

Research Note

Effects of complex organic additives on improving the growth of PLBs of Vanda Kasem's Delight**Pavallekoodi Gnasekaran¹, Ranjetta Poobathy¹, Maziah Mahmood², Mohd. Razip Samian¹ and Sreeramanan Subramaniam^{1*}**¹School of Biological Sciences, Universiti Sains Malaysia (USM), Minden Heights, 11800, Penang, Malaysia²Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), 43400, Serdang, Selangor, Malaysia

* Corresponding author: sreeramanan@gmail.com, sreeramanan@usm.my

Abstract

The effects of potato, papaya, and tomato organic extracts, prepared at various concentrations (0, 5, 10, 20 and 30%) were examined for the successful proliferation of *in vitro* Vanda Kasem's Delight (VKD) orchid's protocorm-like bodies (PLBs). Results obtained indicated that the growth of PLBs treated with organic extracts was significantly different compared to that of the control which lacked both carbon source and plant growth regulators. The use of Vacin and Went (VW) medium supplemented with coconut water and organic extracts appreciably enhanced the proliferation of PLBs of VKD, although no significant differences were detected among the treatments. It was concluded that VW medium, when supplemented with coconut water and 20% tomato extract, produced the highest proliferation rate for PLBs of VKD. Observations from this treatment included the production of healthy, green and fresh-looking PLBs, and a reduction in the occurrence of culture browning. Therefore, this treatment has successfully resolved problems involving the long reproductive cycle and slower proliferation nature of VKD orchid plant.

Keywords: Vanda Kasem's Delight orchid; Organic extract; Protocorm-like bodies (PLB); *In vitro*.**Abbreviations:** PLB-Protocorm-like body; VKD-Vanda Kasem's Delight; VW-Vacin and Went.**Introduction**

Orchids are among the most diverse of the flowering plant families, with over 800 described genera and 25,000 species (Atwood 1986; Chai et al., 2007). Some examples of orchid genera include *Arachnis*, *Ascocentrum*, *Cattleya*, *Cymbidium*, *Dendrobium*, *Laelia*, *Oncidium*, *Paphiopedilum*, *Phalaenopsis*, *Renanthera*, and their intergeneric hybrids (Zettler et al., 1990; Lee and Chang 2008). Orchids are grown primarily as ornamentals. Orchids are an export commodity in countries such as Thailand, Australia, Singapore, and Malaysia. There is a high demand for orchids in the form of potted plants and cut flowers because of their exotic beauty and long shelf life (Chugh et al., 2009). Orchids of the genus *Vanda* are known to produce large, colourful and stunning orchids. *Vanda* orchids are leagues ahead of other orchid genera due to factors such as the range of colours in modern hybrids, blooming frequencies of six or more times per year and lasting inflorescences that remain on the plant for between four to eight weeks (Motes 2004). *Vanda* Kasem's Delight orchid is valued both commercially and in horticulture due to the hybrid's diverse shapes, forms and colours. The hybrid's flower is four inches across and blooms throughout the year. The orchid is exported out of Thailand and Singapore as potted plants and cut flowers. There is a necessity for large scale multiplication of *Vandaceous* orchids using tissue culture techniques. However, micropropagation techniques are limited by problems such as

the exudation of phenolics from explants, and somaclonal variation (Chugh et al., 2009). Orchid culture and propagation media are typically incorporated with commonly-defined carbon sources such as glucose, fructose and sucrose (Faria et al., 2004); a combination of any different members of vitamin B, ascorbic acid as an antioxidant (Szendrak and Eszeki 1993) and standard MS stock solutions (Murashige and Skoog 1962). Occasionally, plant growth regulators are added at various concentrations to improve the treatment (Roy and Banerjee 2002). Successful *in vitro* plant proliferation is influenced by many factors such as plant genotypes, type of explants, media composition, and the cost of raw materials involved in the orchid propagation process. Complex and undefined organic extracts have been applied for decades in the enhancement and improvement of orchid plant proliferation. A number of organic extracts, such as banana pulp juice, peptone, slap honey, and the extracts of beef or taro, are known to be very effective in providing nutrients and growth factors for *in vitro* orchid plantlets. The extracts were reported to be successful in inducing the growth and development of several orchid species and explants (Arditti et al., 1990). The addition of organic additives to orchid tissue culture media generated much interest as it supports the rapid propagation of orchid PLBs. This has been proven for *Phalaenopsis* and *Doritaenopsis* (Islam et al., 2003; Gnasekaran et al., 2010).

