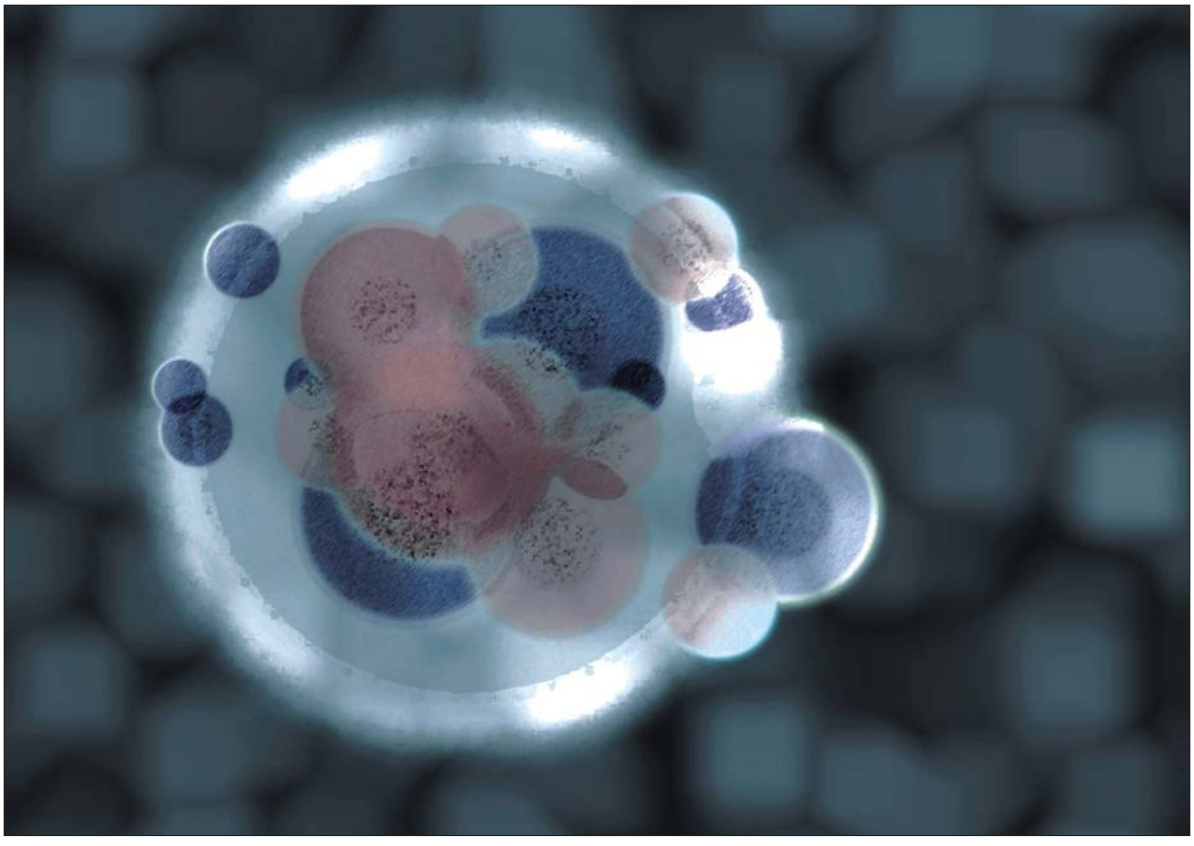

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Evaluation of Natural Exudate Gum from *Sterculia urens* as Gelling Agent in Culture Media for *in vitro* Regeneration of Rough Lemon (*Citrus jambhiri* Lush.) Shoot Tips

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Abstract: Natural exudate gum (sterculia gum) has been successfully used as gelling agent in culture media with an aim to reduce the production cost of tissue culture raised plants. Shoot tips were cultured on agar and sterculia gum gelled media to study the difference in shoot and root regeneration response. Initially, shoot tips were cultured on agar gelled Murashige and Skoog (MS) medium supplemented with different concentrations of 6-Benzyl amino purine (BAP), Kinetin (KN) and Naphthalene acetic acid (NAA). Maximum percentage of cultures showing shoot regeneration (63.19%) was observed on agar gelled MS medium containing BAP 1.5, KN 0.5 and NAA 0.5 mg L^{-1}. Sterculia gum and agar were then evaluated at different concentrations and combinations. The media gelled with sterculia gum was equally transparent as that of liquid medium. The shoot regeneration response on media gelled with 25 and 30 g L^{-1} sterculia gum were 49.99 and 61.11% respectively. Regenerated shoots were rooted on agar gelled MS medium supplemented with different concentrations of Indole-3-Acetic Acid (IAA), NAA and Indole -3-butyric acid (IBA, 0.5-2.5 mg L^{-1}). Maximum rooting response (56.94%) was observed with IBA 2.0 mg L^{-1}. Same medium when evaluated for rooting response using sterculia gum (30 g L^{-1}) as gelling agent, 56.24% cultures showed root regeneration. The difference in regeneration response obtained with agar (8 g L^{-1}) and sterculia gum (30 g L^{-1}) was statistically insignificant. This study indicates that sterculia gum can be used as alternative gelling agent in place of agar for the development of cost effective micropropagation protocol.

