Histological analysis of somatic embryogenesis in Itoh peony

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Itoh peony is one of the popular ornamental plants, which represents an intermediate form between herbs and trees. The present study was aimed at developing a culture growing system for mass multiplication of Itoh peony cv. 'Julia Rose'. Axillary buds were cultured on Murashige and Skoog medium with 6-benzylaminopurine and indole-3-butyric acid for indirect somatic embryogenesis. Nodular callus was derived from the base of the axillary buds. Histological analysis was performed to elucidate the origin and development of somatic embryos.

Keywords: Histology, Itoh peony, morphogenesis, plant regeneration.

PEONIES are known as ornamental and medicinal plants. In China, they have been cultured for more than 3000 years[1]. The genus *Paeonia* L. comprises about 40 species. Most of them are perennial herbaceous plants, which occur in South Europe, Asia and western North America. This genus includes also tree peonies growing in south-western China. Herbaceous peonies, being characterized by cold-hardiness in winter, abundant flower production and simple farming are popular in ornamental gardening. Tree peonies do not possess these features, but they are used in selection as a source of ornamental characters. Inter-sectional hybridization of herbaceous peonies with tree peonies provided plants with valuable characters typical for these groups. In 1948, Toichi Itoh obtained hybrids by crossing the tree peony *Paeonia* × *lemoinei* Rehder with the herbaceous peony *P. lactiflora* Pall. cv. 'Kakoden'. In 1974, four Itoh cultivars with yellow flowers were registered in USA[2]. In the late 1970s, these cultivars were referred to a separate group of Itoh hybrids[3]. Currently, this group of peonies numbers a few hundreds of cultivars.

Propagation of Itoh peonies is difficult. Cultivars of this garden-grown group are triploids and do not produce viable seeds[4]. Itoh peonies are commonly propagated by dividing their rhizomes, but only a small number of plants can be obtained using this method. An alternative method of vegetative propagation is *in vitro* micropropagation. The main technique of micropropagation is direct organogenesis induction[5,6]. Axillary buds and leaves were used as explants[7–9]. Only a few papers have reported about somatic embryogenesis in peonies, e.g. in *P. lactiflora*[10], *P. albiflora* Pall.[11], *P. anomala* L.[12] and tree peonies[13]. Adventitious bud formation was observed in the callus tissue of *P. suffruticosa* Andrews, initiated from the basal part of axillary buds[14]. However, reports on *in vitro* regeneration of Itoh peonies are not available. The present communication describes induction of somatic embryogenesis in Itoh peony cv. 'Julia Rose' from the shoot callus and successful regeneration of plants. The present histological study was designed to analyse the somatic embryogenesis pathway in Itoh peony.

Axillary buds of Itoh peony cv. 'Julia Rose' were collected in late September from a plant cultivated in the collection of the institute. Buds were isolated along with a part of the shoot tissues adjacent to their base (0.5–1.0 cm). Their surface was disinfected with 95% ethanol, and the outer bud scales were removed (Figure 1 *a* and *b*). The explants were thoroughly washed under running tap water (40 min) and then treated with soap emulsion (30 min). The surface was sterilized with 0.5% (w/v) sodium hypochlorite (10% v/v Domestos) for 5 min, shaken in 0.2% (w/v) mercuric chloride for 10 min, and then rinsed thrice in sterile distilled water. Then the bases of the explants were trimmed, and two bud scales were removed before inoculation into half-strength Murashige

