microcutting was recorded on the MS medium containing 2.0 \( \mu \text{M} \) of IBA. The high concentration (4.0 \( \mu \text{M} \)) of all the auxins inhibited the root formation and produced malformation at the microcutting base. Within 30 days of incubation, 85.0\% root formation occurred in hormone-free MS medium. Rooted plantlets were transferred to plastic pots containing Kanuma soil, and successfully established under greenhouse conditions with the survival rate of 90.5\%.

Mass propagation by conventional breeding methods appears difficult. Propagation by \textit{in vitro} culture technique was more easily achieved than that of cuttings and root sprouting. These results presented here suggest potential applications for first multiplication of \textit{P. amurense}.

**Chapter 2:** In order to bring about any genetic manipulation through cell fusion, incorporation of DNA, and transformation, the regeneration of the entire plant through manipulation of protoplasts is a prerequisite. In the present study, isolation and culture of protoplasts, culture of protoplast-derived callus, and plantlet regeneration from the callus were investigated with using \textit{P. amurense}.

Different levels of auxin and cytokinin varied the morphology of callus and the amount of callus formation. Best callus formation occurred from leaves and stem segments on MS solid media containing 2.0 \( \mu \text{M} \) BAP and 4.0 \( \mu \text{M} \) NAA or IBA. The growth of callus was largely promoted in those media without browning throughout the subculture of callus. Suspension cultures have been prepared successfully from the stem-segment-derived friable callus in B5 liquid media containing 2.0 \( \mu \text{M} \) BAP with 4.0 \( \mu \text{M} \) NAA, IBA, or 2,4-D.

Successful protoplast isolation was achieved from the leaves, stem-segment-derived callus, and cell suspension cultures, when enzymatic combination of 1.0\% Cellulase Onozuka R-10 plus 1.0\% Driselase, and 0.6 M mannitol was used. Leaf tissues gave a suitable protoplast yield of 7.1 \( \times 10^5 \) protoplast / g fresh weight and the highest viability of 86.0\% by 6 hrs enzymatic treatment in this combination. On the other hand, satisfactory protoplast yields of 5.5 \( \times 10^5 \) and 4.5 \( \times 10^5 \) protoplast / g fresh weights and viability 90.0\% and 95.0\% were obtained from stem segment-derived callus and cell suspension cultures, respectively, in the same enzyme mixture by 8 hrs enzymatic treatment.

Leaf- and callus-derived protoplasts were cultured in MS liquid medium containing 2.0 \( \mu \text{M} \) BAP and 4.0 \( \mu \text{M} \) NAA with 2.0\% sucrose and 0.6 M mannitol. They gave the highest 0.8\% and 1.2 \% colony formation, respectively, when the plating density 4\( \times 10^3 \) protoplast / ml was used. Microcolony formation was observed within 2-5 weeks of culture incubation. On the other hand, the highest frequency 1.5\% of colony formation was achieved from cell-suspension culture-derived protoplasts in B5 liquid medium containing 2.0 \( \mu \text{M} \) BAP with 4.0 \( \mu \text{M} \) NAA and 3.0\% sucrose at the culture density of 4 \( \times 10^5 \) protoplast / ml. After one month of culture, the microcolony formation was observed in this medium, and the microcolonies were transferred to the same medium for their development into microcalli after 8 weeks of culture.

For the proliferation of callus, microcalli obtained from leaf-, callus-, and cell-suspension culture-derived protoplasts were cultured on MS medium supplemented with 2.0 \( \mu \text{M} \) BAP with 4.0 \( \mu \text{M} \) NAA or IBA. The maximum 80.0\% of callus proliferation was observed from the callus- and cell-suspension culture-protoplast-derived microcalli, while leaf-protoplast-derived microcalli showed only 60.0\% of callus proliferation in.

