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In vitro mutation breeding of *Paphiopedilum* by ionization radiation

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ABSTRACT

The basic medium containing half-strength of MS basal medium, full-strength of vitamin MS, 20 ml l^{-1} coconut water and 2 g l^{-1} activated charcoal supplemented with 170 mg l^{-1} NaH_2PO_4, 10 mg l^{-1} 2,4D and 0.1 mg l^{-1} TDZ was found to be appropriate for callus formation of *Paphiopedilum delenatii* and *Paphiopedilum callosum*. Additional supplementation with 2.0 mg l^{-1} BA and 0.1 mg l^{-1} TDZ in combination with NaH_2PO_4 and 50 mg l^{-1} oligochitosan enhanced PLB multiplication. Shoot bud proliferation of tested orchids was optimally increased in a basic medium supplemented with 0.5 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA. The basic medium supplemented with NaH_2PO_4 was suitable for plantlet regeneration. In addition, the values of LD_{50} (lethal dosage of 50% irradiated samples) of protocorm like bodies, shoot buds and *in vitro* plantlets were determined for *P. delenatii* (20 Gy, 23.7 Gy and 38 Gy, respectively) and *P. callosum* (23 Gy, 27.1 Gy and 40.4 Gy, respectively). No variant line was found for both orchid samples irradiated by gamma-rays, whereas 24 variant lines (which are divided among the two tested *Paphiopedilum*) were screened for samples irradiated by C^{6+} ion-beams (3 Gy). The genetic relationships among six generated variant lines and wild types were analyzed using RAPD techniques. Irradiation-induced variation is promising for developing new mutant varieties of *Paphiopedilum*.

1. Introduction

Paphiopedilum delenatii and *Paphiopedilum callosum* are endemic and endangered rare slipper orchids of Vietnam and other Indochinese countries (Averyanov et al., 2003). Though *Paphiopedilum* is a favorite potted flower in several countries due to their attractive colors and distinctive shapes, they have been protected by the Convention on International Trade of Endangered Species (CITES) of Wild Flora and Fauna. In an effort to commercialize this high-value potted orchid, several scientists have studied the micropropagation technique for propagation but the success with *Paphiopedilum* has been relatively limited due to the difficulty of bacterial and fungal decontamination of *ex vitro*-derived explants and the poor development of explants that survive under *in vitro* condition (Stewart and Button, 1975; Huang, 1988; Lin et al., 2000). Different micropropagation methods for this orchid have been reported, including plantlet regeneration from protocorm like bodies (Lin et al., 2000) shoot multiplication from seedling (Huang et al., 2001; Nhut et al., 2005), direct shoot bud formation from leaf explants (Chen et al., 2004), and shoot generation *via* stem node culture (Nhut et al., 2007). Recently, addition of phosphate to culture medium had been reported to be effective for the micropropagation of slipper orchids. In addition, Huang et al. (2001) enhanced shoot induction and plantlet regeneration from shoot tips of *Paphiopedilum* by supplementing 148.8 mg l^{-1} NaH_2PO_4 to the medium. Oligochitosan a molecular weight of 16 kDa has also been found as a growth promotion compound for micropropagation of plant. This oligomer was also found to improve the survival ratio and growth of transferred plantlets acclimatized in a greenhouse condition (Luan et al., 2005).

Radiation technology had proven to be useful for mutation breeding and has contributed towards improvements in agricultural crops. According to the report of the joint FAO/IAEA programme for nuclear techniques in agriculture, there have been 3100 officially released mutant varieties from 170 different plant species in more than 60 countries. Among the mutant varieties, about 90% of these mutant varieties were generated by using radiation (Lagoda, 2010). Several new flower varieties with high commercial value, such as carnation (Okamura et al., 2003), chrysanthemum (Lamseejan et al., 2000; Nagatomi et al., 2000; Dowrick and Bayoumi, 1966; Nomizu et al., 2005), anthurium (Puchooa, 2005), *Curcuma alismatifolia* (Abdullah et al., 2009), cyclamen (Sugiyama et al., 2008), lily (Chinone et al., 2005; Chiba

