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Admixture of Isubgol Husk together with agar as gelling agent for sugarcane callus induction

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Abstract
The study was conducted on commercially important crop plants like Sugarcane (*Saccharum spp*). 'Isubgol', the mucilaginous husk, derived from *Plantago ovata* was successfully used as a gelling material in tissue culture media. Many attempts have been made to find a substitute for agar as gelling agent. The use of 'Isubgol husk' along with agar can reduce the cost of gelling agents approximately by 47.5% in plant tissue culture media. The response from media gelled with Isubgol husk in sugarcane was similar to that from media solidified with agar. The substitution of conventionally used solidifying agent agar (0.8%) with alternative isubgol (1.5%) induced almost similar response with average callus formed. The properties of 'Isubgol husk', including its polysaccharide like and colloidal nature, reported resistance to enzymatic activity, good gelling ability. The husk even after autoclaving remained suspended and formed a gradient once the medium solidified. There was no softening of the 'Isubgol'-gelled medium during the entire course of culture. The media remained reasonably transparent and offered no serious problems to the explants.

Keywords: *Plantago ovata*, Mucilaginous husk, sugarcane, Isubgol husk, gelling material

Introduction
The 'Isubgol husk' derived from the seeds of *Plantago ovate* a stem less herb of the plantaginaceae family, is used asemollient, demulcent and laxative and in the treatment of dysentery and diarrhoea. The efficacy of 'Isubgol husk' is entirely due to the large quantity of mucilage present in the husk. Like agar, 'Isubgol' mucilage is colloidal and polysaccharide likes in nature and is mainly composed of xylose, arabinose and galacto uronic acid, rhamnose and galactose [2]. Agar has mostly been used as solidifying agent in tissue culture media because of its stability, high clarity, non-toxic nature and resistance to metabolism during culture. Commercially, agar is extracted from species of red algae genera *Gelidium*, *Gracillaria* and *Pterocladia* [6]. The almost exclusive use of agar is resulting in over-exploitation of its sources and the very expensive price of tissue culture grade agar; attempts have been made to identify a suitable alternate gelling agent in view of inexpensive, feasible and easily available in the India.
Recently starches from various sources such as barley, corn, potato, rice, wheat and tapioca have been utilized as gelling agents, either singly or in combination with others, with varying degrees of success [13a, b, c,6, 16, 17, 11]. In spite of remarkable expensive advances in plant cell culture, the cost of tissue culture media is still high. Agar has been the most frequently used as a solidifying agent and the most exclusive constituent of culture media. For commercial purposes cost reduction is mandatory. Many attempts have been made to identify suitable alternative gelling agents. Through this experiment, 'Isubgol husk' was used as a gelling agent to reduce the cost of plant tissue culture media. Locally 'Isubgol husk' is about one twentieth of the price of good quality agar. Through this experiment, an attempt was taken to reduce the cost of gelling agent in the media. The aim of the present investigation, therefore, is to establish a suitable protocol for using 'Isubgol' as a gelling Admixture of Isubgol husk together combined with agar in plant tissue culture media.
By considering the above facts the present investigation was taken up under the objective to standardize and assessment of the protocol for admixture of 'Isubgol husk' together with agar as gelling agent for *in vitro* callus culture of sugarcane.

Materials and Methods
Sugarcane (*Saccharum spp.*) crop were used for their regeneration on 'Isubgol' as a gelling agent together with conventional agar media.

The *in vitro* plantlets were initiated through meristem shoot tip culture. The media used for this present investigation were 'Isubgol husk' in different combinations with agar.

Materials

'Isubgol' husk is mainly derived from the seeds of *Plantago ovata*. Raw 'Isubgol husk' was collected from local market. In the present investigation used the explant as an apical shoot tip meristem of cultivar 'Co-671' collected from demonstration field.

Choice of explants

The selection of the stock plant or plants must be a typical of the variety or species and free from any symptoms of disease. Explants for culture were obtained from stalk apices of 8-12 months old field grown healthy sugarcane cultivars (Co-671).

Explant sterilization

- Actively growing points of sugarcane top, taken as explants from 8-12 months old sugarcane cultivar.
- The outer mature leaves were removed till a spindle of about 1 cm in dia was obtained. The spindle (3.0-4.0 cm) was excised and thoroughly washed under tap water for 30 minutes.
- The explants were treated with aqueous solution of Bavistin (BASF India Limited) [1% w/v] and few drops of Tween-20 for 20 minutes.
- Explants were again washed with sterile double distilled water for 10 minutes.
- The explants were then taken inside the laminar hood for further sterilization. Inside the laminar hood, sterilization with savlon (Johnson & Johnson) (1.5% v/v chlorohexidine gluconate solution and 3.0% w/v cetrimide) and 0.1% mercuric chloride (Ranbaxy) for 7-8 minutes each is carried out. Finally rinsing is done thrice with sterile double distilled water to completely remove any mercuric chloride. pH is usually maintained at 5.8, which is prone to changes over culture duration. The optimum incubation temperature should be in the range of 24-26 °C.

