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Local Sugars Alternatives for Tissue Culture of *Dendrobium* Hybrid CV. *Sonia*

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Abstract

In developing countries, commercial exploitation of tissue culture technology is limited by high cost of production. An assessment of the effectiveness of using low cost alternative sugars for the *in vitro* micropropagation of *Dendrobium* cv. *sonia* was investigated. Protocom-Like Bodies (PLBs) were multiplied in Murashige and Skoog (MS) liquid medium with 3.0mgL^{-1} Benzyl adenine (BA) and 15% (v/v) coconut water. After the fifth subculture, 48.9% of the PLBs showed abnormal growth and 17.0% turned brown. PLBs after the first subculture were transferred to solidified MS medium with 0.1mgL^{-1} BA , 1.0 mgL^{-1} NAA, 15% (v/v) coconut water, 20gL^{-1} analytical grade sucrose and local sugars such as refined brown sugar, refined dark brown sugar, unrefined dark brown sugar, refined white sugar and super refined white sugar. No significant difference ($p>0.05$) was observed between analytical grade sucrose and local sugars. Healthy PLBs were obtained on solidified MS media supplemented with 20gL^{-1} refined brown, refined dark brown, unrefined dark brown and super refined white sugars. Browning of PLBs was obtained on MS medium with 20gL^{-1} refined local white sugars. Results indicated that when local sugars are used, the cost incurred on the importation of analytical grade sucrose can be reduced by more than 99%.

Keywords: sugars, Protocorm-like bodies, Murashige and Skoog medium, cost

Abbreviations: Benzyl adenine-BA, Degree Celsius -° C, and others - *et al.*, Gramme-g, Hour- h, Kilogram- Kg, Litres-L, Milligramme-mg, Murashige and Skoog-MS, α-Napthaleneacetic acid-NAA, Protocorm-Like Bodies-PLBs, Revolutions Per Minute- rpm, Rupees-Rs, United States Dollar-USD, Volume by volume-(v/v), Weight by volume-(w/v)

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INTRODUCTION

Orchids are considered as one of the largest family of the flowering plants and consist of more than 800 genera and 25 000 species (Tee *et al*., 2003; Li *et al.*, 2005). Associated with the numerous number of species is an extraordinary floral diversity. Orchids have complicated flowers and unusual pollination systems. Due to its complicated structure, the orchid flower has given rise to a multitude of hypotheses regarding its homologization and derivation from comparatively simple trimerous monocotyledonous flower (Kull & Arditti, 2002).

Dendrobium, is considered as the second largest genus in the Orchidaceae family and consists of about 1600 distinct known species (Puchooa, 2004). *Dendrobium* is a monocot plant, a perennial herb (Rao, 1977; Hickey and King, 1988) and a sympodial orchid, which may grow on rocks, but most of them, are epiphytic growing on branches of trees (Rittershausen and Rittershausen, 1970). One of the *Dendrobium* hybrids, *Dendrobium* cv. *sonia* has lavender coloured flowers of about three to four inches in width (Kamemoto *et al*., 2001).

Dendrobiums are prized for their beautiful flower sprays, wide colour variation, year-round availability and their long lasting flowering life (Sim *et al*., 2007; Khosravi *et al*., 2008). Moreover*, Dendrobiums* such as *Dendrobium huoshanense* are used in Chinese medicine to treat throat inflammation, cataract and chronic superficial gastritis (Wei *et al.*, 2007). Furthermore, orchids have been used as medicines in Japan, Europe, the Americas, Australia & Africa (Bulpitt, 2005).
Due to high demand for orchid cut flowers and potted plants internationally (Nagaraju and Mani, 2005); production of orchids for commercial purposes has greatly increased throughout the world in the last 25 years (Lopez and Runkle, 2005). In the United States, approximately 90% of the 52 million imported orchids were *Dendrobiums,* which valued a total of 3.6 million United States Dollar in 2005 (Khosravi *et al*., 2008). In the tropics, *Dendrobiums* accounts for 85% of the total orchid cut flower trade (Sheela, 2008).

