

Low-Cost Gelling Agent in Tissue Culture Technology for the Orchid Regeneration

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ABSTRACT

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

Keywords: alternative gelling agent, alternative water source, Orchid subculture

INTRODUCTION

Micropropagation is the practice of rapidly multiplying stock plant material to produce many progenies plant under specific control environment condition. It is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding method. It is also used to provide enough plantlets from a stock plant which does not produce seeds or does not respond well to vegetative reproduction. Using the appropriate growing conditions for each explants type, plants can be induced to rapidly produce new shoots and new roots with the addition of suitable hormones. 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.

A Tissue culture was used for the first time in 1960 (Arditti and Ernst, 1993). Tissue culture technique is highly successful to get virus free plants. Today tissue culture is preferred for 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.

Arditti and Ernst (1993) stated that many protocols have been developed for large- scale propagations of several orchid species (including *Cymbidium*, *Vanda*, *Paphiopedilum* 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 and Mii, 2001). Proliferation of 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 component including growth regulators are often necessary for different types of growth responses in a single explants' material (Álvarez *et al.*, 2019). There are various media compositions which are frequently used for plant tissue culture. A nutrient medium generally contains inorganic salts, vitamins, growth regulators, 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 and 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.

Gel provides a support to tissues growing in static conditions. The media which is used for tissue culture technique is expensive because of media components are costly, such as for chemicals and gelling agent. However, media chemical cost is cheaper than the gelling agent. 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. The gelling agents such as agar contribute 70% of the costs.

Other ingredients in the media - 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.

The growth of cultures and production of shoots or roots is strongly influenced by the physical consistency of the culture medium. Gelling agents are usually added to the culture medium to increase its viscosity because of which plant tissues and organs remain above the surface of the nutrient medium. Many gelling agents are used for plant culture media, e.g., agar, 'Agarose', and 'Gellan gum', and are marketed under trade names such as 'Phytigel, Gelrite' (Sigma Co., Merck & Co. Inc, Kelco division), and 'Gel-Gro' (ICA Biochemicals).

Agar is the most used gelling agent for preparation of solid and semi-solid media. It contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers (Debergh, 1983).

Various brands and grades of agar are available commercially, which differ in the amounts of impurities, and gelling capacity. Agar brands vary widely in price, performance, and

composition. It is the actual use and experience, which ultimately determines the choice of agar brand in a specific system and for a plant species. It is usually unnecessary to use high purity agar for large-scale micropropagation; cheaper brand of agar have been successfully used for industrial scale micropropagation (Boxus and Druart, 1986).

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 rock, 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 a requirement for any special occasion and as a result the *Cattleya* has often been called the Queen of Orchids. While 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 a wide range of plant sizes. Most *Cattleyas* and their relatives are easy to grow. With reasonable care, they can be grown anywhere in the world. The use of alternative media instead of agar is profitable to lower the cost of production of tissue culture technique. Cheaper alternatives to agar include various types of starches and plant gums (Pierik, 1989, Mohapatra and Batra, 2001). 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 and Zapata, 2001). The use of liquid media eliminates the need of agar. Other options include wheat flour, laundry starch, semolina, potato starch, rice powder 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 a ratio of 2:1:1 reduced the cost of gelling agent by 70-82% (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. 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. The shoot proliferation is better on corn starch-medium than on agar. The cost of Corn starch was \$1.8/kg compared with \$200/kg of agar. Find out an alternative gelling agent/combination of gelling agents to replace agar. When practicing the tissue culture technology in Asian countries, people are facing various problems. It needs special equipment and materials. When consider about cost of media, chemicals, gelling agent, and water, these are the continuance cost of tissue culture. In some cases, the cost may be as low as 5% for the medium components. The gelling agents such as agar contribute 70% of the costs. Other components which are used for tissue culture techniques are having minimum influence on the cost of production since they are very cheap. Plantlet producers must reduce the cost for gelling agents and water. With the development of tissue culture techniques there are some pathways to reach low-cost alternative medium components. Agar is the most used gelling agent for preparation of solid and semi-solid media. It contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers. In this study our focus on sago, semolina, corn flour, wheat flour, laundry starch, rice powder and potato powder can be used as alternatives for agar. This study was focused on evaluating the effect of alternative media components on growth and development of *Cattleya* seedlings.

