Integration of Low Cost Micropropagation Technique to Accelerate the Breeding Program of Tea (*Camellia sinensis* L. O. Kuntze)

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ABSTRACT

Crop improvement program of tea [*Camellia sinensis* (L.) O. Kuntze] is expensive and requires nearly 25 years for releasing a new cultivar. Hence, the present study was focused on employing a cost effective micropropagation technique as a supplementary tool to reduce the cost and time taken in conventional tea breeding program. The objective was to identify locally available low cost material as an alternative to agar and to reduce time by adapting *ex-vitro* rooting technique developed earlier at Plant Breeding Division of Tea Research Institute, Talawakelle, Sri Lanka. Results revealed that among the solidifying agents tested, medium containing sago produced good quality micro-shoots comparable to agar and thus sago could be used as a substitute to agar in the culture medium. Micro-shoots could successfully rooted and transferred to nursery and subsequently to the field by growing on *ex-vitro* rooting medium containing coir dust: top soil: sand in the ratio of 1:1:1. Furthermore, it was able to cut down the time period taken for rooting and acclimatization as both the stages were accomplished simultaneously thereby, saving the cost involved in maintaining plantlets under controlled environment. Cost analysis of the proposed protocol showed that the cost of production of a micropropagated plant can be reduced to Rs. 11.00 compared to Rs. 39.00 per plant in the previously developed micropropagation protocol and Rs. 23.00 per plant in the conventional nursery propagation method. The proposed protocol can be effectively integrated into the conventional tea breeding program to accelerate the release of new tea cultivars to the growers.

Key words: Alternative solidifying agents, *ex-vitro* rooting, low cost options, micropropagation, tea, tissue culture

INTRODUCTION

Tea is a perennial plantation crop with high economic value and the most widely consumed beverage in the world. As tea plant is a woody-perennial, it requires 20-25 years to develop new tea cultivars. This imposes constraints in catering to the needs of the growers to achieve their expectations in time. *In-vitro* culture techniques including micropropagation have a vast potential in supplementing conventional breeding programs. Use of *in-vitro* techniques could possibly cut down the number of years required for tea crop improvement and therefore, has a tremendous potential in accelerating the breeding program.

Although an efficient micropropagation method has been developed at the Tea Research Institute of Sri Lanka, (Arulpragasam and Lattif 1986; Sarathchandra *et al.,* 1997; Gunasekare and Evans 2000), its application in mass propagation is cost prohibitive due to high cost involved in obtaining micropropagated tea plants. Hence, this had prevented integrating this technology to supplement the on-going conventional breeding program. At present, the nutrient medium consists of purified agar, analytical grade sucrose and plant cell cultures tested chemicals (ingredients of MS basal medium and growth regulators). *In-vitro* cultures are maintained in artificially lit, temperature and photoperiod controlled culture room which consumed high amount of energy. Therefore, it has become important to work out a cost-effective micropropagation protocol for tea, if this technology to be integrated successfully into the on-going tea breeding and improvement program. Thus the objective of the present study was to identify locally available low cost materials as an alternative to agar and to adapt *ex-vitro* rooting technique to reduce the cost involved in the standard micropropagation protocol.

MATERIALS AND METHODS

Materials

Hybrid seeds from the cross between TRI 3013 x DT 95 were generated through the annual controlled hybridization program conducted by the Plant Breeding Division of the Tea Research Institute of Sri Lanka was used as the starting materials to derive the explants.

Preparation of plant material for inoculation

Seeds were sterilized with 20% (v/v) Clorox two drops of Tween 20 for 10 minutes. Then Clorox solution was decant and seeds were washed two to three times with sterile distilled water. The same procedure was repeated once without Tween 20, thoroughly washed with sterile distilled water and embryos were excised under sterile conditions in side a laminar flow cabinet.