Abbreviations: ANOVA, analysis of variation; CW, coconut water; LD_{50}, lethal dose of 50% sample; PLBs, protocorm like bodies; BA, 6-benzylaminopurine; 2,4D, 2,4-diclorophenoxyacetic acid; IBA, indol-3-butyric acid; LSD, the least significant difference; MS, Murashige and Skoog's medium; Mw, weight average molecular mass; NAA, 1-naphthaleneacetic acid; TDZ, thidiazuron; RAPD, random amplified polymorphic DNA.

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et al., 2005), *etc.* have been generated by gamma-rays and ion-beams. For ionizing radiation, ion-beams are expected to be utilized widely as new mutagens, because high energy can be directed on a target in a densely and focused manner compared to low linear energy transfer (LET) radiations, such as electrons, X-rays and gamma-rays (Kahl and Meksem, 2010). According to Okamura et al. (2003) the mutation frequencies from ion-beams were much higher than those of gamma-rays. The combined method of ion-beams irradiation with tissue culture was useful to obtain the mutant varieties in a short time. Nevertheless, no study on mutation breeding of slipper orchids had been reported. The objectives of this study were to optimize the conditions for the micropropagation of *P. delenatii* and *P. callosum* and to generate *in vitro* mutant lines of these orchids by ionizing radiation in combination with tissue culture.

2. Materials and methods

2.1. Explants and chemicals

The ripe seed capsules of *P. callosum* and *P. delenatii* used in this study were supplied by Langbiang Farm Ltd. (Dalat, Vietnam). These orchids grew naturally at an elevation of about 1500 m above sea level with an average temperature of about 18 °C and a range between 10 and 30 °C. N_6-benzyladenine (BA), 2,4-diclorophenoxyacetic acid (2,4D), indol-3-butyric acid (IBA), α-napthylacetic acid (NAA), thidiazuron (TDZ) and Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962) were supplied by Sigma–Aldrich Co. (St. Louis, MO, USA). Oligochitosan with a molecular weight (Mw) of about 16 kDa was prepared by the irradiation method as described by Luan et al. (2005).

2.2. Initial explant preparation

Half-strength of MS mineral salt, full-strength MS vitamins, coconut water (CW) (20%), sucrose (20 g l^{-1}) and activated charcoal (2 g l^{-1}) were used as the basic medium. The medium pH was adjusted to about 5.8 using KOH or HCl before autoclaving for 15 min at 121 °C and 105 kPa. The ripe orchid seed capsules of the orchids were sterilized for 20 min in a 10% $Ca(OCl)_2$ solution. The seeds were then removed from the capsules and sowed in 250 ml Erlenmayer flasks containing basal medium supplemented with 170 mg l^{-1} potassium dihydrogen phosphate (NaH_2PO_4). Sowed seeds were germinated at 25 °C ± 2 °C under a 16-h photoperiod provided by fluorescent lamp at 45 μmol m^{-2} s^{-1} photosynthetic photon density flux.

2.3. Callus induction

Protocorm like body (PLB) explants about 2 mm in diameter were cultured in Erlenmayer flasks containing basic medium supplemented with 2,4D (1, 5 and 10 mg l^{-1}) and TDZ (0.1, 0.5 and 1.0 mg l^{-1}) as described by Chang and Chang (1998). Samples were incubated in the dark condition at 25 °C ± 2 °C. Callus formation was recorded after 6 weeks incubation. Callus samples were then transferred to a basal medium without plant growth regulator supplementation to induce PLBs.

2.4. Protocorm like body proliferation

The PLB explants were cultured in Erlenmayer flasks containing basic medium supplemented with BA (2.0 mg l^{-1}) in combination with NAA (0.1, 0.2, 0.3 and 0.5 mg l^{-1}), NaH_2PO_4 (170 mg l^{-1}) and oligochitosan (50 mg l^{-1}). The number of PLBs per clump was determined after culturing in light for 6 weeks.

