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Low-cost tissue culture technology for the orchid regeneration

Tecnología de cultivo de tejidos de bajo coste para la regeneración de orquídeas

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Abstract

Orchids are one of the most important flowers grown in Sri Lanka for exports and local market. One of the majors constrains in expanding the orchid cultivation is a limited supply of quality planting materials. Micropropagation techniques can be successfully applied for the mass propagation of orchids. But micropropagation is expensive, thus increasing the cost of production. Therefore, this research was undertaken to replacement of expensive agar for *in vitro* subculturing of *Cattaleya* seedlings. Two-month-old *Cattleya* seedlings were subcultures on Murashige and Skoog media (MS) and Knudson C media (KNC) as basal media. As gelling agents' sago, semolina, corn flour, semolina + agar, corn flour + agar were used. Control treatment was carried out with agar. The cultures were maintained inside the culture room. The number of leaves, number of roots, leaf length, leaf width and percentage of contamination were recorded at four-week intervals. Sago and semolina + agar showed higher performance and low contamination percentage as alternative gelling agents in the MS media. Sago, corn flour, sago + agar, semolina + agar and corn flour + agar showed higher performances and low contamination percentages in KNC media. The results of this study propose that sago, semolina, and corn flour can be used as alternative gelling agents in MS and KNC media for subculture practices of orchids.

Keywords: *Alternative gelling agent, Cattleya seedlings, micropropagation, Orchid subculture*

Resumen

Las orquídeas son una de las flores más importantes que se cultivan en Sri Lanka para la exportación y el mercado local. Uno de los principales obstáculos para la expansión del cultivo de orquídeas es el limitado suministro de materiales de plantación de calidad. Las técnicas de micropropagación pueden aplicarse con éxito para la propagación masiva de orquídeas. Pero la micropropagación es costosa, lo que aumenta el coste de producción. Por lo tanto, esta investigación se llevó a cabo para sustituir el costoso agar para el subcultivo in vitro de plántulas de Cattaleya. Las plántulas de Cattaleya de dos meses de edad se subcultivaron en los medios Murashige y Skoog (MS) y Knudson C (KNC) como medios basales. Como agentes gelificantes se utilizaron sagú, sémola, harina de maíz, sémola + agar, harina de maíz + agar. El tratamiento de control se realizó con agar. Los cultivos se mantuvieron en la sala de cultivo. El número de hojas, el número de raíces,

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la longitud y la anchura de las hojas y el porcentaje de contaminación se registraron a intervalos de cuatro semanas. El sagú y la sémola + agar mostraron un mayor rendimiento y un bajo porcentaje de contaminación como agentes gelificantes alternativos en el medio MS. El sagú, la harina de maíz, el sagú + agar, la sémola + agar y la harina de maíz + agar mostraron mayores rendimientos y bajos porcentajes de contaminación en los medios KNC. Los resultados de este estudio proponen que el sagú, la sémola y la harina de maíz pueden utilizarse como agentes gelificantes alternativos en los medios MS y KNC para las prácticas de subcultivo de orquídeas.

Palabras clave: *Agente gelificante alternativo, plántulas de Cattleya, micropropagación, subcultivo de orquídeas*

Introduction

Micropropagation is the practice of rapidly multiplying stock plant material to produce many progenies plants under specific controlled environmental conditions. It is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plan breeding method. It is also used to provide sufficient plantlets from a stock plant, which does not produce seed or does not respond well to vegetative reproduction (Bhowmik & Rahman, 2020).

The plants can be induced to rapidly produce new shoots and roots with the addition of suitable hormones, when using the appropriate growing conditions for each explant type. These plantlets can also be divided, usually at the shoot stage, to produce large numbers of new plantlets. The new plants can then be placed in soil and grown in the normal manner to get a mother plant (Cardoso et al., 2020).

The tissue culture technique was applied to orchids in 1960. The tissue culture technique is highly successful to get virus-free plants. Today tissue culture is preferred for the commercial propagation of orchids. Both liquid and solid media are used for the orchid tissue culture. The explants after being isolated from the shoots are cultured in or on the desired medium under sterile conditions, offer to produce clones of a plant to mature plant (Calevo et al., 2022).