Effect of alternative low cost materials as a substitute to agar on shoot multiplication

Embryos excised were inoculated on MS basal medium (Murashige and Skoog, 1969) supplemented with 3 mg/ L BAP, 0.5 mg/ L IBA and 30 g/ L sucrose (Gunasekare and Evans 2000). As a gelling/solidifying agent, ten alternative locally available low cost gelling agents as a substitute for agar was compared against agar at 0.8% (W/V) as a control treatment (Table 1). Medium pH was adjusted to 5.8 before autoclaving. Ten embryos were inoculated per treatment. The cultures were incubated at 25 ^{0}C and with 16 hour photoperiod provided with light intensity of 1000 Lux using fluorescent light.

Micro-shoots regenerated from the embryos were sub-cultured on to the same but fresh medium. Sub-culturing was done at 2 month intervals for a period of six month and during the sub-culturing; fresh weights of shoots and number of sub-cultured micro-shoots were recorded. Experiments were laid out as a Completly Randomized Design, each treatment was replicated ten times and experiment was repeated twice.

***Ex-vitro* rooting**

Micro-shoots (2 month-old shoots from 4th subculture) of uniform size (approximately 4 cm in length) were transferred to *ex-vitro* rooting medium consisted of top soil: and: coir dust in 1:1:1 ratio (Ranaweera, 2011) to reduce the cost of *in-vitro* rooting and acclimatization. Basal end of micro-shoots were dipped in 50 mg/ L IBA for 3 hours as a pulse treatment before transferring into clay pots containing the rooting substrates and maintained in a walk-in type sealed propagator (RH-95% and temperature 30$\pm$1 ^{0}C) in the plant breeding nursery. Watering was done when necessary using a sprinkler system. After five days of transferring the micro-shoots, 10 g/ 10 L dosage of fungicide (Rhydomil) was applied to micro-shoots as foliar spray to control fungal diseases. Experiment was laid out in a completly randomized design and experiment was repeated twice. Same day conventional tea nursery block was established using cuttings from same age, uniform and known cultivar as a control. Five plants of each treatment were taken as destructive sample. Rooting performance of micro-shoots was assessed 2 months after planting by counting number of roots and measuring root lengths and fresh/ dry weights of roots (Table 2).

Nursery and field performance of micropropagated plants

Rooted micro-shoots were transferred to the normal nursery polythene bags filled with soil and performance of plants were evaluated over a period of six months by assessing number of leaves, number of branches per plantlet and height compared with plants of the same age obtained through conventional vegetative propagation methods. After nursery evaluation plants were planted in the field at Tea Research Institute of Sri Lanka, Talawakelle and field performances were evaluated at six months for number of leaves, number of branches and stem height compared with plants of the same aged conventionally propagated plants.

Cost-benefit analysis

Cost of production of 1000 plants were calculated for 3 different methods *viz.* (i) Normal micropropagation procedure combined with *in-vitro* rooting (Gunasekare and Evens, 2000), (ii) Low cost micropropagation with sago as a gelling agent and *ex-vitro* rooting step (iii) Conventional nursery vegetative propagation technique. Cost calculation of normal micropropagation procedure consisted of 4 main steps: (i) cost of preparation of culture medium, (ii) cost of sub-culturing, (iii) cost of *in-vitro* rooting and (iv) cost of acclimatization. The steps (iii) and (iv) in the conventional micropropagation technique were combined in the protocol developed in the present study and culture medium components were replaced by low cost materials to reduce the cost of production further.

RESULTS

Alternative low cost materials as a substitute to agar

Plant regeneration, from the zygotic embryos isolated from the hybrid seeds, started 2 weeks after culture initiation on agar and ten alternative low cost medium supplemented with 3 mg/ L BAP and 0.5 mg/ L IBA. Comparatively higher shoot growth and rate of multiplication at each sub culture were observed in media containing coir dust, sago, kitul flour, half burnt rice husk and agar (Table 1). Shoots with dark green leaves and healthy appearance were also observed in above treatments and poor growth and pale colour leaves were observed in media containing refuse tea, cassava flour, and rice polish. Micro shoots on remaining treatments were observed having moderate growth. Significantly higher multiplication rate of micro-shoots was observed in the medium containing sago ($P<0.0004$) as compared to agar containing medium (Table 1 and Figure 1). Furthermore, kitul flour, half burnt rice husks were found as moderately suitable for shoot multiplication.