Plant regeneration was achieved from protoplast-derived calli, when they were transferred to regenerating medium. Leaf-protoplast-derived calli produced 40.0% shoot regeneration on MS media containing 2.0 μM BAP plus 1.0 μM NAA or 4.0 μM BAP plus 2.5 μM IBA, while callus-protoplast-derived calli produced 80.0% shoot regeneration on the same medium compositions. On the other hand, 60.0% shoot regeneration was obtained from protoplast-derived calli induced from cell suspension cultures on MS media supplemented with 4.0 μM BAP plus 1.0 μM NAA or 2.5 μM IBA. Active shoot elongation occurred on MS medium supplemented with 1.0 μM BAP. Microcuttings were rooted successfully on MS medium containing 2.0 μM IBA.

The present investigation is the first report on successful obtaining the whole plantlet regeneration from the protoplast culture of *P. amurense*. The plantlet regeneration system established here is considered to be applicable to other *Phellodendron* species.

Chapter 3: The aim of this investigation is to observe in detail the morphology and histology of callus for the initiation and development of somatic embryo-like-structures, and of shoots from callus tissues. In this study, BAP with 2,4-D or NAA was the suitable combination for producing callus from hypocotyl explants of *P. amurense*. Among the different combinations and concentrations of plant growth regulators in MS medium 0.89 μM BAP plus 4.52 μM 2,4-D and 4.44 μM BAP plus 5.37 μM NAA produced the highest 90.0% and 80.0% of callus, respectively. Embryo-like-structures were found in MS medium containing 0.89 μM BAP plus 4.52 μM 2,4-D, whereas tuber-like-structures were formed when hypocotyl explants were cultured on MS medium supplemented with 2.22 μM BAP and 9.84 μM IBA. On the other hand, some aroid embryo-like-structures were developed in the calli on MS medium supplemented with 2.22 μM BAP and 10.74 μM NAA. Morphological study revealed that different types of callus formation and their structures depended on different cytokinin-auxin combinations and their concentrations.

In this study, histological observation of generative callus showed that cell divisions occurred anticlinally and periclinally in callus tissues. Two-to-five-celled proembryo-like-structures were observed in the callus, which are the prerequisite for somatic embryo formation. Many somatic embryo-like-structures observed on MS medium containing 0.89 μM BAP plus 4.52 μM 2,4-D, where there was no vascular connection with embryo-like-structures. Shoot tip differentiation was also observed in these embryo-like structures when they were transferred to BAP plus NAA-containing medium after 4 weeks of transfer.

A continuous stage of shoot development was observed in histological studies of hypocotyl-derived callus. The results showed that there are two pathways in regeneration system of adventitious shoots from callus, through organogenesis or through embryo-like-structure formation. The meristematic tissues formed a crescent-like cell region in callus after 10 days of incubation, and within 3-4 weeks the meristematic tissue formed a meristematic dome with a procambium. Meristematic clusters and vegetative buds developed from the callus surface after 60 days of incubation, and later developed into shoots. This development was found only in the callus which was obtained from MS medium supplemented with 4.44 μM BAP plus 5.37 μM NAA or 2.22 μM BAP plus 4.92 μM IBA. On the other hand, embryo-like-structures were formed in MS medium containing 0.89 μM BAP and 4.52 μM 2,4-D. This event suggests that adventitious shoot development and embryo formation in callus largely depend upon the cytokinin-auxin combination and their concentration.

Cell division and cell wall development occurred normally in regenerating callus. A continuous cell wall development was observed in surface layer to inner layer of callus. Irregular shapes of thin-walled cell with starchy materials were found in surface layer, whereas thick and well-developed cell walls with bordered pits
were observed in inner layer.

In conclusion, an efficient micropropagation protocol for axillary and adventitious shoots regeneration, *ex vitro* establishment of regenerants, plant regeneration from protoplast culture, and morpho-histological studies has been described in this study. Though successful achievements on plant regeneration from tissues and protoplasts culture have been done, further study is needed to produce somatic hybrids by protoplast fusion and electroporation between *P. amurense* and other species. This study may be open the door for genetical experiments and analysis of *P. amurense*.