Arditti & Ernst (1993) stated that many protocols have been developed for large-scale propagation of several orchid species (including *Cymbidium, Vanda, Phaphiopedilum* and *Phalaenopsis*) through in vitro culture of various plant parts. Tissue culture is one of the most important developments in the commercial production of orchids (Prasad, 2019). Most of the orchids of commercial importance have been propagated using tissue culture through the formation of protocorm-like body (PLB) except for some recalcitrant species such as *Paphiopedilum* (Tokuhara & Mii, 2001). The proliferation of protocorms and protocorm like bodies (PLB) is often the only means of increasing the number of orchids, which produce few seeds or may not germinate well (Murdad et al., 2006).

The success in cell, tissue and organ culture technology depends on the culture medium. As no single medium will support the growth of all tissue cultures therefore modifications in the nutritional components, including growth regulators, are often necessary for different types of growth responses in a single explants' material (Álvarez et al., 2019). Various media compositions which are frequently used for plant tissue culture. A nutrient medium generally contains inorganic salts, vitamins, growth regulators, and a carbon source and gelling agent. Other components added for specific purposes include organic nitrogen compounds, amino acids, antibiotics and plant extracts (Álvarez et al., 2019).

Arditti & Ernst (1993) stated that both solid and liquid media are suitable for orchid proliferation, which is generally faster and more extensive in the latter, both shaken and stationary, except for *Paphiopedillum.* Apart from Knudson C, media used in tissue culture of orchids include Murashige and Skoog medium (MS), Nitsch and Nitsch (NN), Vacin and Went, White, Gumborg, Bergeff etc. commercially available formulations of culture media also used (Bose et al., 2002).

Gelling or solidifying agents are commonly used for preparing semi solid or solid tissue culture media (Álvarez et al., 2019). Washed or purified agar of TC grade or Difco-bacto agar grade is used in tissue culture. In static liquid

cultures the tissues or cells become submerged and die due to lack of oxygen. The gel provides a support to tissues growing under static conditions. The media which is used for tissue culture technique is very costly because of media components are costly, such as for chemicals and gelling agent. But media chemical cost is less than the gelling agent cost. Media chemicals cost less than 15 % of micro-plant production (Prakash et al., 2004). In some cases, the cost may be as low as 5 % for the medium components. Gelling agents such as agar contribute 70 % of the costs. Other ingredients in the media such as salts, sugar and growth regulators have minimal influence on production cost and are reasonably cheap. However, low-cost options are available to replace expensive gelling agents, sugars and reduce the cost of water (Calevo et al., 2022).

Crop growth, and shoot and root production are strongly influenced by the physical consistency of the growing medium. Since plant tissues and organs remain above the surface of the nutrient medium, gelling agents are usually added to the medium to increase its viscosity. Many gelling agents are used in plant growth media such as agar, "agarose" and "zelangum" and are sold under trade names such as "Phytagel, Gelrite" (Sigma Co., Merck & Co. Inc, Kelco division), increase, and "Gel-Gro" (ICA Biochemicals) (Prakash et al., 2004).

Agar is the most widely used gelling agent for the preparation of solid and semi-solid media. It contributes to the potential of the matrix, humidity and affects the availability of water and lysate in the culture vessel (Debergh, 1983). Various brands and grades of agar are available on the market, with different number of impurities and different gelling abilities. Agar brands vary greatly in terms of price, performance and composition. It is the actual use and experience that ultimately determines the choice of agar brand for a particular system and plant species. Large-scale micropropagation usually does not require the use of high-purity agar. The cheapest agar brands have been successfully used in industrial scale micropropagation (Boxus & Druart, 1986).

The lowest concentration of agar, which can be used, depends on its purity and brand. Agar is usually used at 0.6 g per 100 mL to 0.8 g per 100 mL. It is advisable to prepare sample media in small quantities using various concentrations of agar, e.g., 0.7 %, 0.75 %, 0.8 %, 0.85 % and 0.9 %. An appropriate concentration should then be used for large-scale production purposes. In addition to cost saving, there are several other advantages of using low concentrations of gelling agents. A semi-solid medium ensures adequate contact between the plant tissue and the medium. It is beneficial to growth as it allows better diffusion of medium constituents and is easily removed from plantlets before their transfer to in vivo conditions. For these reasons, a semi-solid medium is often preferred over a solid medium (Weckx et al., 2019).