Key words: *Citrus*, rough lemon, rootstock, micropropagation, sterculia gum, agar, shoot tips

INTRODUCTION

Rough lemon (*Citrus jambhiri* Lush.) is the most commonly used rootstock for different *Citrus* species. Locally in Punjab, it is known as "Jatti Khatti" and scions grafted on it produce large trees with high yield of fruits. It develops a deep rooting system, gives long life to scion and forms normal union with scion grafted on it. (Altaf *et al*., 2008). It is an important rootstock for *Citrus* species like mosummi, kinnow, oranges and grape fruits in North Indian states like Punjab, Haryana, Rajasthan and Uttar Pradesh. In Punjab there are only few *C. jambhiri* plants available for seed collection. Further these seeds have a very short life span because they lose their viability (Johnston, 1968), so can not be stored for longer time. When seeds of desired rootstock are not available in sufficient quantities, it becomes very difficult to meet the requirements of rough lemon rootstock for grafting. Moreover, plants regenerated from seedlings are not uniform and some time variations also appear on grafted plants (Hartman *et al.,* 1997). Under such circumstances, *in vitro* propagation techniques hold potential and could offer solution to these problems.

In vitro clonal propagation via direct organogenesis provides large number of uniform plants in a short period of time as compared to conventional breeding methods. Plants regenerated via direct shoot organogenesis are uniform, pathogen free and are clones of parent plant (Saini *et al*., 2010), so they can easily be exchanged between countries. *In vitro* propagation have been described for a number of *Citrus* species (Moreira-Dias *et al*., 2000; Al-Khayri and Al-Bahrany, 2001; Tao *et al*., 2002; Usman *et al.,* 2005; Altaf *et al*., 2009; Laskar *et al*., 2009; Sharma *et al*., 2009; Perez-Tornero *et al*., 2010). However, little work has been carried out on *in vitro* propagation o f rough lemon (Ali and Mizra, 2006; Altaf *et al*., 2008; Savita *et al*., 2010). In our previous study (Savita *et al*., 2011), regeneration of rough lemon plants from callus cultures was reported and here we report cost effective protocol for regeneration of plants from rough lemon shoot tips.

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Micropropagation requires efficient and reproducible protocol along with expensive culture medium. Among different components of the culture medium agar represents one of the most expensive components (Goel *et al*., 2007). Search for low cost alternative gelling agents has become crucial for plant tissue culture laboratories (Daud *et al*., 2011) particularly, for those involved in micropropagation. In earlier reports number of substances like corn starch (Henderson and Kinnersley, 1988; Zimmerman *et al*., 1995), Tapioca (Nene *et al*., 1996), Isubgol (Babbar and Jain, 1998), Guar gum (Babbar *et al*., 2005), Gum katira (Jain and Babbar, 2002) and Xanthan gum (Jain-Raina and Babbar, 2011) has been tested for their gelling ability in plant tissue culture media. Gum exuded from bark of *Sterculia urens* is generally used in food and pharmaceutical industry. The present study describes the possibility of using it as gelling agent in plant tissue culture media.

MATERIALS AND METHODS

Source of explant and gum: Rough lemon plant selected at Govt. Nursery, Department of Horticulture (Punjab), Attari, Amritsar, India was used as a source of explant for raising shoot tip cultures. The bud flushes were induced by defoliating and tipping some shoot branches of a healthy plant in February 2009. These bud flushes were harvested after 10-15 days of tipping and transported to lab in moist bags. Sterculia gum in the form of lumps (Fig. 1a) was purchased from Girijan Cooperative Corporation Ltd., Visakhapatnam, India and stored in airtight polypropylene jars. Gum lumps were powdered using high speed mechanical blender (Bajaj, India) and sieved through 150 size mesh before use.