MATERIALS AND METHODS

The experiments were conducted at the Tissue Culture Laboratory, Floriculture Research Unit, Royal Botanical Gardens, Peradeniya. 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 autoclave with 121°C temperature and 1.06 kg/cm² pressure (wet heat) for 20 minutes. Thoroughly cleaned culture bottles were sterilized using oven under 160°C temperature for 2 hours.

Table 1. Required chemical amounts, need to prepare one 1-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 ₄ NO 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 ₂ O	370	3.7	100
	KH ₂ PO ₄	170	1.7	
MS Micro (100*concentration)	H ₃ BO ₃	6.2	0.62	10
	MnSO ₄ .4H ₂ O	22.3	2.23	
	ZnSO ₄ .7 H ₂ O	8.6	0.86	
	Na ₂ MO ₄	0.25	0.025	
	CuSO ₄ .5 H ₂ O	0.025	0.0025	
	CoCl ₂ .6 H ₂ O	0.025	0.0025	
	KI	0.083	0.083	
MS IS (100*concentration)	FeSO ₄ .7H ₂ O	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				100mg/100ml
2,4-D				100mg/100ml

The tools that have been sterilized were removed from their wrapping, dipped in 100% 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 minutes before start culturing and surface sterilized with 100% alcohol.

Murashige and Skoog (MS) media was used in all the experiments. Exact amounts of chemicals which required preparing the stock solutions (Table 1) 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 up to correct volume and stored in the refrigerator (4°C). Distilled water was used as water source. pH was adjusted to the range of 5.6-5.80. Solution was heated by using gas cooker. 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. 45ml of media was

poured into each bottle, and media height was about 1.5cm. Finally, the media was autoclaved under 121°C and 1.5kg/cm² for 20 minute.

Using sterilized tools under a laminar flow cabinet mature old leaf of two-month-old *in vitro* cultured seedlings were cut and removed. Seedlings were established on different gelling agent media bottles by 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 ± 2°C, 25 % RH and under fluorescent illumination with 16-hour photoperiod. After about 4 weeks (after the plants were well established on the medium showing a better growth) the established plants were transferred to a same media. They were also maintained under the same conditions as described above.

The experiment was arranged as a RCBD (Randomized complete block design) with 10 replicates. Observations and data collection were done accordingly.

Data were analyzed by using SAS computer package to check whether significant differences exist between treatments by ANOVA procedure at 5% probability level. Mean separation was done using Dunnett's test.

RESULTS AND DISCUSSIONS

When using sago as a gelling agent there was no significant difference in growth parameters compared with control (agar) (Table 2). Then contamination occurred in sago medium was 13% (Figure 2). Semolina treated medium was not significantly affected on the growth parameters, except leaf length when comparing with control. Leaf length showed the lowest significant difference compared with agar medium (Table 2). Also, the contamination percentage was quite higher than in other medium. It was 20% contamination occurred in semolina medium (Figure 2). Corn flour treated medium was not significantly affected on growth parameters except number of leaf & leaf length, when compared with agar medium. Number of leaf and leaf length showed lowest significant difference compared with agar medium (Table 2). The contamination percentage was 12% in corn flour medium (Figure 1). Semolina and agar medium were not significantly affected on all growth parameters, when compared with control (Table 2). The contamination percentage was 13 % (Figure 2). Corn flour and agar mixture of gelling agent also not significantly affected on growth parameters except for number of leaves compared with control. Number of leaves showed lowest significant difference compared with control (Table 2), while making the contamination percentage was 11% in this medium (Figure 2).

In this study we also assess the cost of the media which was used for propagation of *Cattleya* seedlings. Semolina shows a huge profit somewhat 87% which is way forward compared with the other gelling agent (Table 3). To reduce the cost, we combined Semolina with the Agar, variant which also shows a 50% profitable with less contamination (Data not shown).