Cattleyas are among the most grown orchids, and their culture is often used as the basis for comparison with other types of orchids. Like most other cultivated orchids, Cattleyas are epiphytes, or air plants. They have well-developed water-storage organs (called pseudobulbs) and large, fleshy roots. They should be potted in a porous, free-draining medium. The most used are fir bark, shredded tree-fern fiber, various types of rocks, processed coconut fiber and, lately, mixes based on peat moss and perlite. Keep out of cold, dry air while in bloom. For a long period, a Cattleya arrangement was required for any special occasion and as a result, the Cattleya has often been called the 'Queen' of Orchids. Although no longer the reigning queen of the orchid floral industry, it is difficult not to be impressed by a well-flowered Cattleya. No longer limited to white and various shades of lavender and purple, high-quality flowers are available in the entire colour scale (except true blue) and in various plant sizes. Most Cattleyas and their relatives are easy to grow. With reasonable care, they can be grown anywhere in the world (Knapp, 2021).

The use of alternative media instead of agar is profitable for lowering the cost of production of the tissue culture technique. Cheaper alternatives to agar include various types of starches and plant gums (Pierik, 1991, Mohapatra & Batra, 2017). The National Research Development Corporation, India (NRDC, 2002) has listed low-cost agar alternatives, which are worth evaluating for routine use in commercial micropropagation. Gelrite can be replaced with starch-Gelrite

mixture (Kodym & Zapata, 2001). The use of liquid media eliminates the need of agar. Other options include wheat flour, laundry starch, semolina flour, potato starch, rice flour, and sago. For micropropagation of ginger and turmeric, the combination of certain gelling agents gave growth as good as on agar-based media. The use of laundry starch, potato starch and semolina in 2:1:1 ratio reduced the cost of the gelling agent by 70 % to 82 % (Ahloowalia et al., 2004). However, the addition of such gelling agents to the medium also has some disadvantages. Some gelling agents contain inhibitory substances that hinder morphogenesis. An appropriate concentration of agar should be used for large-scale production. To lower the cost of production in large-scale alternative gelling agents can be used. Sometimes it is more advantageous than using expensive agar. Shoot proliferation is better on corn starch-medium than on agar, further the cost of corn starch was 1.8 dollars per kilogram compared with 200 dollars per kilogram of agar (Knapp, 2021).

When practicing the tissue culture technology in Asian countries, people are facing to various problems. It needs special equipment and materials. When considering the costs of media, chemicals, gelling agent, and water, this is the continuance cost of tissue culture. In some cases, the cost may be as low as 5 % for the medium components. Gelling agents such as agar contribute 70 % of the costs. Other components which are used for tissue culture techniques have minimum influence on the cost of production since they are very cheap. Plantlet producers must reduce the cost of gelling agents and water. With the development of tissue culture techniques, there are some ways to reach low-cost alternative medium components (Prabowo et al., 2021).

Agar is the most widely used gelling agent for the preparation of solid and semi-solid media. It contributes to matrix potential, moisture and affects the availability of water and lysate in the culture vessel. (Prabowo et al., 2021). In this study our focus on sago, semolina, corn flour, wheat flour, laundry starch, rice powder, potato powder can be used as alternatives for agar. This study is focused on, to evaluate the effects of alternative media components on the growth and development of *Cattleya* seedlings.

Material and Methods

Location

The experiment was conducted at the Tissue Culture Research Laboratory of the Floriculture Research Unit of the Royal Botanical Gardens in Peradeniya.

Cleaning and sterilization of glassware and metal tools

All the glassware, including culture bottles, Petri dishes, and conical flasks, beakers, reagent bottles, measuring cylinders and pipettes, were washed thoroughly using water and detergent solution (Teepol) and finally rinsed with distilled water. Metal tools (Scalpers and Forceps) and petri dishes were covered with papers and were sealed in cellophane bags. Then, all the glassware and metal tools were sterilized using an autoclave at 121 °C and 1.06 kg/cm^2 pressure (wet heat) for 20 min Thoroughly cleaned culture bottles were sterilized using an oven under 160 °C for 2 h.