However, in Mauritius, the flower industry is mostly centered towards the cultivation of anthuriums (Boolell, 2007). Orchids are being imported weekly since local growers are unable to meet the hotel demands (The president of the Republic of Mauritius, 2008). The main reasons for not producing *Dendrobiums* on a commercial scale are that the propagation of orchids via conventional means is slow and only a small number of propagules are produced (Bose *et al.,* 1999; Kamemoto *et al*., 2001; Puchooa, 2004; Runkle, 2005). Other constraints include low germination rate, inability to adapt to physical changes in the environment (Rao, 1977), long juvenile period (Sim *et al.*, 2007), lack of usable genetic variability (Kuehnle and Sugii, 1992) and difficulties to create improvements in orchids (Tee *et al*., 2003).

Propagation of orchids by tissue culture techniques is thus preferred. Even if orchid micropropagation has shown spectacular development in the recent years, the wide spread use of micropropagation is still limited in developing countries due to the high cost of the media components (Demo *et al*., 2008). These include carbon sources, gelling agents, inorganic and organic supplements and growth regulars.

Media chemicals account for less than 15% while the carbon sources such as analytical grade sucrose contribute about 34% of the production cost (Demo *et al*., 2008). Thus, in order to produce *Dendrobium* cv. *sonia* (*Dendrobium* 'Caesar' x *Dendrobium* 'Tomie Drake') on a commercial scale in developing countries like Mauritius, the possibility of replacing the expensive lab sucrose by local sugars was investigated.

Protocorms

Protocorms are referred to as somatic embryos (Batygina *et al.*, 2003; Puchooa, 2004; Anjum *et al.,* 2006; Wei *et al.*, 2007) and its occurrence in orchids might be due to the elimination of some phases of sexual embryo development (Batygina *et al.*, 2003) . Various reports have shown that orchids like *Cymbidium, Ophrys* and *Phalaenopsis* undergo an intermediary callus phase before being differentiated into protocorms (Kitsaki *et al*., 2004; Košir *et al*., 2004; Teixeira da Silva *et al*., 2006).

Coconut water

Coconut (*cocos nucifera)* milk is often added in orchid media and contains a large amount of zeatin riboside which promotes cell division (Moore, 1979; Sim *et al*., 2008). However, formation of protocorm-like bodies (PLBs) on *Dendrobium* buds was observed in medium containing 15 % (v/v) coconut water after 4-5 weeks (Rao, 1977). Multiplication of PLBs was observed in medium supplemented with 1.0mgL^{-1} Benzyl adenine (BA) but the number of PLBs obtained was not sufficient for performing further experiments although it has been reported that 1.0mgL^{-1} BA gave the highest number of PLBs in *Dendrobium* cv. *sonia* (Sheela, 2008).

Carbohydrate source:

Sucrose is mostly used at a concentration of 2-3% in the tissue culture of orchids (Faria *et al*., 2004). Supplying a carbon source to plant grown *in vitro* is fundamental since the photosynthetic activity is reduced, light intensity is low, gas exchange is limited and humidity is relatively high and the plant tissues are not autotrophic (Pierik, 1997; Nowak *et al.,* 2004; Ślesak *et al.,* 2004; Faria *et al*., 2004; Demo *et al*., 2008). An exogenous supply of carbohydrates is also needed as a source of carbon and energy supply (Wolfgang and Wolfgang, 1977; Smith, 1999; Chandran *et al.,* 2006). Moreover, Sugars have signaling functions throughout all the life cycle of a plant and can signal alteration in gene expression and enzymatic activities (Smith, 1999; Ślesak *et al.*, 2004; Teixeira Da Silva, 2004; Chandran *et al*., 2006). Sugars can also act as osmotic agents *in vitro* and thus influencing the uptake of all constituents in the media (Nowak *et al*., 2004). Sugar is important for the proper maintenance of the structure and semi-permeability of the plasma membrane (Chandran *et al.*, 2006) and embryogenesis is supported by osmotic potential provided by sugars (Fuentes *et al*., 2000). However, sucrose which is the commonly used carbon source in plant micropropagation, adds significantly to the media cost.