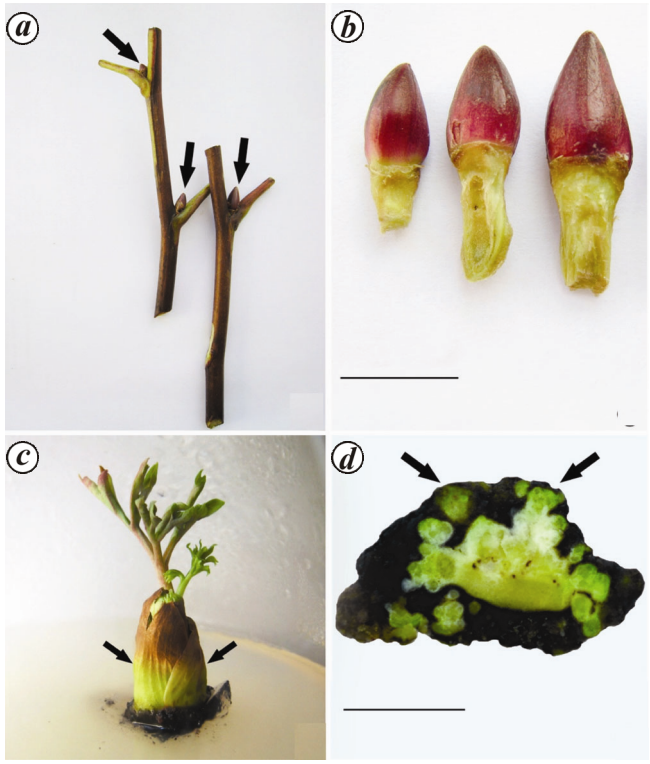

Figure 1. Initial stages of Itoh peony micropropagation (bar = 1 cm). ***a***, Shoots with axillary buds (arrows). ***b***, Isolated axillary buds before culturing. ***c***, Germinating *in vitro* axillary bud with persistent bud scales (arrows). ***d***, Formation of nodules on the embryogenic callus (arrows).

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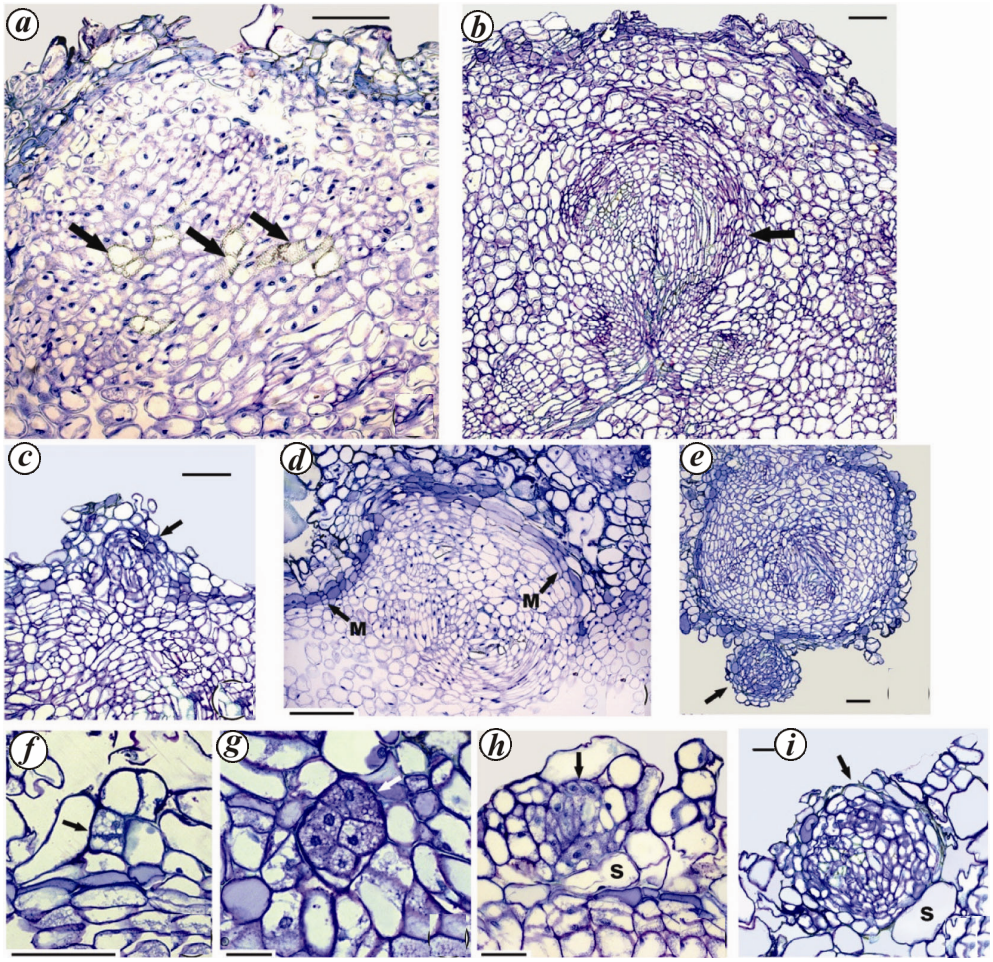

Figure 2. Histological analysis of Itoh peony somatic embryogenesis in longitudinal sections. ***a***, Tracheary elements (arrows) at the callus base. ***b***, Vascular bundle at the callus base (arrow). ***c***, Nodule initiation (arrow). ***d***, Nodule development. ***e***, Differentiated nodule with somatic embryo (arrow) (*a*–*e*, bar = 200 µm). ***f***–***i***, Initiation and further development of somatic embryos (arrows) (*f*–*i*, bar = 100 µm). M, Meristem-like layer; S, suspensor-like cell.