The sterilized tools were removed from their wrapping, dipped in pure ethyl alcohol, and exposed to the heat of a flame. After each step instrument which was used previously was dipped again in ethyl alcohol and reframed. The laminar flow cabinet was switched on 20 min before the start of culture and surface sterilized with absolute alcohol.

Preparation of stock solution

Murashige and Skoog (MS) media and Knudson C (KNC) media were used in the experiments. The exact amount of chemicals required to prepare the stock solutions (Table 1 and 2) were weighted correctly with analytical balance and dissolved in de-ionized doubled distilled water while stirring. After the chemicals were dissolved well, their volume was adjusted to the correct volume and stored at 4 °C.

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Table 1: Required chemical amounts needed to prepare one liter stock solutions of MS media and concentrations of the stock solutions**.**

Stock solution	Included chemicals	Chemical amount per 1L of media (mg)	Required chemical amount (g)	Concentration of stock solution (mL/L)
MS A (10*concentration)	NH_4NO_3	1650	16.50	100
	KNO_3	1900	19.00	
MS B (10*concentration)	$CaCl_2$	440	4.4	100
MS C (10*concentration)	$MgSO_4.7H_2O$	370	3.7	100
	KH_2PO_4	170	1.7	
MS Micro (100*concentration)	H_3BO_3	6.2	0.62	10
	$MnSO_4.4H_2O$	22.3	2.23	
	$ZnSO_4.7H_2O$	8.6	0.86	
	Na_2MO_4	0.25	0.025	
	$CuSO_4.5H_2O$	0.025	0.0025	
	$CoCl_2.6H_2O$	0.025	0.0025	
	KI	0.083	0.083	
MS IS (100*concentration)	$FeSO_4.7H_2O$	27.8	2.78	
	EDTA	37.3	3.73	10
MS Vitamin (10*concentration)	Nicotinic acid	0.5	0.005	100
	Glycine	2	0.02	
	Thiamine	0.1	0.001	
	Pyridoxine	0.5	0.005	
	Inositol	100	1	
Kinetin				100 mg/100 mL
2,4-D				100 mg/100 mL

Table 2: Required chemical amounts needed to prepare one liter stock solutions of KNC media and concentrations of the stock solutions**.**

Stock solution	Included chemicals	Chemical amount per 1 L of media (mg)	Required chemical amount (g)	Concentration of stock solution (mL/L)
KNC A (10*concentration)	NH_4NO_3	1650	16.5	100
KNC B (10*concentration)	$(NH_4)_2 SO_4$	500	5	100
	$MgSO_4.7H_2O$	370	3.7	100
	$MnSO_4.4H_2O$	0.25	0.0025	100
KNC C (10*concentration)	KH_2PO_4	250	2.5	100
KNC IS (100*concentration)	$FeSO_4.7H_2O$	27.8	2.78	10
	Na_2 EDTA	37.3	3.73	10
Sucrose		20 g		
Gelling agent		8 g		
Coconut water		250 mL		
Banana		75 g		

Effect of different gelling agents on seedling growth of Cattleya plants in KNC

Preparation of media

KNC media was prepared as basal media. The medium was solidified while performing a control experiment on 7 g/L agar using different gelling agents at different concentrations to solidify the culture media (Table 3). Distilled water was used as water source. pH was adjusted to the range of 5.6 to 5.8. Solution was heated by using gas cooker, and when the nutrient solution was boiling, gelling agent was dissolved by thoroughly stirring. The media was poured into sterilized empty jam bottles covered by cellophane film. 45 mL of media was poured into each bottle, and media height was about 1.5 cm. Finally, the media was autoclaved under 121 °C and 1.5 kg/cm^2 for 20 min.

