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'Isubgol' as an alternative gelling agent in plant tissue culture media

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Abstract 'Isubgol', the mucilaginous husk derived from the seeds of *Plantago ovata*, was successfully used as a gelling agent in tissue culture media for in vitro seed germination, shoot formation and rooting in *Syzygium cuminii* and anther culture in *Datura innoxia*. For seed germination, Knop's basal medium supplemented with 1% sucrose was employed, whereas for the development of shoots the epicotyl segments excised from in vitro-developed seedlings were cultured on MS basal medium supplemented with 4% sucrose and 1 mg/l 6-benzyladenine. Shoots that developed from the epicotyl segments were rooted on Knop's medium enriched with 2% sucrose and 1 mg/l indole-3-acetic acid. The anthers of *D. innoxia* excised at the late uninucleate to early binucleate stages of microspore development were cultured on Nitsch's basal medium containing 2% sucrose. Media were either gelled with 0.9% agar or 3% 'Isubgol'. The response on media gelled with 'Isubgol' in each of the cases was similar to that on media solidified with agar.

Key words Agar-agar · 'Isubgol' · Gelling agent · Plant tissue culture media

Abbreviations *BA* 6-Benzyladenine · *IAA* indole-3-acetic acid · *MS* Murashige and Skoog (1962) basal medium

Introduction

Since the report of White (1939), agar has been the most frequently used solidifying agent in tissue culture media (Street 1977). The main reasons for its wide use are its stability, high clarity, non-toxic nature and resistance to metabolism during culture (McLachlan 1985; Henderson and Kinnersley 1988). Some investigations have, however, raised doubts about the biological inertness and non-toxic nature of agar (Singha 1980; Debergh 1983; Kohlenbach and Wernicke 1983; Arnold and Ericksson 1989). Commercially, agar is extracted from species of red algae genera *Gelidium*, *Gracillaria* and *Pterocladia* (McLachlan 1985). The almost exclusive use of agar is resulting in over-exploitation of its sources. Because of the above-mentioned reasons and the exorbitant price of tissue culture grade agar, attempts have been made to identify suitable alternative gelling agents.

In the recent past, 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 (Sorvari 1986a, b, c; Henderson and Kinnersley 1988; Tiwari and Rahimbaev 1992; Zimmerman et al. 1995; Nene et al. 1996). However, the use of starch as a gelling agent has been limited, probably because of its inferior gelling quality and tendency to be metabolized, which results in a gradual decrease in the consistency of the medium during the course of the culture period.

'Isubgol', the husk derived from the seeds of *Plantago ovata*, is used as emollient, demulcent and laxative and in the treatment of dysentery and diarrhoea (Chopra et al. 1958). The efficacy of 'Isubgol' is entirely due to the large quantity of mucilage present in the husk. Its action has been found to be purely mechanical as mucilage, which swells into a jelly-like mass, stimulates intestine peristalsis and remains practically unaffected by the digestive enzymes and bacteria (Chopra et al. 1958). Like agar, 'Isubgol' mucilage is colloidal and polysaccharidic in nature and is mainly composed of xylose, arabinose and galactouronic acid; rhamnose and galactose have also been reported. Two polysaccharide fractions have been separated from the mucilage. One fraction (eq. wt. 700; uronic acid 20%) is soluble in cold water and upon hydrolysis yields D-xylose (46%), aldobiouronic acid (40%), L-arabinose (7%) and an insoluble residue (2%). The other fraction (eq. wt. 4000; uronic acid 3%) is soluble in hot water, forming a highly

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viscous solution which sets to a gel when cooled and yields upon hydrolysis D-xylose (80%), L-arabinose (14%), aldobiouronic acid (0.3%) and traces of D-galactose (Laidlaw and Percival 1949, 1950).

This report describes the successful use of 'Isubgol'as a gelling agent. This is illustrated by seed germination, shoot formation, rooting and anther culture on 'Isubgol' medium. The superiority of 'Isubgol' over other gelling agents is discussed.