For developing countries to benefit from tissue culture techniques, the cost of commercial micropropagation has to be reduced but the quality of the micropropagules should not be affected (Savangikar, 2004., Demo *et al.,* 2008). One method to achieve this aim is to replace expensive analytical grade sucrose by low cost local sugars. The growth of protocorm-like bodies (PLBs) was not

affected by the addition of local sugars to the Murashige and Skoog (MS) medium. Local sugars and sucrose AR are not significantly different at the 5% level ($p>0.05$) while fresh and dry weight of *Catharanthus roseus* leaves was found to be higher in media with 4% market sugar compared to those grown on media with analytical grade sucrose (Namdeo *et al*., 2006).

Beneficial effects of brown sugars have been observed in the micropropagation of potato (*Solanum tuberosum*) (Demo *et al*., 2008). It has also been reported that brown sugars contain more quantities of calcium, phosphorus, iron, potassium and sodium , but these elements are present in small concentrations in white sugars (Demo *et al*., 2008).

MATERIALS AND METHODS

Materials and Methods

Plant materials. The experiment was carried out in the plant tissue culture laboratory at the faculty of Agriculture, University of Mauritius. *In vitro Dendrobium* cv. *sonia* Protocorm-Like Bodies (PLBs) obtained from the plant tissue culture laboratory were used as starting materials. The PLBs were separated from each other using sterile forceps and were immersed in 70% ethanol for 1 min and were rinsed 5 times with sterile distilled water.

Media and conditions. The PLBs were transferred to a modified Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962) supplemented with $30gL^{-1}$ sucrose, 15% (v/v) coconut water and with three different concentrations (0, 1.0 and $3.0mgL^{-1}$) of Benzyladenine (BA) to identify the appropriate concentration for the multiplication of *Dendrobium* cv. *sonia* protocorms. The coconut water was prepared from young coconuts obtained from the local market. The coconut water was boiled for 5mins and was filtered using a filter pump (Javac 8524 PVH-11 Vacuum Pump) to remove any traces of proteins. The pH of the medium was adjusted to 5.4 using 0.1M NaOH or 0.1M HCl. 10ml of the culture medium was dispensed in culture jars and autoclaved at 121 ºC for 15-20 mins. After inoculation, the culture jars were sealed with parafilm, placed on an orbital shaker (Edmund Bühler SM 25 DIGI) at 110 rpm and incubated at 26±2ºC and 16h of light daily provided by cool-white fluorescent tubes (36/10Watts). Subculturing was performed every 3 to 4 weeks on the same medium and incubated on the shaker under the same culture conditions

Regeneration of plantlets: After the fifth subculture, the PLBs were separated and cultured on Murashige and Skoog (MS) medium solidified with $3.0gL^{-1}$ phytagel and supplemented with $30gL^{-1}$ sucrose, 15 % (v/v) coconut water, $0.1mgL^{-1}$ Benzyl adenine (BA) and $1.0mgL^{-1}$ NAA for plantlet regeneration. The culture jars were sealed with parafilm and incubated under the same culture conditions.

Effects of alternative low cost sugars: Six different types of sugars namely analytical grade sucrose AR and local sugars such as refined white sugar, super refined white sugar, refined light brown sugar, unrefined dark brown sugar and refined dark brown sugar were compared at 20g/L.

A mass of protocorm-like bodies (PLBs) about 0.5cm in length were transferred on solidified Murashige and Skoog (MS) medium containing 15 % (v/v) coconut water and 0.1mgL^{-1} Benzyl adenine (BA) and 1.0mgL^{-1} NAA and 20gL^{-1} analytical sucrose AR, and local sugars from the local supermarket; refined white sugar, super refined white sugar, refined light brown sugar, unrefined dark brown sugar and refined dark brown sugar.