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### Appendix-1

**Composition of MS (Murashige and Skoog, 1962) Medium Used in the Present Study**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mg/l)</th>
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</tr>
<tr>
<td>KNO₃</td>
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<tr>
<td>NH₄NO₃</td>
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<tr>
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<tr>
<td>FeSO₄•7H₂O</td>
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<td>Na₂EDTA•2H₂O</td>
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</tr>
<tr>
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</tr>
<tr>
<td>myo-inositol</td>
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</tr>
<tr>
<td>Nicotinic acid</td>
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</tr>
<tr>
<td>Pyridoxinic HCl</td>
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</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.10</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.00</td>
</tr>
<tr>
<td>Sucrose (g)</td>
<td>30.00</td>
</tr>
<tr>
<td>Agar powder/Gellan gum (g)</td>
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</tr>
<tr>
<td>pH</td>
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</table>

### Appendix-2

**Composition of MMS (Modified Murashige and Skoog, 1962) Medium Used in the Present Study**

<table>
<thead>
<tr>
<th>Constituent</th>
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<tr>
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</tr>
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</tr>
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<tr>
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<td>Thiamine HCl</td>
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</tr>
<tr>
<td>Glycine</td>
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<tr>
<td>Sucrose (g)</td>
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<td>Agar powder/Gellan gum (g)</td>
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<td>pH</td>
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### Appendix-3

**Composition of MMS (Modified Murashige and Skoog, 1962) Medium Used in the Present Study**

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<th>Constituent</th>
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<tr>
<td>Thiamine HCl</td>
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</tr>
<tr>
<td>Glycine</td>
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<tr>
<td>Sucrose (g)</td>
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</tr>
<tr>
<td>Agar powder/Gellan gum (g)</td>
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<tr>
<td>pH</td>
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### Appendix-4

**Composition of WP (Lloyd and McGown, 1980) Medium Used in the Present Study**

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<th>Constituent</th>
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<tr>
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</tr>
<tr>
<td>H₂BO₃</td>
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<td><strong>Organic nutrients:</strong></td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Sucrose (g)</td>
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<tr>
<td>Agar powder/Gellan gum (g)</td>
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<tr>
<td>pH</td>
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Appendix-5
Composition of B₅ (Gamborg et al., 1968) Medium Used in the Present Study

<table>
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<th>Constituent</th>
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<tr>
<td>ZnSO₄·7H₂O</td>
<td>2.00</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
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</tr>
<tr>
<td>CuSO₄·5H₂O</td>
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<tr>
<td>CoCl₂·6H₂O</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Nicotinic acid</td>
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</tr>
<tr>
<td>Pyridoxinic HCl</td>
<td>1.00</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>10.00</td>
</tr>
<tr>
<td>Sucrose (g)</td>
<td>20.00</td>
</tr>
<tr>
<td>Agar powder/Gellan gum (g)</td>
<td>8.00/2.00</td>
</tr>
<tr>
<td>pH</td>
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</tbody>
</table>

Mustafa Abul Kalam Azad

Summary

*Phellodendron amurense* Rupr. (Kihada), which belongs to the family Rutaceae and is one of the important medicinal woody plants, was used as the material for plant tissue culture. This plant is used as an anti-inflammatory, antipyretic, chologogue, and antibacterial medicine. This plant is also a potential source of industrial cork. The main objective of the present study was to carry out experiments with a view to developing a reproducible protocol for plant regeneration from mature and seedling explants, plant regeneration from protoplast culture, and morpho-histological studies of regeneration system of adventitious bud in *in vitro* produced callus. In the present study, the results obtained from different experiments were discussed in the following paragraphs.