Materials and methods

The media used for the present investigations were either gelled with 3% (w/v) 'Isubgol' (Mashaal Brand, Malhotra Industries, Delhi) or 0.9% (w/v) bacteriological grade agar (Qualigens, Glaxo Fine Chemicals, Bombay). 'Isubgol' husk is mainly derived from the seeds of *Plantago ovata*, a stemless herb of the Plantaginaceae family. Dried seeds of the plant contain over 30% mucilage. The seeds of another species, *P. psyllium*, also yield a similar mucilaginous husk, but these are considered to be inferior because of the low mucilage content (10–12%) and light-brown to moderate-brown colour of the seeds and the husk. The husk, which contains all of the mucilaginous matter, is separated from the seeds by crushing and winnowing. Crushing is done with emery grinders or flat-stone grinding mills which may be hand- or power-driven. After thorough cleaning the seeds are passed through these mills six to seven times for complete removal of the husk. The crushed material consisting of husk and kernel is sieved to remove the kernels, after which it is passed through screens of different mesh size to separate it out into products of different fineness (Anonymous 1969).

The pH of the media was adjusted to 5.8 before autoclaving at 1.05 kg/cm^2 and 121°C for 15 min. For all the experiments, 20 ml of culture medium was dispensed in 25×150-mm culture tubes for culturing the explants. Cultures for in vitro seed germination, shoot formation and rooting were exposed to continuous light at an intensity of 3.86 W m^{-2} provided by cool daylight fluorescent tubes (40 W, Philips, India), whereas anther cultures were exposed to a continuous diffuse light (0.23 W m^{-2}, 450–750 nm). All the cultures were incubated at 25°±2°C.

For in vitro seed germination, seeds of *Syzygium cuminii* were treated with 50% (v/v) ethanol for 5 min, 1 N HCl for 10 min and 0.5% (w/v) mercuric chloride for 20 min. The treated seeds were washed thoroughly with sterile distilled water and left in water for 30 min for imbibition. The seeds were germinated on Knop's basal medium (Knop 1865), containing 1% (w/v) sucrose. One seed was inoculated per tube, and 48 replicate cultures were raised for each treatment.

For experiments dealing with shoot formation, 1-cm-long epicotyl segments excised from 5-week-old in vitro-grown seedlings of *Syzygium cuminii* were explanted on Murashige and Skoog's basal medium (1962) supplemented with 4% (w/v) sucrose and 1 mg/l BA. Two explants were inoculated per tube, and 24 replicate cultures were raised per treatment.

For in vitro rooting, the microshoots that developed from the epicotyl explants were excised and individually transferred to culture tubes containing Knop's basal medium supplemented with 2% (w/v) sucrose and 1 mg/l IAA. The number of replicates raised for each treatment was 24. Plants developed through in vitro shoot formation and subsequent rooting on either 'Isubgol'- or agar-gelled media were transferred to pots containing autoclaved garden soil.

For experiments dealing with anther culture, floral buds containing anthers at stages 3–5 of microspore development (see Sunderland 1974) were collected from plants of *Datura innoxia* growing in the wild. After the buds were processed, as described earlier (Gupta and Babbar 1980), the anthers were dissected under aseptic conditions and inoculated on Nitsch's basal medium (1969) fortified with 2% (w/v) sucrose. Two anthers from each bud were inoculated on 'Isubgol'-gelled medium and the other two on agar-gelled medium. The fifth anther of each bud was fixed in ethanol and acetic acid (3:1) to ascertain the cytological stage of the anthers at the time of inoculation. Twelve replicate cultures were raised for each treatment.

All of the experiments described were repeated at least twice. Each time the same number of replicates as mentioned above were maintained for individual treatments of the experiments. The quantitative results in both treatments of each experiment were not exactly the same. To test whether the observed differences were significant or not, we subjected the data to statistical analysis employing the chi-square test (P=0.05).

Results and discussion

The suitability of 'Isubgol' as a gelling agent was investigated for seed germination, shoot formation, anther culture and rooting, the morphogenic processes for which semi-solid media are commonly employed. For the first three, *Syzygium cuminii*, a fruit tree belonging to family Myrtaceae, was selected because the protocol for its recurrent plantlet production has recently been standardized in our laboratory (Jain and Babbar, in preparation). For in vitro anther culture, *Datura innoxia*, the plant in which this was first reported, was selected (Guha and Maheshwari 1964, 1966).