Sterilization of glassware

The wire mesh basket containing glassware's were autoclaved at 15lbs. at 121 ^{0}C for a 30 min followed by drying in hot air oven at 80–100 ^{0}C for 1hr. for removal of excess moisture. Forceps and scalpel like instruments were sterilized by flame sterilization technique. The culture showing unwanted microbial growth (contamination) was discarded after autoclaving in order to destroy the source of contaminants.

Preparation of stock solutions

The medium consisted of macronutrients, micronutrients, Fe-EDTA, vitamins, amino acids, and sucrose, agar and plant growth regulators. All the stock solutions and final media were prepared [3].

The Growth hormones were dissolved in a few drops of the solvent and then the volume was made up to the required level with double distilled water, filter sterilized. The stock of growth hormones was prepared in 1mg/ml for convenient use and stored at cold condition (4 ºC).

The stock solutions of major and minor elements (8X) and vitamins (50X) were prepared in sterile distilled water. The quality of major elements, minor elements and vitamins were measured as per description given in (Table 1), dissolved thoroughly in sterile H_2O with the help of magnetic stirrer and stored at 4 ^{0}C. These stock solutions were routinely used in preparation of various media composition required in callus induction of sugarcane (Table 2.1).

Carbon source

Sucrose (3%) was used as main carbon source for plant regeneration and rooting. High purity sucrose was used. Sucrose was added before adjusting the pH of the medium.

Organic additives

Coconut water (CW) was used as organic additives in different experiments. This was collected from tender nuts, filtered through Whattman no. 1 filter paper (3mm) and added in medium before autoclaving.

Preparation of media

The appropriate composition of the medium largely determines the success of cultures. Plant material does vary in their nutritional requirements and therefore it is often necessary to modify the medium to suit a particular tissue. The basal medium employed for the culture of Sugarcane is MS medium. A variety of growth regulators such as 6-Benzyl amino purine (BAP), alpha-Naphthalene acetic acid (NAA), 3-Indole Butyric acid (IBA) and 2,4- dichlorophenoxy acetic acid (2,4-D) were added to the medium singly or in combinations at various concentrations and were used for initiating different experiments. The stock solution of macro, micro, trace elements and vitamins were prepared with double distilled water and stored inside the refrigerator at 4 ^{0}C.

Also the stock solution of growth regulators like NAA, IBA, BAP, Kinetin, and GA_3 were prepared by dissolving their required quantity in a few drops of 1N NaOH and final volumes made up with sterile water, filter sterilized and stored in refrigerator at 4 ^{0}C.

Procedure for preparation of MS culture media

1. One liter of MS basal media was prepared by taking 125 ml of stock of major nutrient (MA 8X) was transfer to clean and sterile beaker.
2. To the same beaker 20ml of stock of minor (MB 50X) was added and stirred well with help of magnetic stirrer.
3. The major source of carbon (sucrose) was added at a conc. of 3% and dissolved to the medium while continuous stirring, the volume of solution was made up to 900ml with doubled distilled water.
4. The pH of the medium was adjusted to 5.8 by using 0.1N HCl and 0.1 N NaOH before sterilization.
5. The final volume of solution was made up to one liter by using doubled distilled water.
6. Make four combination of medium 250ml each by adding different concentration of agar + isubgol i.e. Group A, Group B, Group C, Group D.
7. The solidifying agent, Agar-agar was added as (Table 2) to the medium at 0.8% concentration and mixed thoroughly to the medium as proportion given in (Table 1) by gently heating of the media.
8. The required quantity of media approximately 50ml per culture bottle was dispensed.
9. The bottle containing culture media were kept in wire mesh basket, wrapped with aluminium foil and autoclaved at 121 ^{0}C at 15 lbs PSI pressure for 20 min.
10. After sterilization the media was allowed to cool and then required volume of growth regulators (BAP, KIN, GA_3,

NAA) from stock solution were added separately in Group B,C,D in laminar airflow cabinet under aseptic condition.

11. The cultural were sealed with parafilm and stored at 25 ^{0}C until use.

Table 1: Preparation of stock solution of major and minor nutrients (8X) and vitamin nutrients (50X).