The main objective of this study was to establish axillary shoot proliferation from mature and seedling explants, direct and indirect adventitious shoot regeneration, and rooting of the *in vitro* proliferated shoots. Nodal and shoot tip explants of mature and seedling plants were cultured on MS medium containing 2.0 \( \mu \text{M} \) BAP. Among them, nodal segments of seedling showed the best results for axillary shoot proliferation, and the highest percentage of shoot proliferation and the greatest number of total shoot per culture were 100.0\% and 8.5 \( \pm \) 0.6, respectively.

Direct adventitious shoot regeneration occurred in MS medium containing 2.2 \( \mu \text{M} \) BAP with 0.5 \( \mu \text{M} \) NAA from hypocotyl and internode explants, while 6.6 \( \mu \text{M} \) BAP plus 0.5 \( \mu \text{M} \) NAA and 4.4 \( \mu \text{M} \) BAP plus 0.5 \( \mu \text{M} \) NAA combinations produced adventitious shoots from cotyledon and leaf explants, respectively. The suitable medium combination for the induction of fast growing callus from hypocotyl, cotyledon, leaf, and internode explants was 2.0 \( \mu \text{M} \) TDZ with 4.0 \( \mu \text{M} \) 2,4-D. Plant regeneration occurred from these calli, when they were transferred to MS medium containing 1.5 \( \mu \text{M} \) BAP and 1.0 \( \mu \text{M} \) NAA.

Microcuttings obtained from micropropagated shoots were rooted on MS medium containing 2.0 \( \mu \text{M} \) IBA, and the maximum rooting frequency and the total number of root per shoot cutting were 98.8\% and 7.2 \( \pm \) 0.8, respectively. Rooted shoots were acclimatized and established onto the Kanuma soil under *ex vitro* conditions, and the survival rate was 90.5\%.

2. Plant Regeneration from Protoplast of *Phellodendron amurense* Rupr.

The present study was conducted with a view to isolation and culture of protoplasts, culture of protoplast-derived callus, and plantlet regeneration from protoplast-derived callus. In this experiment, expanded leaves and stem segments obtained from *in vitro* grown shoots were used for the isolation and culture of protoplasts. Callus was induced from leaf and stem segments on MS medium containing 2.0 \( \mu \text{M} \) BAP and 4.0 \( \mu \text{M} \) NAA. Suspension cultures were obtained from stem-segment-
derived friable callus in B5 liquid medium supplemented with 2.0 μM BAP plus 4.0 μM NAA.

The protoplasts were isolated from leaves, stem-segment-derived callus, and cell suspension cultures. The suitable enzyme solution for protoplast isolation was 1.0% Cellulase Onozuka R-10 plus 1.0% Driselase, and suitable osmoticum was 0.6 M mannitol. Leaf tissue produced satisfactory protoplast yield of $7.1 \times 10^5$ protoplast / g fresh weight with the highest viability of 86.0% by 6 hrs enzymatic treatment. On the other hand, stem-segment-derived callus and cell suspension cultures produced suitable yield of $5.5 \times 10^5$ and $4.5 \times 10^5$ protoplast / g fresh weight, and viability of 90.0% and 95.0% in the same enzyme mixture, respectively, by 8 hrs enzymatic treatment.

Leaf- and stem-segment-derived protoplasts were cultured in MS liquid medium containing 2.0 μM BAP plus 4.0 μM NAA, 2.0% sucrose, and 0.6 M mannitol, where they produced 0.8% and 1.2% of colony formation, respectively. On the other hand, the highest frequency 1.5% of colony formation was obtained from cell-suspension-culture-derived protoplasts in B5 liquid medium containing 2.0 μM BAP with 1.0 μM NAA and 3.0% sucrose. In both cases, culture density was $4 \times 10^5$ protoplast / ml.

Protoplast-derived microcalli obtained from leaf, callus, and suspension cultures were cultured on MS medium containing 2.0 μM BAP with 4.0 μM NAA for proliferation of callus. The highest 80.0% callus proliferation occurred from the callus- and cell-suspension-culture-protoplast-derived microcalli, while leaf- protoplast-derived microcalli showed 60.0% callus proliferation.