Table 2. Effect of different gelling agent on growth parameters in MS media

Treatment	No of roots	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 - Control	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 **

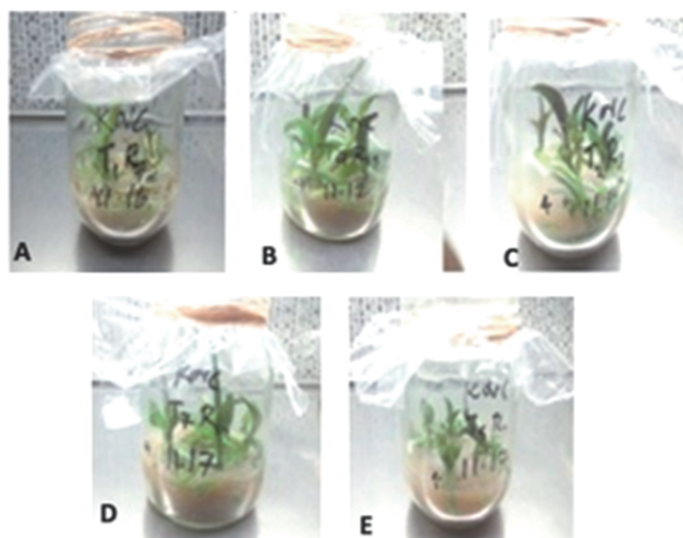


Figure 1. Growth of *Cattleya* seedlings in different mediums of MS A-Sago, B-Semolina, C-Corn flour, D-Agar, E-KNC control media

Table 3. 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 (Euro)	Total cost (Euro)	Profit (Euro)	Profit %
Sago	120	8.3	17.2	0.86	14.77	42.48	74
Semolina	90	11.1	12.9	0.57	7.39	49.87	87
Corn flour	90	11.1	12.9	1.29	16.62	40.63	70
Agar	7	142.8	1	57.25	57.25	0.00	0

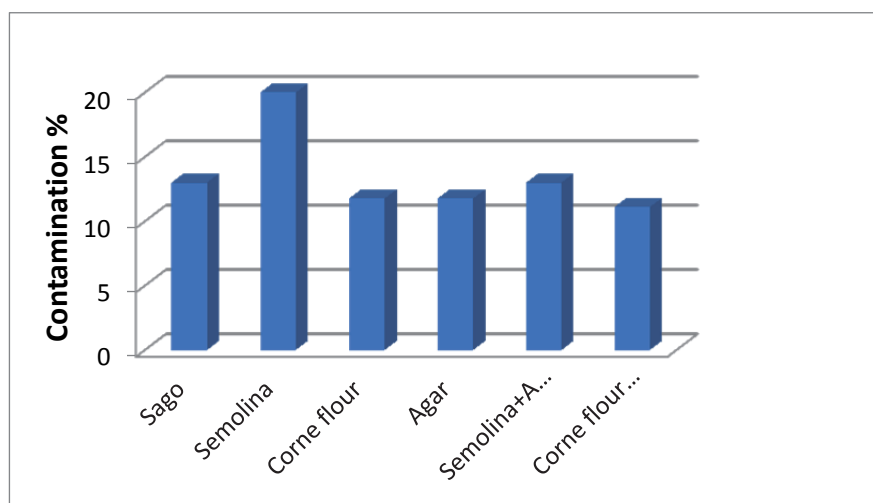


Figure 2. Contamination percentage of different gelling agent on MS media

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

Norhayati *et al.*, (2011) used four kinds of commercial starch or flour as alternative for 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 to the original gelling agents. In low-cost media, tapioca was used as substitute of agar and replacing sucrose with sugar cane, because of low cost and easy availability (George and Manuel, 2013). Calcium ammonium nitrate, Single super phosphate 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 shoots and proliferation.

CONCLUSIONS

It has been stressed time and again that in the long-term agriculture and forestry need to be sustainable, using 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 to produce plants of superior quality, but this potential has not been fully exploited in the developing countries. According to the results it can be concluded that agar can be replaced with sago and Agar + Semolina as gelling agent in MS media and agar used in KNC media to reduce the production cost and obtain quality material. In this study we found an alternative gelling agent. Sago and semolina + agar showed higher performance and low contamination percentage as 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.

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