Constituents	Quantity(mg/lit)
Macronutrients	
$MgSo_4.7H_2O$	7400
KH_2PO_4	3400
KNO_3	38000
NH_4NO_3	33000
$CaCl_2 2H_2O$	8800
Micronutrients	
H_3BO_3	1240
$MnSO_4 4H_2O$	4460
$ZnSO_4 7H_20$	1720
$Na_2MoO_4 2H_2O$	50
$CuS0_4 5H_2O$	5
$CoCl_2 6H_2O$	5
Chelating agent	
$FeSO_4 7H_2O$	5560
$Na_2EDTA.2H_2O$	7460
Vitamins	
Inositol	20000
Thiamine HCl	100
Pyridoxine HCl	100
Nicotinic Acid	100
Glycine	400

Establishment of Medium:

All media contained 0.7% agar + 'Isubgol husk' (in different combinations) as gelling agent and 3.0% sucrose, pH of the culture media was maintained 5.8 before autoclaving. All cultures were grown in the growth chamber illumination by 40W (~2500 lux) white fluorescent tubes fitted at a distance 25 cm from culture shelves and controlled temperature maintain 25 ± 2 ^{0}C with air cooler. Four different combinations of Agar and 'Isubgol husk' were used as gelling agent (Table 2). Besides, the experimental set was divided into four groups based on different combination of agar + Isubgol applied in the culture media. Group-A MS media contained no hormone. Group-B,C,D MS media contained 3.0 mg/l 2,4-D + 0.5 mg/l Kn. Twenty cultural bottle, on average, were taken in the study for all four groups. Equal amount of twenty cultural bottles were taken to study of sugarcane explants. Twenty explants of sugarcane, on average, were sub cultured into each cultural bottle.

Table 2: Preparation of different combination media by using 'agar and Isubgol husk' as gelling agent.

Culture Group MS media	Hormones Con^c.	Agar(%)+ Isubgol husk (%)	Agar + Isubgol husk (gm)/250ml
Group A	-	100+0	1.75 +0.0
Group B	3 mg/l 2,4-D + 0.5 mg/l Kin	50+50	0.85+0.85
Group C	3 mg/l 2,4-D + 0.5 mg/l Kin	30+70	0.52+1.23
Group D	3 mg/l 2,4-D + 0.5 mg/l Kin	100+0	1.75+0.0

Initiation of explants

1. Take out a meristem in a sterile petriplate containing 100 ml 0.2% bavistin.
2. Remove the outer layer of leaf bases with the help of forcep and scalpel carefully and inoculate the meristem tip on establishment medium in a culture bottle and incubate at 25±2 ^{0}C in a dark for a week.
3. Transfer the culture in a light (100 lux) and incubate giving 16 hrs photoperiods 25±2 ^{0}C. Transfer these cultures after 7 to 8 days of the incubation to a fresh medium in the laminar air flow chamber.
4. Clean the base of the explant wherever required.

Results and Discussion

Gelling performance of used combinations was compared (Figure 1). Culture media contained combination no.3 gelling agent did not gel properly. Height and health of plantlets grown in Isubgol husk+ agar gelled media was almost the same as plantlets grown in agar media (Table 2).

Hormones for multiple shooting were applied in Group B culture media but not in Group A. These experiments were repeated for more than 50 times. Multiple shooting (4.5) was observed only in the plantlets on group B culture media (Table 2). So it could be concluded that 'Isubgol' does not hamper the effect of hormones. It was also observed that culture media contained Combination no. 3 gelling agent was not appropriate for the growth of both sugarcane. So, combination no.3 was excluded from the experiment later. On economic feasibility combination no. 2 and 3 were not suggested for commercial uses. Later Group A& D culture media were prepared with combination of equal amount of agar and 'Isubgol husk' (i.e. Combination no.-1 as it is less expensive than Combination no. - 2 and 3). Concentrations of 2,4-D and Kinetin applied to that culture media were same as Group-B. Growth of callus in both media was almost the same. The growth of callus in group B and group D culture bottle same due to same concentration of hormones and gelling of media. So, Isubgol husk + agar gelled media did not hamper hormone activity. The genotypic changes in Sugarcane were not expected because callus cultures were grown with normal life regulatory factors such as same levels of plant growth regulators and nutrient medium, light source, and growth temperature, during the course of experiment. The suitability of 'Isubgol husk' as a gelling agent was investigated for shoot formation. The time of callus induction (3 weeks) and their subsequent growth were comparable on agar and 'Isubgol'-gelled media (Table 2). The growth and morphology of callus were almost similar in both treatments Group B and D (Figure 2). Thus, the development of callus on 'Isubgol husk' medium did not appear to have any adverse effect on callus induction. In this observation, there was no softening of the 'Isubgol'-gelled medium during the entire course of culture, indicating that it is not metabolized during culture.