and Skoog (MS)[15] medium supplemented with 2 mg l^{-1} 6-benzylaminopurine (BAP). After six months of cultivation, the medium was supplemented with 100 mg l^{-1} ascorbic acid, 0.5 mg l^{-1} ferulic acid, 1 mg l^{-1} BAP and 0.2 mg l^{-1} indole-3-butyric acid (IBA). For *in vitro* rooting, MS medium was supplemented with IBA at 1.0–2.0 mg l^{-1}. The medium was adjusted to pH 6.0 with 1N KOH before addition of agar, dispensed into culture flasks, and autoclaved at 121°C and 1×10^5 Pa (1.1 kg cm^{-2}) for 20 min. The cultures were incubated at 24° ± 2°C under cool-white fluorescent light (16/8 h photoperiod, 2000–3000 lx). After 4–6 weeks, the plant organs and tissues were sub-cultured in a fresh medium.

For histological studies, the morphogenic callus was fixed in FAA (formalin/glacialacetic acid/70% ethanol, 7 : 7 : 100, v/v). After washing in 70% ethanol and dehydration, the material was embedded in Technovit 7100 (Heraeus Kulzer, Germany). Serial (10 µm thick) sections were cut using a HM360 microtome (Microm, Germany), and stained with 0.05% (w/v) aqueous toluidine blue. Histological observations were made through a light microscope (Axioplan 2, Carl Zeiss, Germany) equipped with a CCD-camera (AxioCam ICc 3). Morphological features were observed using a stereo microscope (Stemi 2000, Carl Zeiss, Germany).

Establishment of explants *in vitro* was complicated due to exudation of phenolics. Relocation of explants *in vitro* every seven days within the first month of culture mitigated the negative effect of phenols and increased the survival rate of buds. At the initial stage of cultivation, 33% of explants survived. Axillary buds germinated *in vitro* within 7–8 weeks of cultivation (Figure 1 *c*). Callus formation was observed at the base of the buds (area of embryonic shoot) after six weeks. The obtained compact callus had a zonal structure and bore embryogenic nodules on the surface (Figure 1 *d*). The inner zone of the callus was formed by parenchyma cells and vascular tissue in the form of isolated tracheary elements, vascular cords, or concentric vascular bundles (Figure 2 *a* and *b*). The outer zone of the callus was formed by several rows

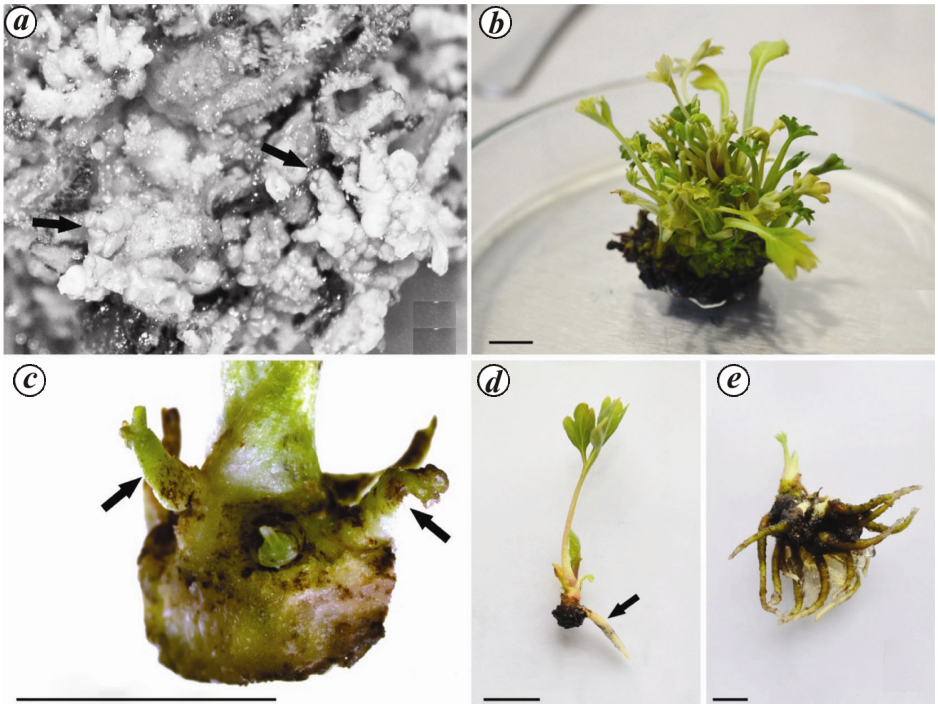

Figure 3. Micropropagation of Itoh peony hybrid (bar = 1 cm). ***a***, Somatic embryos (arrows) and their germination. ***b***, Shoot multiplication. ***c***, Development of adventitious buds (arrows) on the shoot base. ***d***, Regenerated plantlet with adventitious root (arrow). ***e***, Development of adventitious roots in callus.