Each treatment consisted of 5 replicates and each replicate consisted of only one mass of PLBs. The culture jars were incubated at 26±2ºC and 16h of light provided by cool-white fluorescent tubes (36/10 Watts). The number of leaves of each treatment was noted after 6 weeks in culture and the data was subjected to the analysis of variance to test the significant differences between the treatments at the 5% level using the statistical software Minitab (Version 11). The mean number of leaves was compared using the Tukey test at the 5% level. A cost analysis of the different sugar treatment was also performed.

RESULTS

Effects of subculturing on multiplication and growth of PLBs: After the fifth subculture of PLBs to medium with 3.0mgL^{-1} Benzyl adenine (BA), abnormal growth of 48.9% of the PLBs was observed. The PLBs underwent accelerated growth and development and the developed leaves were turgid and thickened (Fig 2B). In addition, 17.0% of the PLBs turned brown. Further proliferation of normal grown PLBs was noted. 51 plantlets and more than 3127 PLBs were obtained.

***In vitro* mass multiplication of *Dendrobium* cv. *sonia* PLBs.**

***Fig 1*: Multiplication of PLBs in liquid Murashige and Skoog medium with Benzyl adenine (BA) (3.0mgL^{-1})**

Effects of Benzyl adenine (BA) on multiplication of protocorm-like bodies (PLBs)

From fig 2, it can be observed that 3.0mgL^{-1} Benzyl adenine (BA) gave the highest number of Protocorm-like bodies (PLBs) after 3 weeks in culture.

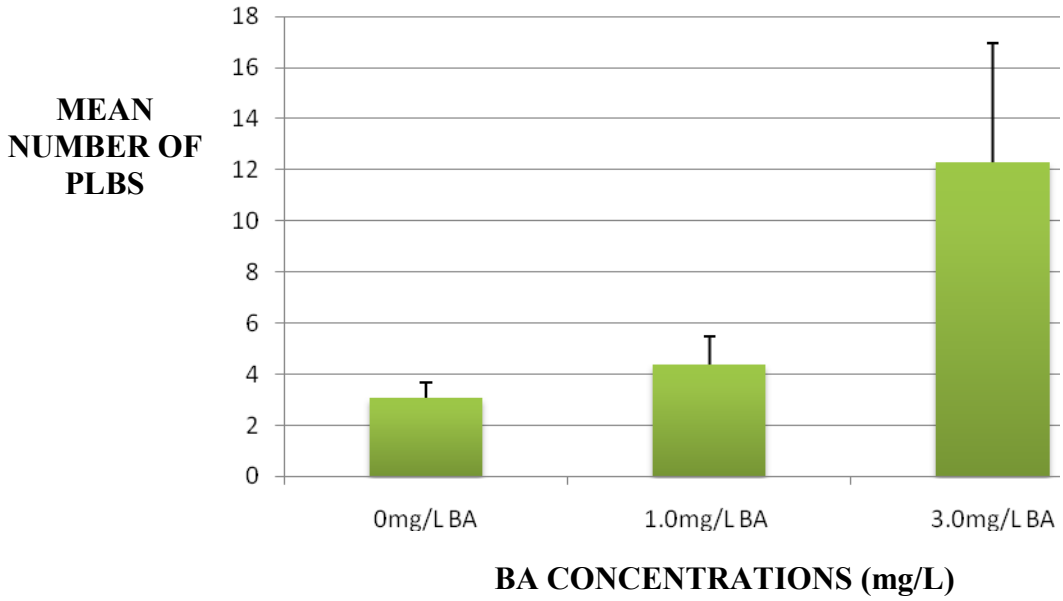


Fig 2: Proliferation of protocorm-like bodies in liquid Murashige and Skoog medium with 0, 1.0 and 3.0mg/L Benzyl adenine (BA).