In-vitro culture of sugarcane in agar media cracking, which is a frequent problem in case of agar and gelrite solidified media. The results are in agreement with Babbar and Jain (1998) who had very similar observations on *in vitro* culture of Syzygium cuminii and Datura innoxia. Local price of 'Isubgol' is about one-twentieth that of agar used in this study.'Isubgol' reduced price of gelling agent approximately by 47.5% in plant tissue culture media (Table 3).

Fig 1: Gelling result of MS media by using agar+Isubgol.

Fig 2: Response of explants on 'Isubgol husk' + agar gelled culture media

Table 3: Response of explants on Isubgol husk + agar gelled culture media

Plants	Groups	Combinations Agar + Isubgol husk (%)	Frequency (%) of Callus generation
Sugarcane	Group A	100+0	12
	Group B	50+50	90
	Group C	30+70	-
	Group D	100+0	92

Agar has remained to be the most expensive constituent of culture media anywhere in any time. The use of 'Isubgol' along with agar can reduce the cost of gelling agents in these laboratories. Besides, the study was conducted on commercially important crop plants like sugarcane. So, from the local and global perspective, study shows an economic feasibility of plant tissue culture media. At the generated callus in the four combinations Group A, B, C, D in which the group B, and D shows same callus formation.

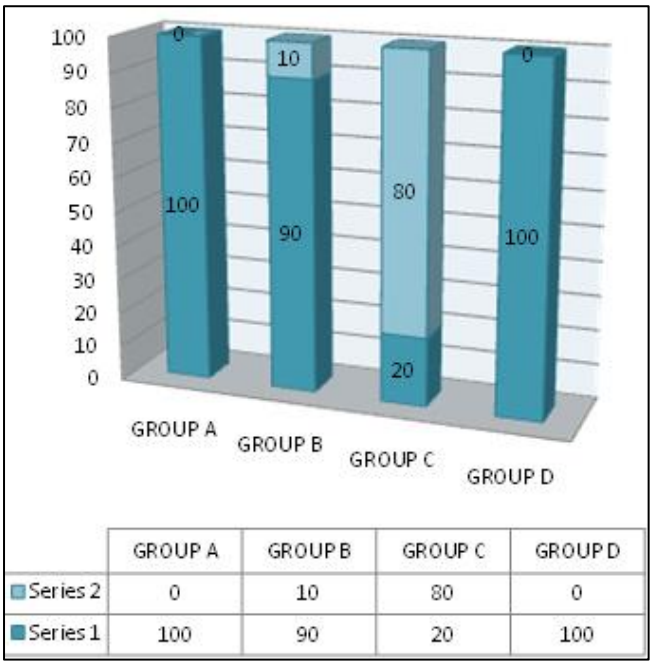

Plate 1: Gelling result of MS media by using agar + Isubgol.

Attempts have been made to find a substitute for agar as gelling agent. The study was conducted on commercially important crop plants like Sugarcane (*Saccharum spp*). 'Isubgol' the mucilaginous husk, derived from *Plantago ovata* was successfully used as a gelling material in tissue culture media. The price of 'Isubgol husk' is cheaper than the conventionally used agar and it had reduced the price ofgelling agent approximately by 47.5% in plant tissue culture media. The use of 'Isubgol husk' along with agar can reduce the cost of gelling agents. The response from media gelled with Isubgol husk in sugarcane was similar to that from media solidified with agar. The substitution of conventionally used solidifying agent agar (0.8%) with alternative isubgol (1.5%) induced almost similar response with average callus formed.

The properties of 'Isubgol husk', including its polysaccharide like and colloidal nature, reported resistance to enzymatic activity, good gelling abilityeven in cold water, and reasonable clarity in gelled form. There was no softening of the 'Isubgol'-gelled medium during the entire course of culture. The media remained reasonably transparent and offered no serious problems to the explants. The plants produced using LC media was consistently better for shoot and proliferation. It is concluded that through reduction of the cost on the techniques, the cost of the product also be reduced and farmers get benefited using low cost, disease free and clonal planting material with high production and also saving land resources.

Gelling agent	Amount of agar of per liter MS (gm)	Amount Isubgol per liter MS (gm)	Cost (%)	Cost (reduction)
Agar	7.0	0.00	70.00	-
Agar + Isubgol	3.5	3.5	36.75	47.5

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