Explant sterilization and inoculation: Bud flushes (2-3 cm) collected from the healthy rough lemon plants were washed with detergent to remove any external microbes and soil particles. After this they were washed thoroughly under running tap water so as to remove the detergent completely. The sterilization of the explant was done under aseptic conditions in a laminar flow hood. The explants were disinfected with 0.05% aqueous solution of mercuric chloride ($HgCl_2$) for 4 min and then rinsed three times with sterilized double-distilled water to remove any traces of disinfectant. Shoot tips (0.5 cm) were excised inside a laminar flow cabinet with the help of sterilized sharp fine scalpel blade.

Shoot regeneration: Sterilized shoot tips of rough lemon were cultured on MS medium (Murashige and Skoog, 1962) gelled with sterculia gum and agar

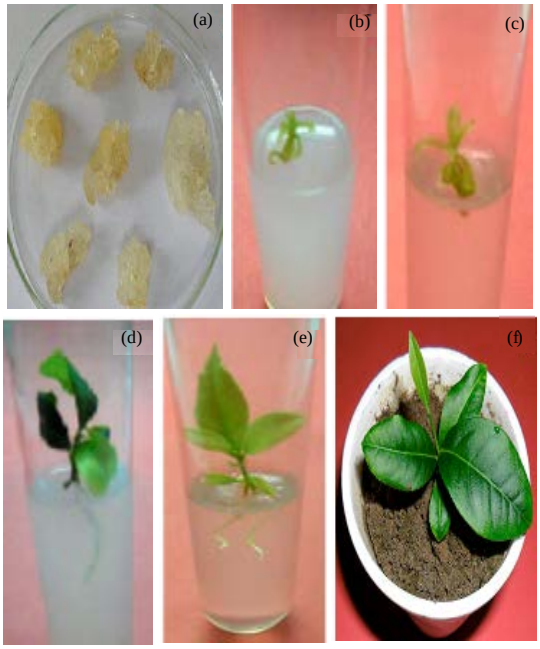

Fig. 1(a-f): *In vitro* propagation of rough lemon (*Citrus jambhiri* Lush): (a) Intact lumps of sterculia gum (b) *In vitro* regeneration of shoot tip on agar (8 g L^{-1}) gelled medium containing BAP 1.5 mg L^{-1}, KN 0.5 mg L^{-1} and NAA 0.5 mg L^{-1} (c) *In vitro* regeneration of shoot tip on sterculia gum (30 g L^{-1}) gelled medium containing BAP 1.5 mg L^{-1}, KN 0.5 mg L^{-1} and NAA 0.5 mg L^{-1}. (d) *In vitro* root regeneration on agar (8 g L^{-1}) gelled medium containing IBA (2 mg L^{-1}) (e) Complete plantlet development after 45 days of culture on sterculia gum (30 g L^{-1}) gelled medium containing IBA (2 mg L^{-1}) (f) Plant development after transplantation of regenerated plantlets into a sand/soil mixture

(HiMedia, India) either alone or in combination. Initially, the shoot tips were inoculated on MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar. Different concentrations of BAP, KN and NAA in combinations were tried in agar gelled media to find out the best suitable medium for shoot regeneration (Table 1). The media were adjusted to pH 5.6 with 1N NaOH and autoclaved at 121°C and 15 lb in^{-2} pressure for 20 min. After finding out the best suitable concentration of plant growth regulator in MS medium, the sterculia gum (20, 25 and 30 g L^{-1}) alone and in combination with agar (2, 4 and 6 g L^{-1}) was tested as gelling agent. MS medium supplemented with suitable plant growth regulator and gelled with 8 g L^{-1} agar served

as control in gum gelled experiments. For preparing sterculia gum gelled media, overnight soaked gum and sucrose were mixed with other constituents of the medium and the volume was raised to the required level on a magnetic stirrer before adjusting the pH to 5.6. For all the experiments, 20 mL of culture medium was dispensed in 25×150 mm culture tubes which were closed with cotton plugs. All cultures were maintained at 26±1°C with a luminous intensity of 40 μmole/m^2/sec and 16 h photoperiod. For each treatment percentage of cultures showing shoot regeneration were recorded 30 days after initial culturing.