Poor growth rates and delays in the *in vitro* proliferation of PLBs of recalcitrant orchids could be time consuming and may not fulfil the increasing demand for the orchid. The enhancement of PLB or callus growth rates through the supplementation of organic additives always secures a special interest in plant tissue culture technology. The incorporation of organic additives does not cause morphogenetic changes in newly-budding PLBs or regenerating plantlets. The present study was undertaken to evaluate the proliferation of PLBs of the recalcitrant orchid, *Vanda Kasem's Delight*, through the supplementation of organic additives to the tissue culture media, in order to overcome both low PLB formation rates and long PLB formation periods. It is important to identify suitable organic additives that enhance and sustain the proliferation of *Vanda Kasem's Delight* orchid for large-scale utilisation and propagation of the orchid.

Results and Discussion

Micropropagation via rapid plantlet regeneration from PLBs is possible for only a few orchids. The recalcitrant PLBs of VKD displayed low growth rates as cell growth depends on the supply and subsequent utilisation of the provided nutrients.

The effect of tomato extract

Modified VW medium supplemented with organic compounds was found to be beneficial for the proliferation of PLBs of VKD (Fig. 1). All PLBs treated with tomato extract remained green, viable and appeared to be very fresh even at the 12th week. Based on the growth response and visual observations of the PLBs, VW medium supplemented with 20 and 30% tomato extract supported vigorous proliferation of PLBs of VKD. Newly-developed PLBs from this treatment were green and possessed a higher differentiation capacity compared to PLBs treated with potato and papaya extracts. In addition, PLBs treated with tomato extract did not turn brown during the culture period, and well-developed and healthy PLBs with improved proliferation rates could be harvested after 12 weeks of culture. Tomato juice contains 93.1% moisture, 4.89% carbohydrate, vitamins and minerals, and is low in protein and fat (Abdel-Rahman and Abdel-Hamd 1982). According to Markovic et al. (2006) and Gebhardt et al. (2009), 100g of tomatoes contain a total sugar content of 2.62g, with glucose (in the form of dextrose), fructose and lycopene constituting 1.25g, 1.37g and 1.82-11.19mg respectively. Glucose and fructose are easily-absorbed sugars and hence promote both cellular maintenance and cellular division during PLB proliferation. Lycopene is a strong antioxidant which counteracts free radical formation, hence assisting in the repair of wounded cells and helping to inhibit DNA oxidation as well (Halliwell, 1996). Apart from lycopene, tomato contains other antioxidants such as ascorbic acid (12.7mg/100g), α -carotene (101 μ g/100g) and β -carotene (449 μ g/100g) (Gebhardt et al., 2009). The presence of sugar and strong antioxidants plays an integral role in the proliferation and production of healthy PLBs. The presence of the antioxidants also successfully prevented browning of the PLB culture. Newly-formed and healthy PLBs displayed green colour (Fig. 2a).

The effect of potato extract

The use of potato extract on PLBs of VKD produced similar effects as observed in the use of tomato and papaya extracts:

the rate of PLB proliferation increased with the supplementation of the potato extract. The best result was obtained when the modified VW medium was supplemented with 20% potato extract. Higher concentrations of the potato extract tended to be inhibitory to the proliferation of the PLBs. Potato is a carbohydrate-rich food which contains about 80% water and 20% dry matter, with 60 to 80% of the latter composed of starch (Prokop and Albert 2008). The potato extract was prepared from unsprouted potato tubers to prevent post-culture fermentation of the medium because potato sprouting leads to conversion of starch into sugar. Sprouted tubers also contain toxic glycoalkaloids such as solanine and chaconine, which are concentrated under the skin and occur at low levels in tubers (Prokop and Albert, 2008). These compounds are not thermally destroyed during cooking or frying (Bushway and Ponnampalam, 1981). The toxic effects of the glycoalkaloids in the potato extract could be neutralised by the antioxidants present in potatoes. Examples of such antioxidants include ascorbic acid, certain carotenoids and anthocyanins. Ascorbic acid stabilises free radicals and prevents cellular damage via iron absorption, which promotes the healing of wounds and cuts on the surface of the PLBs. Apart from antioxidants, notable amounts of proteins of high biological value could also promote the proliferation of PLBs. Substantial amounts of alkaline salts in the form of potassium may thwart acid overdose (Demigne et al., 2004) and prevent medium fermentation, which in turn supports the proliferation of PLBs. In addition, the presence of vitamin B6 in the potato extract could encourage the proliferation of PLBs by inducing the production of essential amino acids. Lower concentrations (5 and 10%) of the potato extract could not provide sufficient amounts of some of the biological compounds present at trace levels in the pure extract. Therefore, PLB growth halted after six weeks of culture. The PLBs obtained from this treatment were dark green in colour, typically considered as a sign of stagnant growth. Furthermore, the newly-emerged PLBs were smaller in size (<0.5mm), similar to that of PLBs treated with papaya extract (Fig. 2b).

