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Elimination of mixed '*Odontoglossum ringspot*' and '*Cymbidium mosaic*' *viruses* from *Phalaenopsis* hybrid 'V3' through shoot-tip culture and protocorm-like body selection

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ABSTRACT

Phalaenopsis is one of the most popular orchid plants in the global market. Several viruses have been reported to negatively impact its growth, yield and quality. A mixed infection of *Odontoglossum ringspot virus* and *Cymbidium mosaic virus* was detected in *Phalaenopsis* hybrid "V3" (Phal. Yukimai × Phal. Taisuco Kochdian) plants in Taiwan. In the present communication, we report a relatively simple protocol for elimination of these two viruses using shoot-tip culture, and early isolation and selection of virus free protocorm-like body (PLB) through subcultures. The indexing of viruses in the field grown plants, PLBs and tissue culture plants was carried out by the Indirect enzyme-linked immunosorbent assay (ELISA), and one-step multiplex reverse transcription polymerase chain reaction (RT-PCR). The induction of PLBs in shoot-tips was achieved on 1/2× Murashige and Skoog's basal medium supplemented with 1% sucrose and 0.9% Bacto-agar. PLBs could be maintained, proliferated and converted to plantlets on 1 g/L Hyponex medium supplemented with 1% sucrose, 6% potato pulp, 0.5 g/L tryptone, 0.25% activated charcoal and 0.6% Bacto-agar. The regenerated plantlets from virus-free PLBs acclimatized easily in a greenhouse and showed 100% survival rate. All the tissue culture-raised *Phalaenopsis* plants in the greenhouse tested negative for the two viruses. Our study demonstrates that some PLB lines selected at the first subculture as virus-free were found to be infected with virus at second subculture, however, re-occurrence of virus was never found in PLB lines at third subculture onwards. Hence, at least 3 subcultures are necessary to authenticate that the cultures are free of viruses. Simple culture media without plant growth regulators used in the present study minimizes the chances of somaclonal variations and ensures genetic uniformity of cultured plantlets, a highly desirable trait in the orchid industry. The method developed in the present study has potential of virus elimination, mass propagation of genetically uniform and virus-free *Phalaenopsis* plants.

1. Introduction

Phalaenopsis (moth orchid), belonging to family *Orchidaceae* is regarded as one of the most popular and commercially important orchids. *Phalaenopsis* is valued worldwide for its beautiful flowers and as an attractive potted plant. Most *Phalaenopsis* commercial cultivars can bloom for at least 2–3 months. Not only its flowers, but also its fleshy, distichous evergreen leaves as well as its adaptability to the room environment make this orchid both attractive and distinctive in the floriculture industry. Taiwan is one of the leading *Phalaenopsis* producing countries in the world. Of the total US$ 164.70 million export of orchids from Taiwan in the year 2012, *Phalaenopsis* had the largest share of 69% (Lee et al., 2014). The genus *Phalaenopsis* comprises about 60 species. The plant is a monopodial epiphyte characterized by its longer blossom phase and different colors. Due to its high economic value, a large number of hybrids with attractive combinations of spray length, bud number, flower color and type, fragrance, seasonality, and compactness have been developed (Paek et al., 2011).

Phalaenopsis Sogo Yukidian "V3", a hybrid of Phal. Yukimai × Phal. Taisuco Kochdian, characterized by its large and white-colored flowers (Fig. 1A) is a popular commercial cultivar in the market. Like other *Phalaenopsis* commercial cultivars, young plantlets were mass propagated vegetatively by tissue culture.

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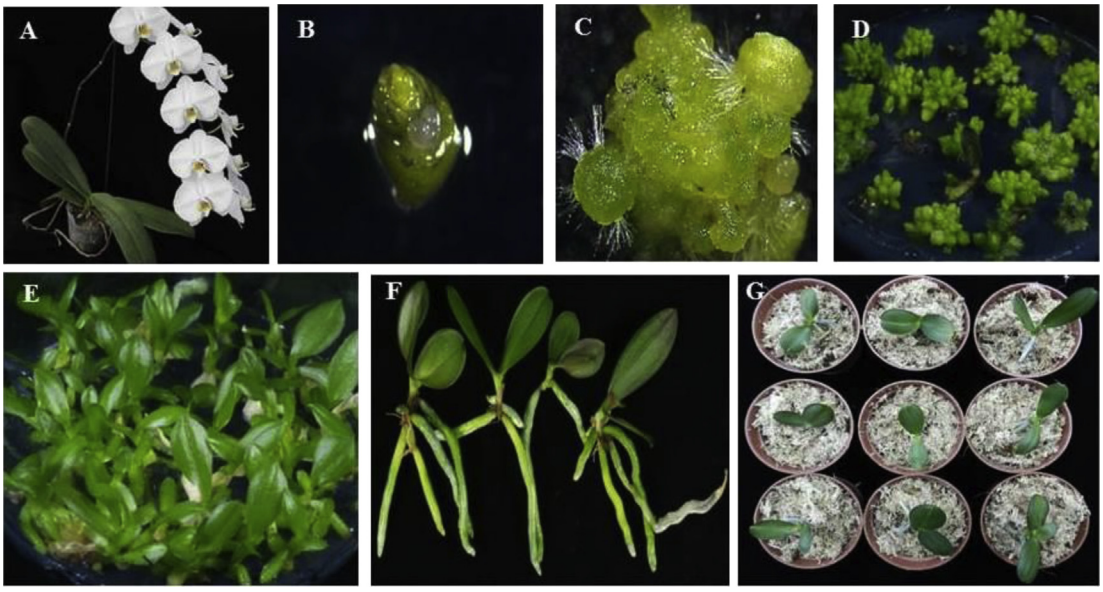