Table 1. Influence of different medium components (agar substitutes) on growth and multiplication of micro-shoots at the time of sub-culture in comparison to agar

Medium component	Percentage increase of weight per sub culture	Mean number of micro-shoots per sub culture
Coir Dust	30.16 BCD	1.77 ABC
Sand	21.67 CD	1.16 BC
Sand+Coir Dust	27.35 BCD	1.54 BC
Refuse Tea	15.37 EF	1.00 C
Sago (150 g/ L)	66.45 A	2.10 A
Cassava Flour (140 g/ L)	30.47 F	1.00 C
Rice Polish	5.80 F	1.20 BC
Compost	33.35 DE	1.30 BC
Kitul Flour (*Caryota urens*) (140 g/ L)	52.96 AB	1.56 ABC
Half burnt rice Husk	47.0 CD	1.70 AB
Agar (Control) 8g/ L	53.82 ABC	1.54 BC

Values followed by the same letters are not significantly different -($P<0.05$)

Figure 1. a) Micro-shoots growing on medium containing agar substitutes
b) Shoot multiplication in medium with sago

***Ex-vitro* rooting**

In agreement with the results obtained in an earlier study (Ranaweera, 2011), micro-shoots obtained from *in-vitro* medium containing sago as the gelling agent could successfully be rooted on *ex-vitro* rooting medium consisting of coir dust: top soil: sand at the ratio of 1:1:1. At two months after planting, 100% survival was observed for micro-shoots grown on this medium (Figure 2).

Figure 2. Profusely growing rooting system in *ex-vitro* rooting substrate

Nursery and field performance of micropropagated plants

At the end of the nursery period and at the field, growth performances of micropropagated and conventionally propagated plants were found to be significantly different ($P<0.01$) and in general micropropagated plants performed well compared to conventionally propagated plants. Micropropagated plants produced taller plants with more branches and leaves than that of conventionally propagated plants (Ranaweera, 2011). However, rooting performances (root lengths, root number and fresh and dry weight of roots) were found to be not significantly different between micropropagated and conventionally propagated plants after 8 months in the nursery (Ranaweera, 2011) (Figure 3).