The effect of papaya extract

The present study indicated that papaya extracts prepared above the optimal concentration of 10% tended to impede PLB growth (Fig. 1). Papaya is a rich source of antioxidants such as beta-carotene, vitamins A, B and C, flavonoids, folate and pantothenic acid. It also contains trace amounts of calcium, chlorine, iron, phosphorus, potassium, silicon and sodium. The saccharides, total soluble sugar and protein contents in papaya extracts are about 9.8%, 7.3% and 0.1% respectively (Ojokoh and Uzeh 2005). The study was conducted using fully-ripened papayas since 100g of papaya fruit contain about 8g of sugar that consists mainly of invert sugar such as glucose and fructose resulting from sucrose hydrolysis. Apart from antioxidants and sugars, papaya contains large amounts of phenolic compounds, organic acids and sterols (Asano et al., 1996). However, the accumulation of phenolic compounds during culture results in both tissue browning and the loss of growth capacity, eventually leading to tissue death (Kaewubon and Meesawat, 2009). Proliferation rates reduced when PLBs of VKD were treated with papaya extract. This was proven in the visual observations of such PLBs in which PLB browning was

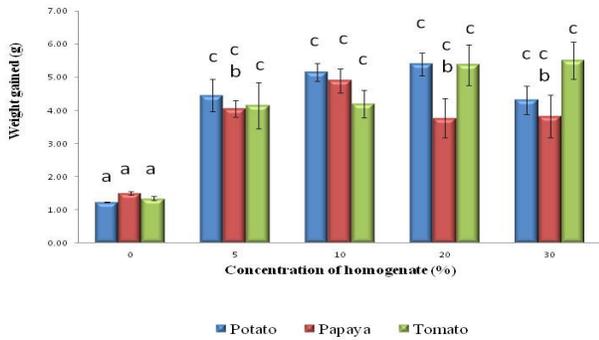


Fig 1. Effect of the different organic extracts and concentrations on the proliferation of PLBs of VKD. Each concentration consisted of three replicates containing ten PLBs. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p < 0.05$).

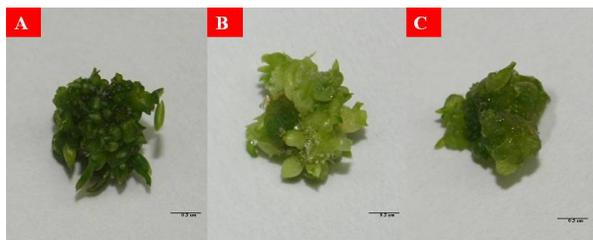


Fig 2. PLBs treated with organic additives at 30%. (A) PLBs treated with 30% potato extract produced dark-green PLBs which were smaller than 0.5mm. (B) PLBs treated with 30% papaya extract displayed a pale and unhealthy appearance and produced secondary PLBs which were smaller than 0.5 mm. (C) PLBs treated with 30% tomato extract remained green and appeared very fresh.

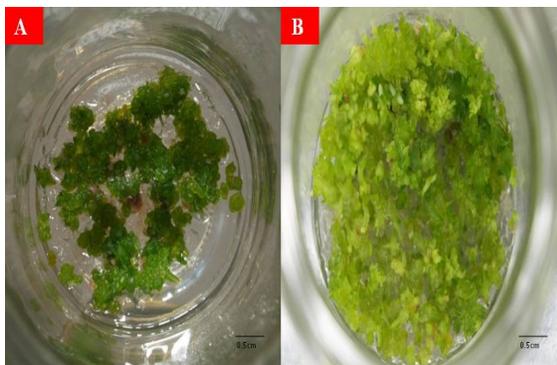


Fig 3. Organic additives significantly increased PLB proliferation of the recalcitrant VKD orchid. (A) Tomato extract enhanced the proliferation of PLBs of VKD by providing sufficient nutrient supply. PLBs treated with the tomato extract appeared healthy, and tissue death due to the browning effect was rare. (B) The utilisation of nutrient and growth factors provided by tomato extract subsequently augmented the regeneration of PLBs into intact VKD orchid plantlets within eight weeks.