Fig. 1. Production of virus-free *phalaenopsis* plants using shoot-tip culture. (A) Flowering plant of *Phalaenopsis* Sogo Yukidian "V3" hybrid. (B) Shoot-tip explant. (C) Protocorm-like bodies induced in a shoot-tip after 12–16 weeks of culture. (D) Proliferation of PLBs. (E) Regenerated plantlets from PLBs at 3 months of culture. (F) Rooted plantlets at 6 months of culture. (G) *In vitro* plantlets transferred to pots containing sphagnum moss as substratum.

Therefore, if the mother stocks of "V3" were virus-infected all the propagated plantlets will also carry the same virus from their mother plants. However, "V3" hybrids in Taiwan, are severely affected by a mixed infection of ORSV, Tobamovirus, and CymMV, Potexvirus. These two viruses adversely affect the growth, vigor and market value of *Phalaenopsis*. In recent years, Taiwan's *Phalaenopsis* industry has significantly suffered from the high virus incidence rate. Therefore, it was imperative to find a simple and workable solution to this virus problem.

Shoot-tip culture has been used to develop plants free of various pathogens including viruses, viroids, mycoplasmas, bacteria and fungi (Walkey, 1978; Bhojwani and Razdan, 1983). The main approach in the shoot/meristem-tip culture is the excision of the organized apex of the shoot from a selected donor plant for subsequent *in vitro* culture. The technique has been used widely for the production of virus-free plants of chrysanthemum (Kumar et al., 2009; Verma et al., 2004) and several other species (Ayabe and Sumi, 2001). Shoot tips of flower stalk buds have been used as explants in culture to produce *Phalaenopsis* and *Doritaenopsis* plants (Tokuhara and Mii, 1993). However, to rescue virus-free plantlets from an infected mother stock only by shoot tip culture of *Phalaenopis* was found to be very difficult by many laboratories (Kang et al., 2011). Problems such as slow growth and high browning rate of excised shoot tips were encountered. Furthermore, both orchid viruses tend to move very closely to the apical dome of shoot tips leading to the difficulty in obtaining virus-free tissues for *in vitro* culture (Kang et al., 2011). Hence, the approach of induction of protocorm-like bodies (PLBs) from *Phalaenopsis* tissues. It was found that a certain percentage of PLBs induced from virus-infected Phalaenopsis mother plant were virus free (Hsieh et al., 2003). Also, by incorporation of anti-virus drugs such as Ribavirin in the culture medium, the percentage of virus-free PLBs could be increased and eventually virus-free plantlets after PLB regeneration could be obtained (Hsieh et al., 2003). However, most of these results could not be transferred to the orchid industry for practical application partly because the time frame needed for virus elimination was too long and its reproducibility was still unsatisfactory. Moreover, high somaclonal mutation rate in horticultural traits of regenerated plants from PLBs was another drawback (personal communication with Prof. F.C. Chen).

In the present communication, we report a relatively simple and rapid *in vitro* shoot tip culture in combination with a protocol for early isolation and selection of PLBs for regeneration and production of *Phalaenopsis* hybrid "V3" plants free of ORSV and CymMV. We have estimated that by this protocol, virus free plants regenerated from selected PLB will be ready for true to type examination at their flowering stage about 14 months after the initiation of cultures. The novelty and distinction of our protocol in comparison with other reports are discussed.

2. Materials and methods

2.1. Plant materials

Potted plants with flower stalks, and aseptic cultures in form of protocorm-like bodies (PLBs) of *Phalaenopsis* Sogo Yukidian "V3" (Phal. Yukimai × Phal. Taisuco Kochdian) naturally infected with *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV) were procured from the Yu-Pin Biotech Co., Ltd., Chiayi County, Taiwan. These were used as plant materials to initiate *in vitro* cultures. Plants that tested positive for both ORSV and CymMV served as mother plants for shoot-tip culture.

2.2. Explant preparation

Nodal segments of flower stalks were washed thoroughly under running tap water and submerged in sodium hypochlorite solution (1%) containing Tween-20 (0.02%; v/v) for 10 min for surface sterilization. Treated explants were washed 3 times with sterile distilled water in a laminar hood to remove the adhering sterilizing solution. The shoot tips (2–3 mm long) (Fig. 1B) excised from nodal segments under a binocular microscope were used as explants. The apical dome though visible, was partially covered by 1–3 leaf primordia. A total of 100 shoot-tips (50 each in two sets) were used to initiate the PLBs.

2.3. Culture media and culture conditions

In an initial trial, we tested five different basal media for induction of PLBs in shoot tips, however, only Murashige and Skoog's