Table 3. Different gelling agents and their weight for treatments in KNC media

Treatment	Agar (g)	Sago (g)	Semolina (g)	Corn flour (g)
T1	7			
T2		120		
T3			90	
T4				90
T5	3.5	60		
T6	3.5		45	
T7	3.5			45

Establishment of seedlings in culture media

Using sterilized tools under a laminar flow cabinet mature old, leaves of two-month-old *invitro*-cultured seedlings were cut and removed. Seedlings were established on different gelling agent media bottles using forceps. Four seedlings were established in one jam bottle. The culture bottles were sealed well with cellophane films and maintained in an air-conditioned growth room at 26 °C ± 2 °C, 25 % RH and under fluorescent illumination with 16 h photoperiod. After about 4 weeks (after the plants were well established on the medium showing a better growth) the established plants were transferred to the same medium. They were also maintained under the same conditions as described above.

Experimental design, observation, and data collection

The experiment was arranged as a RCBD (Completely Randomized Block Design) with 10 replicates. Observation and data collection were performed scientifically accordingly.

Statistical analysis

The data was analyzed by ANOVA followed by Duncan's Multiple Range Test (DMRT) using SAS 6.12 statistical software.

Results and Discussion

Effect of different gelling agent on growth parameters of Cattleya seedlings in MS media after three months

When using sago as a gelling agent, there was no significant difference in growth parameters compared with the control (agar) (Table 4). Then the contamination occurred in sago medium was 13 % (Figure 1). Semolina treated medium had not significant effect on the growth parameters, except for leaf length when compared to the control. Leaf length showed the lowest significant difference compared with agar medium (Table 4). Also, the contamination percentage is quite higher than in other medium. A 20 % of contamination occurred in semolina medium (Figure 1). Corn flour treated medium had not significant effect on growth parameters, except for the number of leaves and leaf length when compared with the agar medium. The number of leaves and the leaf length showed the lowest significant differences compared with the control (Table 4). The contamination percentage was 12 % in corn flour medium (Figure 1). Semolina and agar medium had not significant effect on all growth parameters, when compared with the control (Table 4). The contamination percentage was13 % (Figure 2). The mixture of corn flour and agar as gelling agent also had not significant effect on the growth parameters when compared with the control, except for number of leaves. The number of leaves provoked a contamination of 11 % in this medium (Figure 2).

In this study it was also assessed the cost of the media, which is used for the propagation of Cattleya seedlings. Semolina showed a huge profit somewhat 87 % that is way forward compared with the other gelling agents (Table 5). To reduce the cost, it was combined semolina with agar, this also showed a 50 % profitable with less contamination (Table 6). Using sago as the substitute for agar helped to the Cattleya plant propagation and contamination decrease (Table 5 and 6).

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Effect of different gelling agent on growth parameters of Cattleya seedlings in KNC media after three months

When using sago as a gelling agent, there was no significant difference in growth parameters compared with the control (agar). The contamination occurred in sago medium was 7 %. Semolina treated medium did not significantly affect the growth parameters compared with the control. However, the contamination percentage was considerable higher than other media (Table 7). Corn flour treated medium did not significantly affect the growth parameters compared with the control, and its contamination percentage was 7 %. Sago + agar medium did not significantly affect the growth parameters compared with the control (Figure 3). Figure 4 shows that the highest contamination percentage (20 %) is obtained in Semolina, and the. lowest (5 %) was observed in Sago. The contamination rate is lower even in Sago with Agar and Semolina with Agar, which is 5 % (Figure 4).

Table 4. Effect of different gelling agent on growth parameters in MS media

Treatment	No of root	No of leaves	Leaf length (mm)	Leaf width (mm)
Sago	4.81	5.125	12.25	7.15
Semolina	4.625	4.55	11.675***	7.575
Corn flour	4.395	4.175***	11.725***	6.375
Agar	4.975	5.1	16.1	7.325
Semolina + Agar	5.775	5.125	14.775	8.265
Corn flour + Agar	4.85	4.225***	14.775	7.2