Description of growth and development of protocorm-like bodies after fifth subculture:

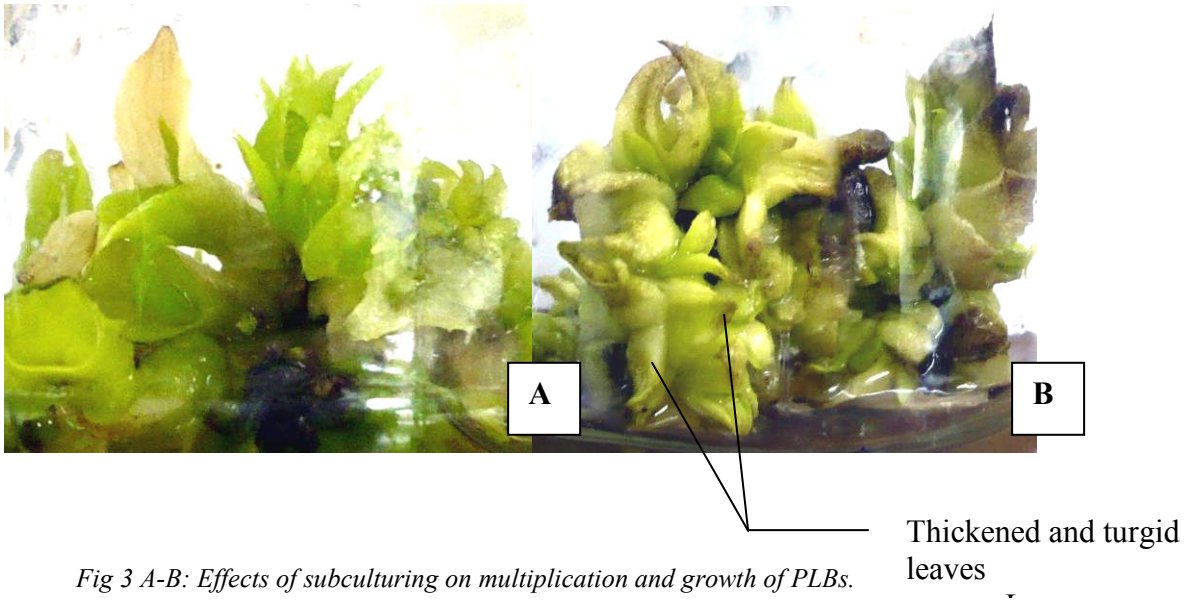
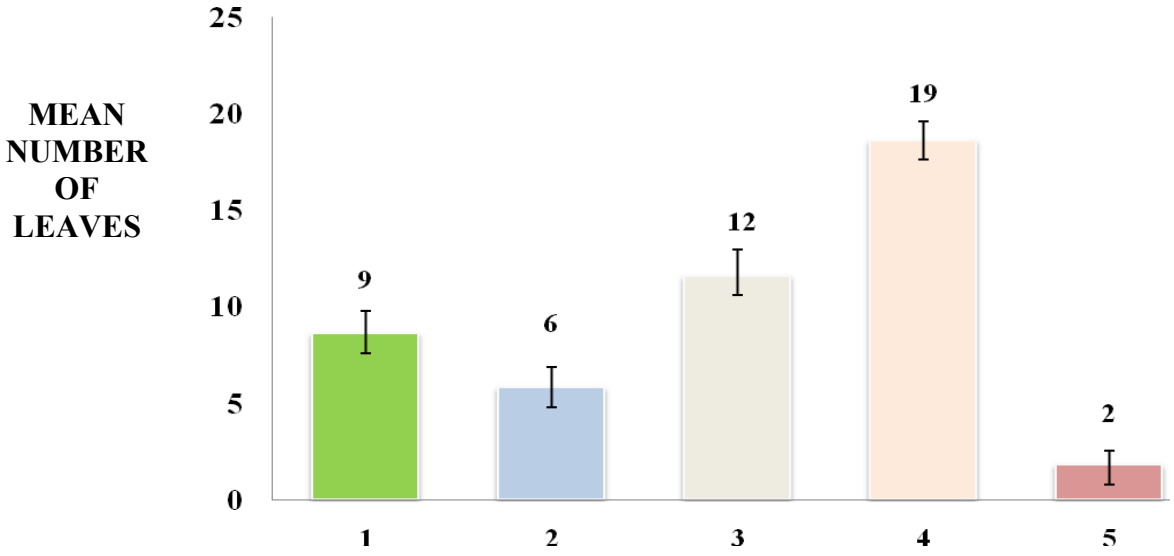


Fig 3 A-B: Effects of subculturing on multiplication and growth of PLBs.