Seeds of *Syzygium cuminii* cultured on agar or 'Isubgol' media started germinating 3 weeks after inoculation. The growth of roots as well as shoots of the seedlings was similar in both cases (Fig. 1a, b). The percentage of seeds germinated on these media was not significantly different (Table 1). The epicotyl segments excised from the in vitro-grown seedlings, when cultured on shoot multiplication medium, developed three to six shoots per explant. The time of initiation of shoots (4 weeks), their subsequent growth and the percentages of explants developing shoots

Table 1 Response of explants on 'Isubgol'- and agar-gelled media[a]

Experiment on	'Isubgol'-gelled media	Agar-gelled media
Seed germination		
Number of seeds	48	48
% germination	87.4[a]	93.7[a]
Shoot formation		
Number of explants	48	48
% responding	79.1[a]	87.4[a]
Average no. of shoots per responding explant	4.1[a]	4.3[a]
Rooting		
Number of microshoots	24	24
% rooting	83.3[a]	91.7[a]
Anther culture		
Number of anthers	24	24
% responding	33.3[a]	45.8[a]
Average no. of embryos per responding anther	10.1[a]	13.3[a]

[a] Values followed by the same letter within rows are not significantly different by Chi-square analysis (P=0.05). Each experiment was repeated at least twice

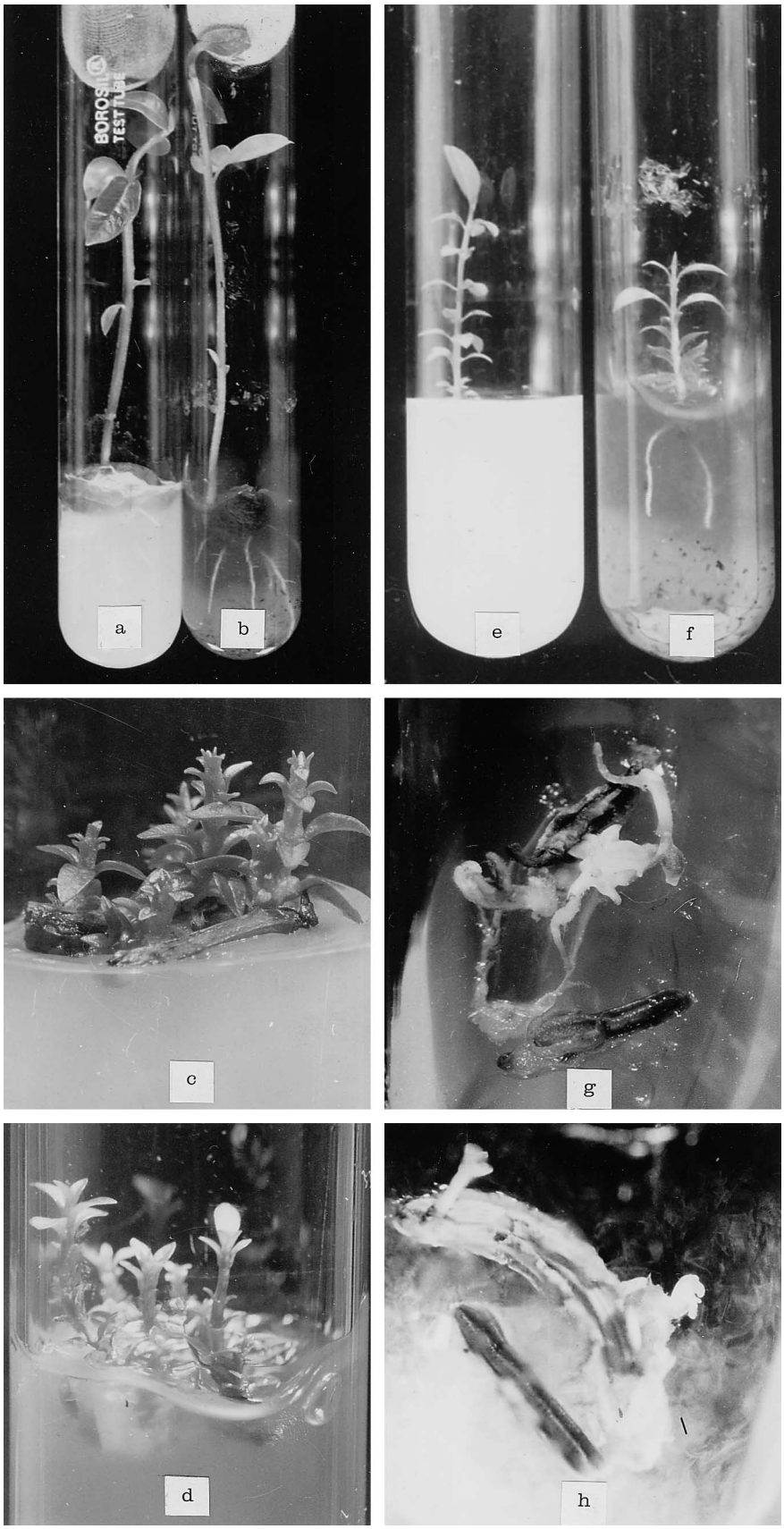

Fig. 1a–h Response of explants on agar- and 'Isubgol'-gelled media. **a, b** Seedlings of *Syzygium cumini* grown on agar- (**a**) and 'Isubgol'- (**b**) gelled media; **c, d** development of shoots from *S. cuminii* epicotyl explants on agar- (**c**) and 'Isubgol'- (**d**) gelled media; **e, f** rooting of in vitro-developed shoots of *S. cuminii* on agar- (**e**) and 'Isubgol'- (**f**) gelled media; **g, h** anther culture of *Datura innoxia* on agar- (**g**) and 'Isubgol'- (**h**) gelled media

were comparable on agar- and 'Isubgol'-gelled media (Table 1, Fig. 1c, d). The shoots developed from the epicotyl explants were cultured on rooting medium, and root initiation took place 3 weeks after inoculation on media solidified with either gelling agent. The growth, morphology and the percentage of shoots developing roots were similar in both treatments (Table 1, Fig. 1e, f). When transferred to soil, plants developed from the shoots regenerated on 'Isubgol' medium and then rooted on 'Isubgol'-gelled rooting medium grew normally, as did those that developed on corresponding agar-gelled media. Thus, the development of shoots as well as the rooting of these shoots on 'Isubgol' medium did not appear to have any adverse effect on subsequent growth of the regenerated plants. Likewise, no difference was discernible in the quantitative or qualitative androgenic response of anthers cultured on agar- and 'Isubgol'-gelled media (Table 1, Fig. 1g, h). In all the experiments, there was no softening of the 'Isubgol'-gelled medium during the entire course of culture, indicating that it is not metabolized during culture. While the 'Isubgol'-gelled media performed statistically as well as the agar-gelled media in all experiments (Table 1), there was a numerical trend in favor of agar media over 'Isubgol' media in all experiments.