Comparisons significant at the 0.05 level are indicated by ***

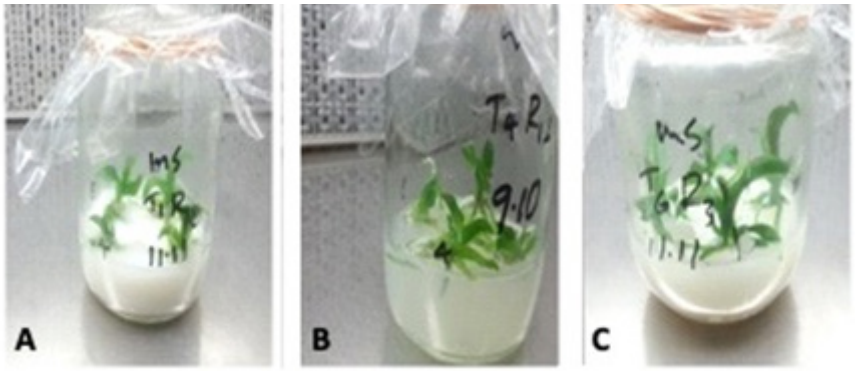

Figure 1. Growth of Cattleya seedlings in different mediums of MS (A-Sago-Agar, C-Semolina +Agar mediums)