A Normal growth **B** Abnormal growth; thickened and turgid leaves

Effects of local sugars on growth of protocorm-like bodies (PLBs).



1- Analytical grade sucrose AR
2- Superrefined white sugar
3- Refined light brown sugar
4- Refined dark brown sugar
5- Unrefined dark brown sugar

Fig 4: Effects of alternatives to analytical grade sucrose on growth of PLBs

In all the above treatments except in MS media with refined white sugar, further multiplication of the protocorm-like bodies (PLBs) was observed. The PLBs were green in colour and initiation of leaves was observed after five weeks in culture (Fig 5). The PLBs turned completely brown after two weeks in Murashige and Skoog (MS) medium with $20gL^{-1}$ refined white sugar. There is no significant difference between analytical sucrose AR and local sugars at the 5 % level of significance (p value =0.147) despite the differences among the mean number of leaves.

Description of PLBs in media with local sugars

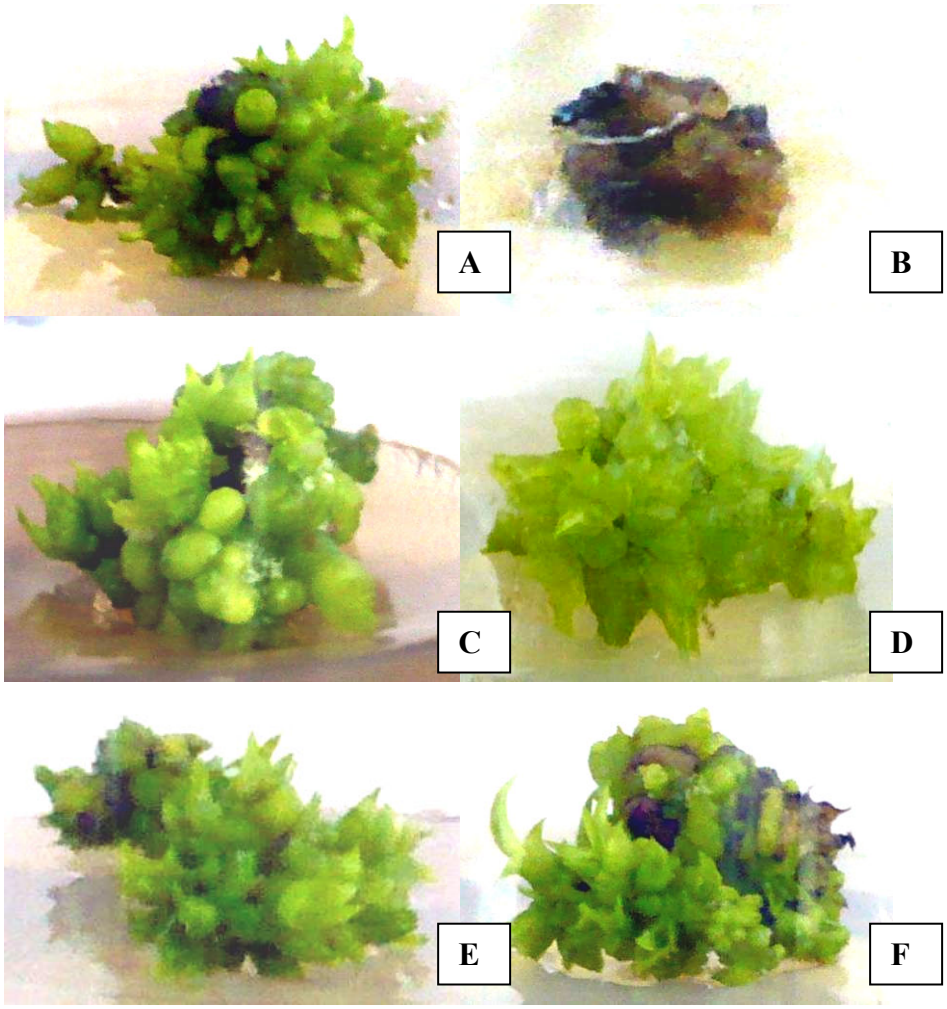


Fig 5 A-F: Effects of alternatives to analytical grade sucrose on growth of PLBs after 7 weeks in culture. ***A*** *MS media supplemented with 20g super refined white sugar,* ***B*** *Complete browning in media with refined white sugar,* ***C*** *Unrefined dark brown sugar,* ***D*** *Refined light brown sugar,* ***E*** *Refined dark brown sugar &* ***F*** *Analytical grade sucrose AR*

DISCUSSION

Effects of local sugars on plantlet regeneration from protocorm-like bodies (PLBs):

Of all sugars tested, only refined white sugars resulted in complete browning of the explants after two weeks in culture. The commercial refined white sugar may contain some toxic compounds that may not be appropriate for the tissue culture of *Dendrobium* cv. *sonia*. Browning of the explants has been found to be related to the oxidation of phenolic compounds which produce toxic quinines and

polymerised substances resulting to darkening of the media and the explants in culture jars (Ozyigit *et al*., 2007) and the concentration of phenolic compounds produced *in vitro* is affected by the type of carbon source added to the medium (Ozyigit *et al*., 2007). Thus most probably, super-refined white brought an increase in the concentration of phenolic compound resulting in browning of the explants.

However, no harmful effect has been observed with super refined white sugar, most probably because the sugar was further processed so that only small traces of toxic compounds were present in the sugar. The growth of protocorm-like bodies (PLBs) was not affected by brown sugars. The utilization of the brown and super refined white sugars by the PLBs could be explained by the fact that many sugars can be transported in plants (Garcìa *et al*., 2002; Taiz and Zeiger, 2006; Demo *et al*., 2008).