Rooting of regenerated shoot: For rooting, the regenerated shoots were cultured on MS medium supplemented with various concentrations (0.5-2.5 mg L^{-1}) of Indole-3-Acetic Acid (IAA), Naphthalene Acetic Acid (NAA) and Indole-3-Butyric Acid (IBA) along with 3% (w/v) sucrose. Initially, 8 g L^{-1} agar was used as gelling agent to find out the best suitable medium for rooting. After finding out the best suitable concentration of plant growth regulator in MS medium, the sterculia gum (25 and 30 g L^{-1}) alone and in combination with agar (2, 4 and 6 g L^{-1}) was used as gelling agent. A total of 48 regenerated shoots were inoculated for individual treatment and the experiment was repeated 3 times. The percentage of cultures showing root regeneration was recorded after 30 days of inoculation.

Hardening and acclimatization: The well-developed plantlets were washed with water in order to remove adhering agar and transferred to autoclaved plastic pots containing a mixture of garden soil, sand and vermiculite in the ratio of 3:1:1. Hardening of potted plantlets was accomplished in a culture room set at 26±1°C, 16 h day-length (40 μmole/m^2/ sec) and covered with polyethylene bags to maintain high humidity. After 12-15 days, polyethylene bags were removed initially for a short duration (15-30 min) daily for about one week. Gradually, the daily exposure time was increased by 30 min for each day. Polyethylene bags were completely removed after 20 days; subsequently the plantlets were transferred to the earthen pots containing only garden soil and kept in the polyhouse for one month and there-after transferred to the field conditions.

Statistical analysis: For each treatment, 48 culture tubes were inoculated and the experiments were repeated thrice. The data pertaining to the effect of gelling agent and plant growth regulators on shoot and root regeneration were subjected to one way Analysis of Variance (ANOVA) and the differences among means ($p = 0.05$) were compared by High-range Statistical Domain (HSD) using Tukey's test (Meyers and Grossen, 1974).

RESULTS

Shoot regeneration: Shoot tips of rough lemon cultured on agar gelled (8 g L^{-1}) MS medium supplemented with different concentrations of plant hormones (BAP, KN and NAA) showed signs of shoot regeneration after 6-8 days of inoculation. The percentage of cultures showing shoot formation was recorded after 30 days of inoculation (Table 1). Among different concentrations of plant hormones tested, maximum percentage of cultures showing shoot formation (63.19%) was observed with MS medium containing BAP 1.5. KN 0.5 and NAA 0.5 mg L^{-1} (Fig. 1a). A further increase in concentration of BAP (2.0-3.0 mg L^{-1}) resulted in a decrease in percent shoot regeneration. Different concentrations (0.5-2.0 mg L^{-1}) of BAP or KN alone in combination with NAA (1.0 mg L^{-1}) were not effective for shoot regeneration from shoot tip explants. The effect of different concentrations of sterculia gum and agar, alone and in combination, on shoot regeneration in MS medium supplemented with BAP 1.5, KN 0.5 and NAA 0.5 mg L^{-1} is shown in Table 2. The shoot regeneration response on media gelled with 25 g L^{-1} sterculia gum was less (49.99%) than those of 30 g L^{-1} (61.11%). The culture tubes containing gum gelled medium at a concentration of 20 g L^{-1} often resulted in sinking of the shoot tips explant to subsurface levels. Such submerged explants also responded positively indicating that sufficient aeration was available in gum gelled media. The media gelled with sterculia gum was as equally transparent as liquid medium (Fig. 1c). Regenerated shoots had yellow green leaves on gum gelled medium as compared to dark green leaves on agar containing medium. Addition of agar (2 g L^{-1}) along with gum (25 g L^{-1}) increased the firmness of the media but not showed significant improvement in shooting response. Shoot regeneration response on gum gelled media along with all the concentrations of agar was less than those of control (agar 8 g L^{-1}). Among all the concentration of gum tested the maximum shoot regeneration was observed with 30 g L^{-1} gum gelled media.

Root regeneration: Regenerated shoots were transferred to the rooting medium gelled with agar (8 g L^{-1}). The effect of different concentrations of NAA, IBA and IAA (0.5-2.5 mg L^{-1}) on root regeneration after 30 days of inoculation is shown in Table 3. The initiation of roots took place after 2 weeks of inoculation. In MS medium supplemented with IBA 2 mg L^{-1}, maximum of 56.94% cultures showed root regeneration (Fig. 1d). A decrease or increase in concentration of IBA resulted in decrease in rooting response. Among IAA and NAA, the later was more effective with a maximum of 35.41% cultures

Table 1: Response of shoot tips on agar (8 g L^{-1}) gelled MS medium containing different concentrations of plant hormones