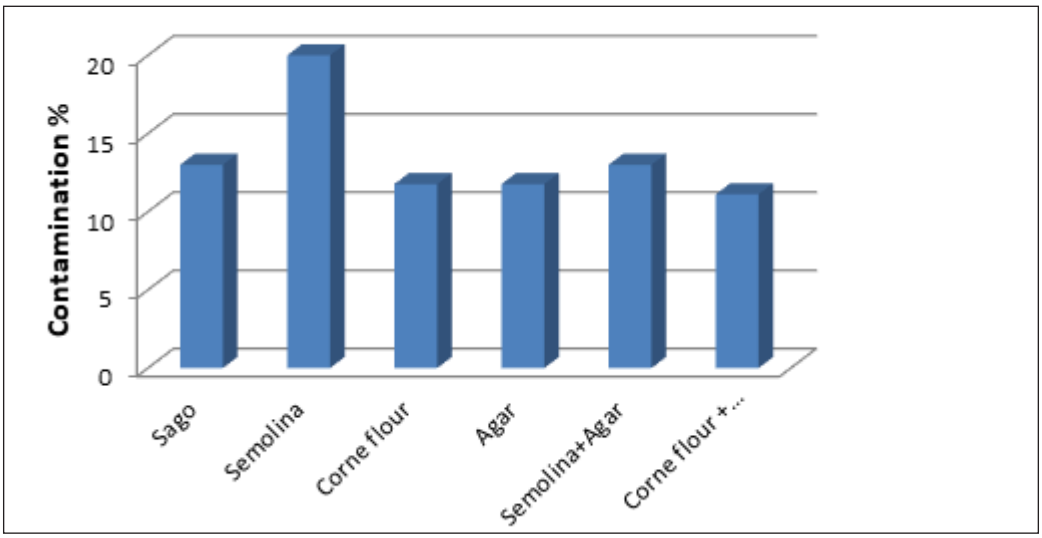

Figure 2. Contamination percentage of different gelling agent on MS media

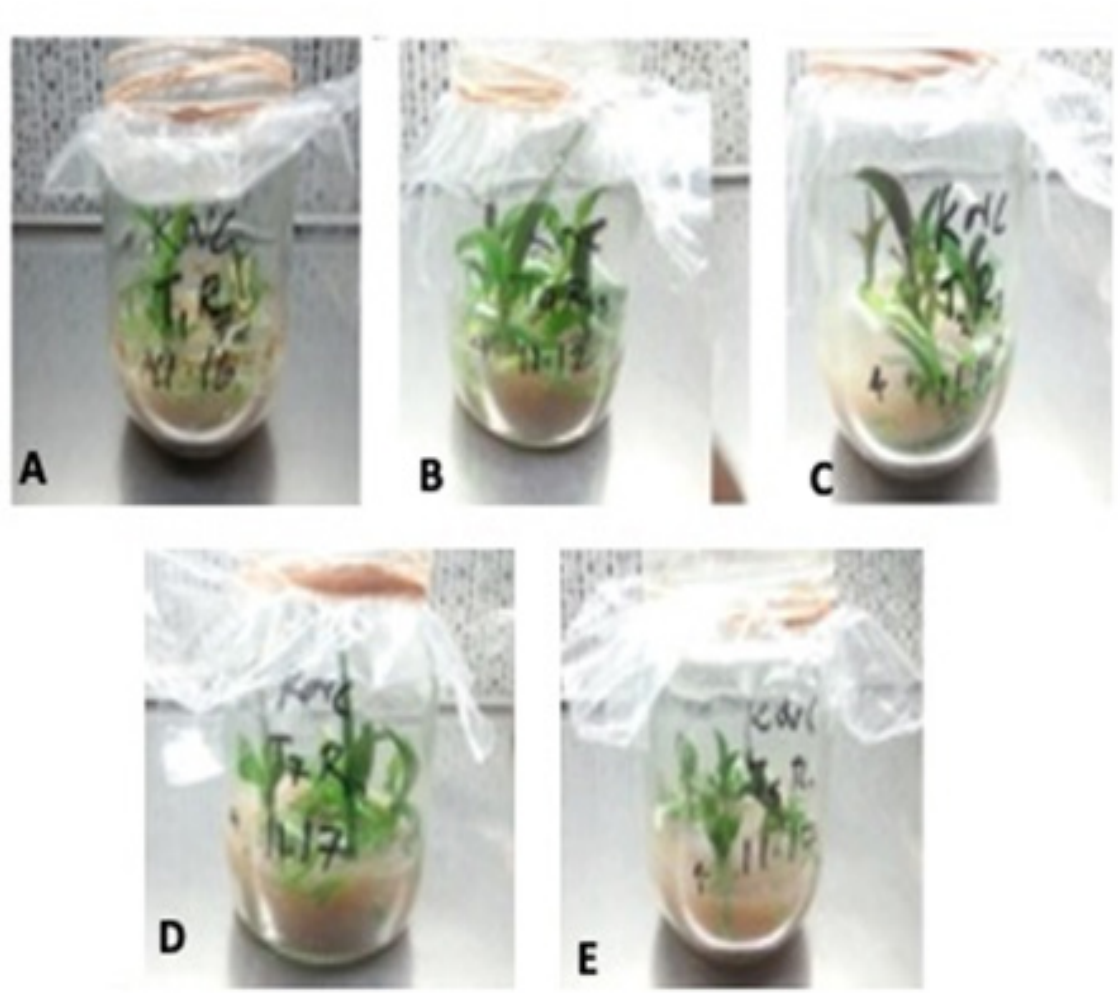

Figure 3. Growth of Cattleya seedlings in different mediums of KNC A-Sago, B-Semolina, C-Corn flour, D-Agar, E-KNC control media