Statistical significance:
With respect to the effects of local sugars on growth of protocorm-like bodies, it has been observed that there is no significant difference between analytical sucrose AR and local sugars at the 5 % level of significance (p value =0.147) despite the differences among the mean number of leaves. Hence, the two sugars are comparable and can be used interchangeably.

Cost analysis:

Sugars	Cost/Kg (Rs)	Cost reduction
Analytical grade sucrose AR	11 600	
Super refined white sugar	47.60	99.6%
	54.00	99.5%
Unrefined dark brown sugar	44.00	99.6%
Refined dark brown sugar	44.00	99.6%
Refined light brown sugar		

Table 1: Comparative costs of culture medium for micropropagation of Dendrobium cv. sonia using local sugars

The cost of the sucrose AR and the table sugars used in the experiment was the actual price in the Mauritian local market.

Mauritius is one of the countries of the African regions where sugar cane is a major commercially grown agricultural crop. Its production in Mauritius varies between 5.0 to 5.5 million tones per annum, 35% of which are produced by about 28, 000 small growers and around 550,000 to 625,000 tonnes of sugar are recovered from the cane (Deepchand, 2005). Since the cost of analytical grade sucrose is too high to be used in large-scale micropropagation of *Dendrobium* cv. *sonia*, the possibility of using local sugars instead of importing analytical sugars required to be studied. From the current study, it has been shown that by replacing lab sucrose by local sugars such as superrefined and brown sugars, the cost spent on sugars for *Dendrobium* cv. *sonia* can be reduced by more than 99% (Table 1). The production cost of potato (*Solanum tuberosum*) was cut off by more than 34-51% when lab sucrose was replaced by table sugar (Demo *et al.,* 2008) while that of banana was reduced to 90% (Demo *et al*., 2008).

Development of Protocorm-like bodies (PLBs) in culture

Protocorm like Bodies were used as starting material. In the current study, the protocorms adopted a spherical shape and the formation of the large number of secondary protocorms occurred from epidermal cells of a single protocorm confirming the statement made by Rao (1977) and Batygina *et al*. (2003). No callus phase has been observed in *Dendrobium* cv. *sonia* during this study. However, Anjum *et al*. (2006) observed the formation of callus at shoot base, apical meristem or at the node of *Dendrobium* Malones but the callus further differentiated into protocorms like bodies. These main differences may be attributed to the type of explant used, the age of the explant and the specific developmental pathways adopted by each *Dendrobium* species.