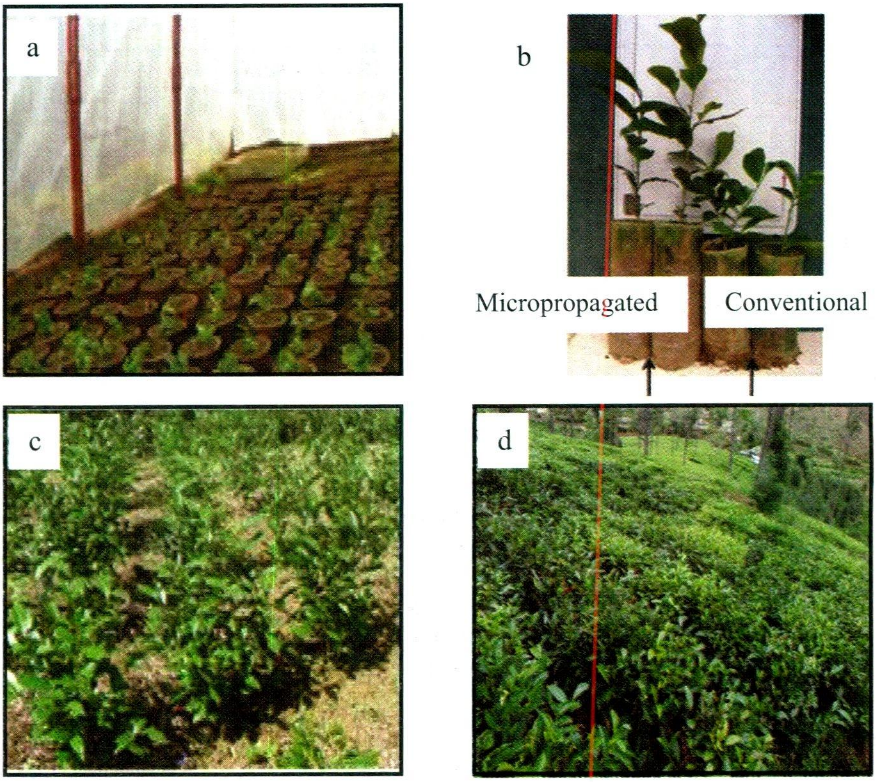

Figure 3. a) *Ex-vitro* rooting
b) Plants produced from micro-shoots and conventional method at nursery stage
c) Field performance of six months old tissue cultured plants
d) Tissue culture plants in the harvesting stage

Cost-benefit analysis

According to the cost calculation of the standard micropropagation protocol, where plants were produced through *in-vitro* shoot multiplication followed by *in-vitro* rooting and acclimatization as described by Gunasekare and Evens, (2000), the cost of production per plant was around Rs. 39.00. In that, the cost involvement of labour, supplies, materials, equipment, and electricity were very high when compared to low cost method mainly due to adopting *in-vitro* condition in stages, shoot multiplication and rooting (Table 2). The cost of production per plants produced through *in-vitro* multiplication using low cost alternative materials followed by simultaneous *ex-vitro* rooting and acclimatization in the present method was about Rs. 11.00 (Table 2). This was nearly $\frac{1}{4}^{th}$ of the cost of production/plant of standard micropropagation protocol.

Table 2. Cost of production per 1000 plants raised through conventional tissue culture method and low cost tissue culture method

Item	Conventional tissue culture method (Rs.)		Low cost tissue culture method (Rs.)	
	Amount	%	Amount	%
Labour	8160.00	20%	2040.00	19%
Supplies	6684.58	17%	1591.70	15%
Materials	11155.00	29%	113.54	1.06%
Equipment and building	4068.48	10%	2084.25	19.4%
Building and installation	1722.80	4.42%	861.40	8%
Electricity	6021.00	15%	3405.00	32%
Maintenances of building and equipment	15.00	0.04%	7.00	0.06%
Interest @15% annual	963.63	2.47%	277.58	2.6%
Total cost/1000 plants	38916.49	-	10721.00	-
Unit cost	**39.00**	-	**11.00**	-

In the conventional nursery practices, plants are maintained for about one year period which incurred material and labour cost. This process is highly labour intensive and according to the results the highest contributory factor (55%) to the total cost was the labour whereas 22% of the total cost was for mother bush maintenance (Table 3). In this method, the cost per plant was about Rs. 23.00. If plants were produced through micropropagation technique, then nursery period is only eight months to obtain plants that are ready for field planting. Thus, protocol developed in the present study is a viable option to reduce the cost of production of propagation of tea plants while preserving the advantage of micropropagation.

Table 3. Cost involved in producing 1000 plants using conventional nursery propagation method in tea

Item	Cost (Rs.)	
	Amount	%
Labour	12650.70	55%
Supplies	664.34	2.91%
Materials	2404.34	10.53%
Transport	800.00	3.5%
Mother bush maintenance	6400.00	28%
Total cost/1000 plants	22830.00	100%
Unit cost	**Rs 23.00**	

DISCUSSION

Micropropagation technology is more expensive than the conventional method of plant propagation. The major reason is high cost of production which requires a constant monitoring of the input costs of chemicals, media, energy, labour and capital. Consumables such as media, culture containers, and electricity make a comparatively greater contribution to production costs. For example, the cost of medium preparation (energy and labour) can account for 30-35% while media chemicals cost is less than 15% of micro propagated plant production (Prakash, 1993). Of the various media components, the gelling agents such as agar contribute to 70% of the chemical cost. Other ingredients in the media such as salts, sugar and growth regulators have minimal influence on production cost and are reasonably cheap. Therefore, low cost gelling agents such as sago, casava flour and kitul flour can be used as an alternative to replace expensive gelling agents such as agar and phytagel.

