Histological studies of shoot regeneration system in hypocotyl-derived callus of *Phellodendron amurense* Rupr.

Abstract An efficient in vitro plant regeneration was achieved from hypocotyl-derived callus of a medicinal tree *Phellodendron amurense*. The expected morphogenic response was obtained on the media containing 0.89 μM 6-benzyl aminopurine (BAP) plus 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.44 μM BAP plus 5.37 μM α-naphthaleneacetic acid (NAA), or 2.22 μM BAP plus 4.92 μM indole-3-butyric acid (IBA). A detailed histological study was undertaken to gain a better understanding of cellular changes that take place during the plant regeneration process from callus tissue. This study led to the identification of the cellular origin of shoot regeneration. Histological studies revealed that the new vegetative buds originated from callus that completely altered the morphology of the callus tissues by the 60th day of culture. It also revealed that BAP with NAA or IBA induces plant regeneration through organogenesis whereas BAP with 2,4-D induces embryo-like-structure (ELS) through hypocotyl-derived callus. The presence of ELS lacking root poles was also observed.

Key words Proembryo-like structure · Embryo-like structure · Unicellular origin · Multicellular origin · Regeneration pathway

Introduction

*Phellodendron amurense* Rupr. (common names: kihada, huang bai, Amur cork tree), which belongs to the Rutaceae family, is a dioecious tree native to Eastern Asia (northern China, Manchuria, Korea, Ussuri, Amur, and Japan), and has remarkable medicinal properties. The bark of this tree contains medicinally important compounds applicable to anti-inflammatory, antipyretic, cholagogue, and antibacterial medicines. It is also effective for purging heat, detoxifying, clearing damp heat, and lowering blood sugar. *Phellodendron amurense* is an effective herb used topically for sores and damp heat conditions of the skin (Schechter 1943; Starshova 1972).

The wood of *P. amurense* has a beautiful color and high-quality grain, and is very easy to process. For these reasons, the wood of this species has been used for making furniture and industrial art. This tree also provides industrial cork and a yellow dye (Ôta et al. 1965). However, in Japan at least 80% of the current consumption of phellodendri cortex is import dependent. Therefore, to increase the stock of the cork tree, more productive uses for the wood must be sought, considering that the sale of phellodendri cortex (endodermis) is not economically feasible (Kira et al. 1993).

Although this tree is propagated with seeds, the germination rate is very low (Read 1974; Dirr 1990). In addition, propagation by stem cutting is very difficult, because of poor rooting ability (Kamiya et al. 2000). Kamiya et al. (2000) also reported that about only 35% of the trees are regenerated successfully by grafting. On the other hand, the in vitro micropropagation technique possesses potential and could offer solutions to these problems.

Although there are a few reports on micropropagation through axillary buds of *P. amurense* (Ariyoshi 1986), so far there is no report on adventitious shoot regeneration and histological studies of callus from this valuable medicinal plant. Further information regarding developmental stages of embryogenesis and organogenesis are needed to study the developmental pattern of in vitro morphogenesis in *P.*
amurense. Such work may lead to a better understanding of in vitro development in *P. amurense*, and consequently result in higher regeneration rates which should benefit clonal propagation and transformation work. This article describes the developmental pattern of organogenesis at the cellular level in *P. amurense* callus induced from the hypocotyl explant.

**Materials and methods**

Collection and sterilization of source materials

Fruit of *Phellodendron amurense* Rupr. was collected from a 50-year-old tree growing at the Medicinal Plant Garden of Kumamoto University, Japan. Flesh was removed from the fruit and seeds were recovered. They were washed for 15 min in a household detergent solution (1 ml l⁻¹ in tap water), and then rinsed thoroughly with running tap water for 20 min. After the seeds were surface-sterilized with 70% ethanol for 3 min, they were moved to a laminar-air-flow cabinet and transferred to a sterilized conical flask. Subsequent surface disinfection was done with 3% (v/v) sodium hypochlorite (Wako, Osaka, Japan) solution 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 (Murashige and Skoog 1962) supplemented with 2.0 μM 6-benzyl aminopurine (BAP) in culture tubes (120 × 25 mm, Asahi Techno Glass, Japan).