Multiplication of protocorm-like bodies

The protocorm-like bodies (PLBs) on medium devoid of Benzyl adenine (BA) only swelled after three weeks in culture. Cytokinins present in coconut milk might have contributed to the growth of the PLBs but was in too low concentration to initiate cell division.

The mean number of protocorm-like bodies (PLBs) in Murashige and Skoog (MS) medium supplemented with 3.0mgL^{-1} Benzyl adenine (BA) was thrice of that obtained with 1.0mgL^{-1} BA (Fig 2) showing an increase.

Effects of subculturing on multiplication and growth of protocorm-like bodies (PLBs):

After the fifth subculture on media supplemented with 30gL^{-1} sucrose, 15% (v/v) coconut water and 3.0mgL^{-1} BA, abnormal growth of 48.9% of the protocorm-like bodies (PLBs) was observed (Fig 3B). The occurrence of malformation of leaves in the current study might be due to the high concentration and long exposure time of Benzyl adenine (BA) (Moncaleán *et al*., 2001). A similar result was observed with the *in vitro* micropropagation of papaya (Burikam *et al*., 1988).

The addition of Benzyl adenine (BA) to liquid media has been reported to be more deleterious to plant grown *in vitro* than solid media with BA (Moncaleán *et al*., 2001). This is because plant hormones are more readily available to explants in liquid media (Mehrotra *et al.*, 2007). In this investigation, solid medium was not used because it has been found that liquid medium is often used to induce PLBs in orchids (Rao, 1977; Goh, 1983) and the greater number of PLBs in *Dendrobium* cv. *sonia* was obtained when liquid medium was used (Puchooa, 2004). The concentration ($3.0mgL^{-1}$) Benzyl adenine (BA) used in this experiment could have caused a rapid increase in cell division leading to genetic changes (Krikorian, 1995) which might also explain the morphological changes in this study.

The 17.0% browning of protocorm-like bodies (PLBs) observed after the fifth subculture might be due to lack of oxygen and submergence of the explants with the liquid medium (Sandal *et al*., 2001; Prasad and Gupta, 2006; Kuria *et al*., 2008). Browning of explants could also be explained by the accumulation of the cytokinin Benzyl adenine (BA) in the plant tissues *in vitro* (Moncaleán *et al*., 2001). Also, it has been suggested that explants that cannot produce antioxidants against activated forms of oxygen are more likely to be affected by hyperhydricity (Saher *et al.*, 2005).

Although, it has been reported that several plant species lose their ability to multiply after various subcultures (Demo *et al*., 2008), in the present study, after the fifth subculture, the protocorm-like bodies (PLBs) continue to divide without visible morphological changes.

CONCLUSIONS

In the present study, it has been demonstrated that by substituting analytical grade sucrose by super-refined white and brown sugars, the cost spent on importation of sucrose can be reduced by more than 99%. A major limiting factor towards tissue culture in developing countries is the cost of setting up and maintaining a tissue culture laboratory. By using local sugars, developing countries like Mauritius can significantly reduce the cost of production and thus exploit tissue culture technology for the micropropagation of *Dendrobium*. Producing this genus on a commercial scale could provide local growers a profitable alternative to anthurium flowers since anthurium production has gone down locally. It will be necessary to pursue further research in sub-culturing and propagating *Dendrobium* cv. *sonia* using local sugars while testing if the local sugars do not induce additional somaclonal variations in protocorm-like bodies relative to the use of laboratory grade sugars. If no additional somaclonal variation is found, there is thus the possibility of replacing the expensive laboratory grade sucrose to table sugar. However, so far, experiments conducted show that such a possibility is feasible. There are other possibilities to reduce the cost of production including replacing expensive conventional gelling agents by sugar-cane bagasse, coir, cotton fiber, white flour, potato and cassava starch are all feasible alternatives that require testing.

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