2.5. Shoot bud multiplication

For investigating the multiplication of shoot buds, PLBs were cultured in Erlenmayer flasks containing basic medium supplemented with BA (1.0, 2.0 and 3.0 mg l^{-1}), TDZ (0.5, 1.0 and 1.5 mg l^{-1}), NAA (0.1, 0.2, 0.3, 0.5 and 1.0 mg l^{-1}), NaH_2PO_4 (170 mg l^{-1}) and oligochitosan (50 mg l^{-1}). The number of shoot buds per clump was determined after 6 weeks culture under light.

2.6. In vitro plantlet regeneration

Individual shoot buds with two expanded leaves detached from 6-week old shoot bud clumps were cultured in 250 ml Erlenmayer flasks containing basic medium supplemented with 0.1 mg l^{-1} NAA, 170 mg l^{-1} NaH_2PO_4, and 50 mg l^{-1} oligochitosan. The shoot height and root length of *in vitro* plantlets were determined after culturing for 6 weeks in the light.

2.7. Determination of LD_{50}

The radiosensitivity test was carried out by irradiating PLBs, shoot buds and *in vitro* plantlets with gamma radiation from a Co-60 source at a dose rate of 0.2 Gy s^{-1}. Doses in the range of 10–80 Gy were applied and 500 samples were used for each irradiation dose. The optimal dose for radiosensitivity, *i.e.* LD_{50} (lethal dosage of 50% irradiated samples) was determined by measuring the survival rate of samples for 4 months after irradiation (Abdullah et al., 2009; Kahl and Meksem, 2010). Since linear energy transfer (LET) of ion-beams is 9.4 times higher than that of gamma-rays, the investigated LD_{50} of samples by gamma-rays was applied for ion-beams by reducing the dose 9.4 times (Kahl and Meksem, 2010).

2.8. Induction of in vitro variation and RAPD analysis

2.8.1. Variation induction

To generate *in vitro* variation, a large number of samples of each orchid species were irradiated with gamma-rays (one thousand of samples were applied for each dose) and ion-beams (four hundreds of samples were applied for each dose). Since the effective doses for mutant induction were proposed to be approximate or lower than LD_{50} (Abdullah et al., 2009; Kahl and Meksem, 2010), doses in range of 10–30 Gy were applied for gamma-rays irradiation of PLBs, shoot buds and *in vitro* plantlets. In case of ion-beams irradiation, PLBs that were uniform in size and about 2 mm diameter were chosen and transferred into sterile 6 cm petri dishes containing basal medium and then covered with kapton film (0.8 μm thickness). Samples were then irradiated by 320 MeV $^{12}C^{6+}$ ion-beams accelerated with an AVF cyclotron at lower doses of 2–4 Gy, since the linear energy transfer (LET) of ion-beams (86 keV μm^{-1}) was approximately 9.4 times higher than that of gamma-rays (0.91 keV μm^{-1}) (Okamura et al., 2003; Kahl and Meksem, 2010).

2.8.2. Extraction of total DNA

Total genomic DNA was extracted from frozen young leaves following the modified CTAB (cetyl trimethyl ammonium bromide) procedure described by Li et al. (2007). One hundred milligrams of frozen leaf tissue were ground to powder in liquid nitrogen using a mortar and pestle. The powder was transferred into 10 ml centrifuge tubes and was mixed with 3 ml of preheated (65 °C) 2× CTAB extraction buffer (100 mM Tris–HC1 (pH 8.0), 1.4 M NaC1, 20 mM EDTA (ethylene diamine tetra acetic acid), 2% CTAB, 2% (w/v) P-mercaptoethanol, 1% (w/v) polyvinylpyrrolidone (PVP40)). The mixture in the tubes was incubated at 65 °C for 30 min, then cooled and mixed with 500 μl Tris–phenol, and held at 65 °C for 15 min. The tubes were gently inverted upside down for several