Agar is a gelatinous complex polysaccharide obtained from marine algae such as *Gelidiella* and *Gracilaria* species. It does not provide any nutrient for growth of micro-shoots. However, the present study showed that a significantly higher number of shoots were produced on media gelled with sago. This may be due to the fact that sago acts as an additional carbon source and adds other ionic supplements (35% carbohydrates, 1% mineral matter) (Bhattacharya *et al.,* 1994) to the medium which most probably resulted in improved cell growth and morphogenesis.

A large part of the electrical energy in tissue culture is used for autoclaving, lighting of the growth room, air filtration in laminar-flow cabinets and air conditioning. Major obstacle in clonal *in-vitro* propagation of woody species is the initiation of rooting and hardening of plantlets for successful field transfer (Gunasekare and Evans, 2000). Debergh *et al.,* (1992) reported that roots induced *in-vitro* are rarely functional, lack root hairs, fragile and are generally damaged during transferring to soil. From a commercial point of view, initiation of rooting *in-vitro* may account for 30-75% of the total cost of plants propagated *in-vitro* (Debergh *et al.,* 1992). The results of the present study showed that micro-shoots treated with IBA, at 50 mg/ L as a pulse treatment facilitated *ex-vitro* root initiation.

Physical properties such as total porosity, bulk density, air space, water holding capacity and available water content of the rooting media can also have a profound effect on the supply of water and air to the growing plant. Of these, aeration and moisture supply appear to be the two properties of major concern in a rooting media which affect the emergence and vigor of roots with consequent effect on quality of rooted cuttings (Nyomora *et al.,* 2009). The differential responses to different rooting media suggest that the existence of significant variation in the physical characteristics particularly differences in total porosity, water holding capacity and aeration porosity of the media used. River sand had the highest aeration porosity which was 31.3%, while the lowest aeration porosity of 9.1% was in the top soil (Nyomora *et al.,* 2009). The media component coir originates from ground-waste coconut husks, very similar to peat in appearance and particle size ranges from about 0.5 to 2 mm. Pore is greater than 80% space and air-filled pore space at container capacity is 9-13%. Unlike sphagnum peat, there are no sticky substances or other extraneous matter in coir dust. Coir has a higher water holding capacity, than peat (Evans *et al.,* 1996) and can be easily rewetted after drying better than peat. In the present study *ex-vitro* rooting substrate which contained coir dust, top soil and sand at a ratio of 1:1:1 may have provided sufficient porosity to allow good aeration that ensures adequate oxygen availability while maintaining the pH and water status favorable for the development of good root system. *Ex-vitro* rooting is an attractive option in micropropagation because of simultaneous rooting and hardening of plants, which reduce the cost of production and time required to produce large number of planting materials from a newly developed hybrid.

CONCLUSIONS

The use of sago as a low cost gelling agent coupled with *ex-vitro* rooting of micro-shoots produced *in-vitro* reduced the cost of production of micro propagated plants by 75%, over the standard micro-propagation thus making the plants produced by the low cost micro-propagation technique cheaper (Rs. 11.00 per plant) than the plants produced by standard micro-propagation method (Rs. 39.00 per plant). In addition, the proposed method is also cheaper than the conventional nursery propagation method where cost per plant is about Rs. 23.00. The proposed method can be used to speed up the conventional tea breeding program and to increase its efficiency towards early release of new cultivars to the growers.

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