observed within six weeks of culture in the medium. The number of non-surviving PLBs from this treatment was proliferation of the recalcitrant VKD orchid. (A) Tomato extract enhanced the proliferation of PLBs of VKD by providing sufficient nutrient supply. PLBs treated with the tomato extract appeared healthy, and tissue death due to the browning effect was rare. (B) The utilisation of nutrient and growth factors provided by tomato extract subsequently augmented the regeneration of PLBs into intact VKD orchid higher compared to those treated with potato extract. The viable PLBs were pale green in colour and the newly formed PLBs were smaller than 0.5mm, which is a sign of poor growth (Fig. 2c).

Beneficial effects of coconut water on proliferation of PLBs

The addition of coconut water from the tender nut to the culture medium even promoted proliferation of PLBs of VKD in the control medium, which was not supplemented with any organic extracts. The liquid endosperm of coconut (*Cocos nucifera L.*) accelerated the growth rates of micropropagated plantlets (Caplin and Steward 1948; Caplin and Steward 1949). Previous studies had proven that the supplementation of coconut water in both liquid and solid medium enhanced the survival of PLBs of *Cattleytonia* (Uesato and Sagawa 1986). This could be attributed to the notable biochemical compounds of the tender coconut milk such as potassium, sodium, calcium, phosphorous, iron, copper, sulphur, magnesium, ascorbic acid and the B group vitamins. In addition, 70% of the free amino acids of coconut milk are made of glutamine, arginine, asparagine, alanine and aspartic acid.

Materials and methods

Plant material and culture conditions

Previously-developed PLBs of VKD maintained by monthly subcultures on $\frac{1}{2}$ MS (Murashige and Skoog, 1962) medium supplemented with 1mgL^{-1} BAP and 20gL^{-1} sucrose were used as explants for the PLB proliferation study. Three to four mm single PLBs were isolated from clumps of PLBs and cultured with the basal section down on solid VW medium in culture jars ($25 \times 250\text{mm}$) containing 25ml of medium. The media were supplemented with 15% (v/v) coconut water and different concentrations of the homogenates of organic extracts at 5%, 10%, 20% and 30% (w/v). The media were solidified with 8gL^{-1} Gelrite (Duchefa). The pH of the VW medium was adjusted to 4.8-5.0. All media were autoclaved at 110kPa for 15 minutes at 121°C . All cultures were incubated at $25 \pm 1^\circ\text{C}$, and under cool-white fluorescent light of $30\mu\text{molm}^{-2}\text{s}^{-1}$ for 16 hours per day.

Preparation of organic extracts

The potato, papaya and tomato were sourced from Penang, Malaysia. The organic materials were cut into cubes of 1cm^3 . The potato and papaya were peeled before being sliced into cubes. Thirty grams of each freshly-diced material were ground with 200ml of liquid VW medium using kitchen blender (Panasonic, MX-899TM) for two minutes. These extracts were immediately added to VW medium supplemented with 15% coconut water.

Experimental design and data analysis

Experiments were performed in a randomised design and were repeated twice. Each treatment had six replicates consisting of 10 explants per culture vessel with the total weight of the explants per replicate set at 1g. Morphogenetic response (PLB formation) from explants was evaluated after 12 weeks of culture, expressed as the weight gain in PLBs with respect to the concentration of the organic extracts. The data were statistically analysed using one-way ANOVA and the differences contrasted using Duncan's test.

Conclusion

Besides enhancing the proliferation of PLBs of VKD, VW supplemented with coconut water and 20% tomato extract supported the regeneration of PLBs into healthy intact plantlets (Fig. 3). Tomato extract contains carbohydrates, protein, vitamins, amino acids, organic acids and strong antioxidants. The formulated media can be employed for the rapid propagation of VKD orchid plantlets. This study has developed a simple, economical and reliable protocol to propagate PLBs of VKD, an economically important orchid. The best culture conditions for propagating PLBs of VKD include the addition of 20% tomato extract in modified Vacin and Went medium supplemented with 10% coconut water. The synergistic effect of the tomato extract and coconut water enhanced the large-scale production of PLBs of VKD, with PLB samples displaying normal morphology in a short period of time.

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