Induction and culture of callus

Hypocotyl explants were excised from in vitro grown seedlings after 4 weeks of culture, and placed in petri dishes (9 × 1.5 cm, Asahi Techno Glass, Chiba, Japan) with 30 ml MS medium supplemented with different concentrations and combinations of BAP and α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), or 2,4-dichlorophenoxyacetic acid (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 2.69, 5.37, and 10.74 μM NAA, 2.46, 4.92, and 9.84 μM IBA, or 2.26, 4.52, and 9.05 μM 2,4-D were used for inducing calli. All media were adjusted to pH 5.7 ± 0.1, fortified with 30 g/l sucrose, and gelled with 2.2 g/l gellan gum (Wako). The cultures were grown at 25° ± 1°C under a 16-h photoperiod with a molar light intensity of 50 μmol m⁻² s⁻¹. Continuous propagation of callus was obtained by subculturing it on the same fresh callus medium every 8 weeks. Initial callus (6–8 mm in diameter) was then subdivided into three to four small calli (3 mm in diameter), and were separately cultured on the callus medium for a new culture cycle. These calli were used for histological studies.

Histological studies

Hypocotyl-derived callus, which was 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 histological studies. In this study, calli were collected at weekly intervals from culture initiation, and then 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 a graded ethanol series (aqueous ethanol solution from 50% to 100% (v/v), 5 min at each concentration). After replacing ethanol with xylene, the calli were embedded in paraffin, and cut into sections that were 10–15 μm thick by a rotary microtome (1512, Leitz, Japan). The sections were fixed with 3% gelatin (v/v) on a slide glass, deparaffined with xylene, and then double stained with safranin and fast green. Finally, stained sections were observed by a light microscope (BH-2, Olympus, Japan).

Data analysis

Twenty replicates were used for all experiments. Experiments were repeated four times. The effect of different treatments was quantified, and this data was analysed by analysis of variance (ANOVA). Tukey’s multiple comparison was used to distinguish differences between treatments.

**Results and discussion**

Induction and culture of callus

The morphogenic potential of the callus depended upon the supplemental plant growth regulators (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 4.44 μM BAP plus 5.37 μM NAA showed the highest performance to produce callus with the frequencies of 90% and 80%, respectively. In contrast, 2.22 μM BAP plus 4.92 μM IBA showed 60% callus formation (Table 1). 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 a small amount of callus. These combinations produced only green-colored hard compact callus, whereas 0.44 μM BAP with 2.26–9.05 μM 2,4-D produced white-colored hard compact callus. The combinations of 2.22 μM BAP and 2.69–10.74 μM NAA or 2.46–9.84 μM IBA produced tuber-like callus, and the callus production rates were 25%, 70%, 50%, and 30%, 60%, 50%, respectively. In addition, 4.44 μM BAP in combination with 2.69–10.74 μM NAA or 2.46–9.84 μM IBA produced green-colored soft callus. On the other hand, 0.89 μM BAP in combination with 2.26–9.05 μM 2,4-D produced embryogenic-like callus and embryo-like structures (ELSs; Pauk et al. 2000; Wassom et al. 2001), and the frequencies were 50%, 90%, and 70%, respectively. On the contrary, 2.22–4.44 μM BAP in combination with 2.26–9.05 μM 2,4-D produced only a few percent of embryogenic-like callus. The combination of 0.89–4.44 μM BAP with 2.69–10.74 μM NAA produced few ELSs. The highest number (40 ± 0.2) of ELSs was found on MS medium containing 0.89 μM BAP with 4.52 μM 2,4-D. Some ELSs showed a tendency to