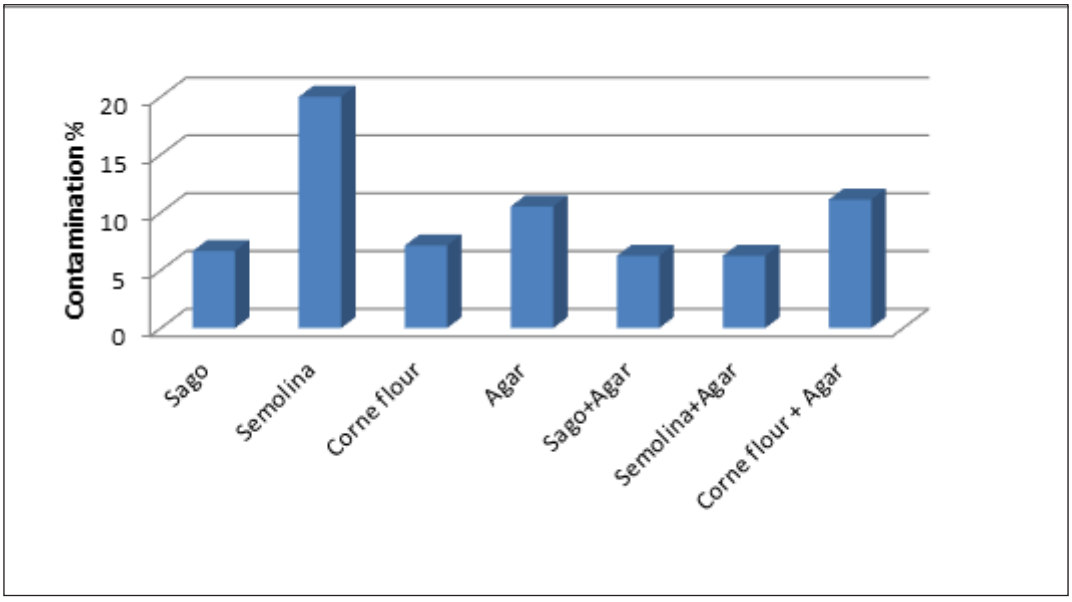

Figure 4 - Contamination percentage of different gelling agents in KNC medium

Table 5. Cost reduction in MS media for different gelling agent

Treatment	g/L	L/kg	Wt. of Agar need to produce 142.8 L of media (kg)	Price/kg (Rs)	Total cost (Rs)	Profit (Rs)	Profit %
Sago	120	8.3	17.2	300.00	5161.40	14839.00	74 %
Semolina	90	11.1	12.9	200.00	2580.00	17420.00	87 %
Corn flour	90	11.1	12.9	450.00	5805.00	14195.00	70 %
Agar	7	142.8	1	20 000.00	20 000.00	0.00	0 %

Table 6. Cost reduction in MS media combination of different gelling agent

Treatment	g/L	Price/L (Rs)	price of 142.8 L (Rs)	Profit (Rs)	profit %
Semolina + Agar	45+3.5	79.00	9996.00	10004.00	50
Corn flour + Agar	45+3.5	90.00	12887.00	7113.00	35

Table 7. Effect of different gelling agent on growth parameters in KNC media

Treatment	No. of root	No. of leaves	Leaf length (mm)	Leaf length (mm)
Sago	5.18	4.90	18.13	6.80
Semolina	4.70	4.90	18.33	8.05
Corn flour	5.23	4.50	15.73	9.08
Agar	4.78	4.43	17.97	8.48
Sago + Agar	3.93	4.28	15.30	8.55
Semolina + Agar	3.88	4.33	16.98	8.43
Corn flour + Agar	4.15	4.35	17.00	9.15

Comparisons significant at the 0.05 level are indicated by ***

Discussion

Worldwide, people are really enthusiastic about micropropagation and face a great difficulty in reducing the cost of production. In Africa sweet potato (*Ipomoea batatas* Lam) is the second most important root crop after cassava. Ogero et al. (2012) has focused on reducing the cost of sweet potato tissue culture nutrients by using affordable alternative nutrient sources (Kodym et al., 2012). Authors developed a new medium with conventional sources of Murashige and Skoog (MS), and salts were substituted with Easygro® vegetative fertilizer containing both macro and micronutrients. Low-cost banana tissue culture was performed by substituting the following ingredients such as normal tap water, table sugar, agar is replaced by isabgol in MS medium.

Norhayati et al. (2011) used four kinds of commercial starch or flour as alternative gelling agents and coconut water as an organic additive in the culture medium has reduced the cost of the medium and there is no significant growth difference compared with the original gelling agents. Use to the low cost and availability of low-cost resources, tapioca was used instead of agar, and sucrose was replaced with sugar cane (George & Manuel, 2013). Calcium ammonium nitrate, single superphosphate Muriate of potash and sugar cane were used as low-cost media in place of MS salts. The plants produced using LC media were consistently better for shoot and proliferation.

It was found an alternative gelling agent. Sago, and semolina + agar showed higher performance and low contamination percentage as an alternative gelling agent in the MS media. Sago, corn flour, sago + agar, semolina + agar and corn flour + agar showed higher performances and low contamination percentage in KNC media.

Conclusions

It has been highlighted that time and again the long-term agriculture and forestry need to be sustainable. They use little or no crop-protection chemicals, have low energy inputs and yet

maintain high yields, while producing high-quality material. Biotechnology-assisted plant breeding is an essential step to achieve these goals. Plant tissue culture techniques have a vast potential of producing plants of superior quality, but this potential has not been fully exploited in the developing countries. According to the observed results, it can be concluded that agar can be replaced with sago, or agar + semolina as gelling agent in MS and KNC medium.

Author contributions

Elaboration and execution, development of methodology, conception and design; editing of articles and supervision of the study have involved all authors.

Conflicts of interest

The signing authors of this research work declare that they have no potential conflict of personal or economic interest with other people or organizations that could unduly influence this manuscript.

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