Treatment (mg L^{-1})			Agar (8 g L^{-1})
BAP	KN	NAA	Shoot regeneration (%)*(Mean±SE)
0.5	-	1.0	11.09±0.69^e
1.0	-	1.0	20.82±1.19^b
1.5	-	1.0	33.31±1.20^a
2.0	-	1.0	31.94±1.39^a
			$F_{3,8}$ = 44.89*; HSD = 6.28
-	0.5	1.0	9.07±1.38^e
-	1.0	1.0	12.48±1.20^b
-	1.5	1.0	22.91±1.19^a
-	2.0	1.0	22.21±0.70^a
			$F_{3,8}$ = 34.16*; HSD = 4.64
1.0	0.5	0.5	53.46±0.70^a
1.0	0.5	1.0	52.08±1.20^a
1.0	0.5	1.5	35.41±1.21^b
1.0	0.5	2.0	24.99±1.19^c
			$F_{3,8}$ = 156.13*; HSD = 4.43
1.5	0.5	0.5	63.19±1.40^a
2.0	0.5	0.5	55.55±1.85^b
2.5	0.5	0.5	53.46±0.70^b
3.0	0.5	0.5	52.77±0.70^b
			$F_{3,8}$ = 14.56*; HSD = 5.05

Data shown are Mean±SE of three experiments. Each experiment consisted of 48 replicates. *Significant at p≤0.05. Values followed by the same letter with in a column are not significantly different using HSD multiple comparison test. Observations recorded after 30 days of inoculation

Table 2: Response of shoot tips on medium (MS medium + BAP 1.5 mg L^{-1}+KN 0.5 mg L^{-1} + NAA 0.5 mg L^{-1}) gelled with sterculia gum and agar either alone or in combination

Gelling agent (g L^{-1})		
Sterculia gum	Agar	Shoot regeneration (%)*(Mean±SE)
25	-	49.99±1.21^c
25	2	52.08±1.20^b
25	4	57.63±1.85^b
25	6	50.69±0.70^b
30	-	61.11±1.40^a
30	2	61.10±1.39^a
30	4	61.80±0.71^a
30	6	59.02±0.70^a
-	8	63.19±1.40^a
		$F_{8,18}$ = 17.58*; HSD = 5.56

Data shown are Mean±SE of three experiments. Each experiment consisted of 48 replicates. *Significant at p≤0.05. Values followed by the same letter with in a column are not significantly different using HSD multiple comparison test. Observations recorded after 30 days of inoculation

showing root regeneration at 2 mg L^{-1} concentration. The effect of different concentrations of sterculia gum and agar, alone and in combination, on root regeneration in MS medium supplemented with IBA (2 mg L^{-1}) is shown in Table 4. Rooting response on media gelled with 25 g L^{-1} sterculia gum was less (53.46%) than those of 30 g L^{-1} (56.24%). Addition of agar (2, 4 and 6 g L^{-1}) along with sterculia gum (25 and 30 g L^{-1}) in media resulted in decrease in percentage of cultures showing root formation. The quantitative response obtained on media fortified with gum and agar was not significantly different. The growth and morphology of regenerated roots were similar on both the media. The elongation of

Table 3: Rooting response of regenerated shoots in agar gelled MS medium containing different concentrations of plant hormones

PGRs	Concentration (mg L^{-1})	Root regeneration (%)* (Mean±SE)
NAA	0.5	18.05±1.40^c
	1.0	27.08±1.21^b
	1.5	31.94±1.39^a
	2.0	35.41±0.70^a
	2.5	34.02±1.20^a
		$F_{4,10}$ = 34.22*; HSD = 5.20
IBA	0.5	25.69±1.40^d
	1.0	31.94±1.38^c
	1.5	39.58±1.21^b
	2.0	56.94±1.85^a
	2.5	52.08±1.20^a
		$F_{4,10}$ = 85.82*; HSD = 6.16
IAA	0.5	17.36±1.19^b
	1.0	28.46±0.69^a
	1.5	29.16±1.18^a
	2.0	31.24±0.67^a
	2.5	30.55±1.20^a
		$F_{4,10}$ = 22.39*; HSD = 5.21

Data shown are Mean±SE of three experiments. Each experiment consisted of 48 replicates. *Significant at p≤0.05. Values followed by the same letter with in a column are not significantly different using HSD multiple comparison test. Observations recorded after 30 days of inoculation

Table 4: Rooting response of regenerated shoots on MS medium supplemented with IBA (2 mg L^{-1}) and gelled with sterculia gum and agar either alone or in combination