Leaf-protoplast-derived calli produced 40.0% of shoot regeneration on MS medium containing 2.0 μM BAP and 1.0 μM NAA, whereas 60.0% and 80.0% of shoot regeneration occurred on the same medium combination from the cell-suspension-culture-protoplast-derived calli and callus-protoplast-derived calli, respectively. For multiplication and elongation of protoplast-callus-derived shoots, they were transferred to 1.0 μM BAP-containing MS medium. Proliferated shoots were rooted on MS medium containing 2.0 μM IBA.


The present investigation was undertaken to observe the morphology and histology of callus for the initiation and development of somatic embryo-like-structures, and of shoots from callus tissue. In this study, hypocotyl explants were excised from in vitro grown seedling and cultured on MS medium containing different combinations and concentrations of BAP and NAA, IBA, or 2,4-D for inducing callus. Hard-compact amorphous callus was produced from hypocotyl explants on MS medium containing 2.22 μM BAP and 4.92 μM IBA. Soft-friable amorphous callus developed on the MS medium containing 4.44 μM BAP and 5.37 μM NAA.

Embryo-like structures (ELSs) were formed in MS medium containing 0.89 μM BAP plus 4.52 μM 2,4-D. Some ovoid ELSs were found in the calli, when they were induced on MS medium containing 2.22 μM BAP plus 10.74 μM NAA. Moreover, tuber-like-callus was produced, when hypocotyl explants were cultured on MS medium containing 2.22 μM BAP and 9.84 μM IBA.

Histological observation showed that anticlinal and periclinal cell divisions occurred in generative callus of P. amurense. Within 10-15 days of culture incubation, two to five-celled proembryo-like-structures were formed. Numerous somatic ELSs were found in generative callus. Procambium and leaf primordium were also found in ELSs.

Adventitious shoot differentiation system was observed histologically in callus tissues. After 10 days of culture incubation on MS medium containing 4.44 μM BAP plus 5.37 μM NAA, the meristematic tissues formed crescent-like cell region in callus tissues. The meristematic cells formed a meristematic dome with a procambium, and meristematic dome produced leaf
initial within 3-4 weeks and 5 weeks of culture, respectively. Many vegetative buds were developed on the callus surface within 60 days of culture. Histological studies revealed that BAP with NAA or IBA produced adventitious shoots, whereas BAP with 2,4-D induced ELSs. These ELSs developed shoots through organogenesis when they were transferred to the MS medium containing BAP plus NAA. The results obtained here showed that there are two pathways in regeneration system of adventitious shoots, that is through organogenesis or through ELS formation. These findings are the first observed in *Phellodendron* spp.

A continuous cell wall development was observed in callus tissues. Irregular shapes of cell with thin cell wall containing starchy materials were found in the surface layer of callus, whereas thickened-wall cells with lacking of starchy materials gradually occurred from the middle to inner layer of callus. Three-layered secondary wall was also found in inner layer of callus tissues.

In conclusion, commercial exploitation for production and conventional propagation of *P. amurense* is hampered due to its poor seed viability, low germination, and poor rooting ability of vegetative cutting. The micropropagation protocols described in the present investigation could offer solutions to these problems. This protocol can produce large qualities of uniform plants from the selected trees.

Moreover, a method for the isolation and culture of protoplasts of *P. amurense* has been developed in this investigation. So far, this the first study of protoplast culture of *P. amurense*. This method is expected to contribute to the production of somatic hybrids by protoplast fusion and electroporation between *Phellodendron* and other species.

Novel findings and interesting results were found in histological studies. These results indicated the pathway of plant regeneration system and the effects of cytokinin and auxin combination, and their concentration for organogenesis or ELS formation from the plant tissues.

(2005年6月30日受理)