of vacuolated cells, which emitted phenolics after dying. This caused the callus and culture medium to be coloured black. The meristem-like layer consisting of 2–3 rows of flattened cells with dense cytoplasm was located between the inner and outer zones of the callus. Nodules were formed on the surface of the callus. Nodule formation is related to inward proliferation of meristem-like layer cells (Figure 2 *c* and *d*). Vascular bundles having no vascular connection with the maternal tissue of the main callus were formed in the central part of the nodule. The formed nodules (2310 ± 142 µm in diameter) copied the zonal structure of the callus tissue and could detach from the maternal tissue.

Somatic embryos developed on the surface of the nodules (Figure 2 *e*). The initial cells of the somatic embryo were formed through periclinal division of some meristem-like layer cells outward. As a result of a series of divisions of the initial cell, a complex of small-sized isodiametric cells with large nucleus and dense cytoplasm was formed (Figure 2 *f*–*h*). Initiation of shoot and root apices occurred at the subsequent stage of development of somatic embryos. The apical part grew in size and gained a globular shape, while the basal part became tapered (Figure 2 *i*). Somatic embryos did not have vascular connection with the maternal tissue and were connected with it via large suspensor-like cells. Somatic embryo formation from globular structures, as described here was also observed in *Terminalia arjuna*[16].

According to the literature, supplementing the MS medium with auxins and cytokinins promotes development of peony shoots[1,17]. A large percentage of adventitious shoot induction was recorded[18] when BAP was added at a concentration of 0.5–1.0 mg l^{-1}. In the present study shoots developed from somatic embryos in the presence of 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} IBA (Figure 3 *a* and *b*). Adventitious buds, which participated in the formation of shoot clusters, were formed in the basal part of the shoots (Figure 3 *c*). A similar development of axillary buds has been observed in *Paeonia lactiflora*[1].

It is shown that clusters of shoots are formed in a peony culture *in vitro*[19,20]. These clusters were divided into parts and cultured on the same medium for further development of shoots. After 7–10 passages, the shoots were placed on the rooting medium. Adventitious roots were formed at the base of the regenerated plantlets; development of the primary root was not observed (Figure 3 *d*). The average root length attained 0.3 cm by the 30th day. After four months, plants had 1–5 adventitious roots (2.5–3.5 cm in length). A long-term subcultivation on the medium with 2.0 mg l^{-1} IBA facilitated development of primary roots from somatic embryos (Figure 3 *e*). The process of plant regeneration from the moment of planting the explant *in vitro* until rooting took 12–14 months.

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Nature of forest fires in Uttarakhand: frequency, size and seasonal patterns in relation to pre-monsoonal environment

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Man-made forest fires in the traditionally populated zone (about 800–2000 m altitude) are common in much of the Central Himalaya, and are a major topic of environmental debate. This study based on an analysis of data of the State Forest Department at Uttarakhand on incidence of forest fires shows that these are high-frequency, low-severity surface fires of small size, largely determined by the moisture conditions of the pre-monsoon season (from March to mid-June), and the traditional practices of biomass collection by local people.

Keywords: Biomass collection, forest fire, pre-monsoon season, moisture conditions.

FOREST fires remain a major hazard in many ecosystems of the world, and a fire event may burn areas, influencing the species composition and ecosystem processes[1]. In California, USA, a lightning-induced fire may burn thousands of hectares of forest, with rotation period ranging from 95 to 974 yrs (ref. 2).

In the Central Himalaya, particularly Uttarakhand, frequent man-made fires are an integral part of the chir-pine (*Pinus roxburghii*) – banj oak (*Quercus leucotrichophora*) forest zone (generally, between 800 and 2000 m altitude), and promote the regional domination of chir-pine at the expense of broadleaf oak forests[3,4]. These man-made forest fires are also a major source of pollutants including black carbon which is regarded as a major cause of glacier melt in the Himalaya[5], and has the capacity to influence regional climate[6]. Fire regime the world over is likely to change in many regions because of the global climate change and change in land-use patterns[1].

Fires in the Himalaya occur during the pre-monsoon summer period of moisture stress[3,7], which is intensified because of global warming and resultant depletion of snowmelt water[8]. Despite being referred to as a major environmental factor, studies on forest fires in the Himalaya, particularly from an ecological standpoint are limited[9]. One of the initial steps in this direction could be assessing their nature.

The main objectives of the present study are to: (i) characterize the nature of the manmade forest fires of the

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