Gelling agent (g L^{-1})		
Sterculia gum	Agar	Root regeneration (%)* (Mean±SE)
25	-	53.46±0.71^a
25	2	52.77±0.70^a
25	4	52.07±2.10^b
25	6	51.38±1.39^b
30	-	56.24±1.21^a
30	2	55.55±1.40^a
30	4	53.47±1.39^a
30	6	50.69±1.41^b
-	8	56.94±1.85^a
		$F_{8,18}$ = 2.88*; HSD = 4.93

Data shown are Mean±SE of three experiments. Each experiment consisted of 48 replicates. *Significant at p≤0.05. Values followed by the same letter with in a column are not significantly different using HSD multiple comparison test. Observations recorded after 30 days of inoculation

roots was much better on gum-gelled medium than on agar medium. The plantlets responded positively in gum gelled medium and there was no softening of the medium even after 45 days of culture (Fig. 1e). The plantlets, thus developed where transferred to pots containing a mixture of garden soil, sand and vermiculite for acclimatization (Fig. 1f) which showed 64 % survival rate after transfer to field conditions.

DISCUSSION

In the present study plantlets were regenerated via direct organogenesis from *in vitro* cultured shoot tips of rough lemon (*C. jambhiri* Lush). Direct organogenesis does not involve intervening callus formation and plants produced by direct organogenesis may exhibit greater genetic stability than those produced from callus

(Khan *et al*., 2009). Plantlets were regenerated on sterculia gum and agar gelled medium supplemented with various combinations of cytokinin and auxin. Shoot formation can easily be induced from shoot tips of rough lemon plant on MS medium supplemented with BAP, KN and NAA and subsequently rooted on IBA-containing medium. Role of cytokinin in shoot regeneration has been studied in many plant species (Usman *et al*., 2005; Zhang *et al*., 2008) and was considered as an exclusive element for shoot formation. NAA along with BAP and KN was found to be beneficial for direct organogenesis from shoot tips explants. These results are in conformity with some of the earlier studies on citrus (Liu *et al*., 2000; Ghorbel *et al*., 2000; Guo *et al*., 2002) and pineapple (Mercier *et al*., 2003) where low concentration of NAA along with cytokinins was found to be effective for organogenesis. It was noted that by increasing the concentration of cytokinins the regeneration response was decreased which may be due to toxic effects of high concentration of the hormone (Wang *et al*., 2007). Among different plant growth regulators tried for rooting, IBA was found to give better results as compared to NAA and IAA. Similar results have been reported by Carimi and De Pasquale (2003) and Perez-Tornero *et al*. (2010) in *Citrus* species. On the contrary, there are some reports indicating NAA to be better rooting hormone for *Citrus* species (Chakravarty and Goswami, 1999; Usman *et al*., 2005).

Agar is the most widely used gelling agent for plant tissue culture media. The properties of agar like stability, clarity, non-toxic nature and resistance to metabolism during culture make it a gelling agent of choice (Henderson and Kinnersley, 1988). During last two decades, there has been an increase in the efforts to look for cost effective suitable substitutes (Zimmerman *et al*., 1995; Babbar and Jain, 1998; Jain and Babbar, 2002; Babbar *et al*., 2005). In this report, effort has been made to evaluate sterculia gum as gelling agent in plant tissue culture media. Sterculia gum is a dried tree exudate from *Sterculia urens* growing in the regions of Central and Northern India. Sterculia gum gelled medium (30 g L^{-1}) was found to be effective for shoot organogenesis and rooting. It was observed that the initial response for organogenesis was better on agar gelled medium as cultures establish on this medium grow faster as compared to sterculia gum gelled medium. But once the cultures establish themselves on gum gelled medium they exhibit normal growth and proliferation. Sterculia gum forms more transparent medium as compared to agar, therefore, it can be used as gelling agent of choice for the experiments that require regular observations of the cells, tissues or organs growing inside the medium. Moreover it is highly cost effective gelling agent as its price in India is about one-fourth of the agar. Therefore, the cost of commercial micropropagation can be reduced if less expensive alternatives are used. The quality of sterculia gum varies with climatic conditions of plant like moisture, temperature, soil composition and use of herbicides, which may limit its use as standard gelling agent. However, if demand for its use as gelling agent arises, efforts can be made to motivate suppliers to supply good quality gum in branded packs.

CONCLUSION

Agar represents one of the most expensive used media components. The present study describes possibility of using sterculia gum in place of agar as gelling agent to reduce production cost of tissue culture raised plants. Sterculia gum forms a transparent medium which supported regeneration response comparable to agar.

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