Many attempts have been made to look for substitutes to agar as gelling agents. Sorvari (1986a, b, c) reported the use of starches from barley, corn, wheat, potato and rice as alternative gelling agents for barley anther culture and the cultivation of potato tuber discs for shoot differentiation. Barley starch yielded the best result, and the response was better than that for agar-gelled media. However, the starch-gelled media were weakly solidified, and polyester nets were used to prevent the sinking of tissues into the medium. Henderson and Kinnersley (1988) compared the responses of tobacco and carrot cell cultures on media gelled either with corn starch or agar singly or with a combination of the two. They observed that the response on starch-gelled medium was invariably better than that on agar medium. According to them the problem of softness of the starch-gelled medium could be eliminated by increasing the concentration of starch up to 10%. However, Tiwari and Rahimbaev (1992), investigating the effect of barley starch, agar and agarose used individually or in combination in anther cultures of barley, reported that a combination of agarose and barley starch provided a better gelling effect as it provided a firm gel surface throughout and prevented the sinking of tissues even after the enzymatic degradation of starch. The response on starch-supplemented media was always better than that with the agar-gelled media (Tiwari and Rahimbaev 1992). Likewise, Zimmerman et al. (1995) utilized corn starch in combination with gelrite in tissue cultures of some fruit crops. In their studies, starch-gelled media usually yielded better results. However, in some cultivars of pear, the response was significantly reduced because of the hyperhydricity of the explants, which could be controlled only partially using hydration control agents. Another problem with starch-gelrite-gelled media was their opacity. Most recently, tapioca starch has been utilized as an alternative gelling agent for tobacco and chickpea cultures (Nene et al. 1996).

From the above, it becomes evident that the problems which may prevent the universal acceptance of starch as an alternative gelling agent are its inferior gelling quality, lower clarity than agar and metabolizable nature which leads to softening of the media during the culture period. Moreover, upon autoclaving, starches yield sugars which will have their own effect, osmotic or metabolic, on the response of cultures. As starches and their hydrolytic products are not biologically inert, they are expected to have limited use and that only for explants whose response is not adversely affected by the presence of starch in the medium.

The properties of 'Isubgol', including its polysaccharidic and colloidal nature, reported resistance to enzymatic activity, good gelling ability even in cold water, and reasonable clarity in gelled form, are indicative of its potential to become an universal gelling agent in tissue culture media. Its price in India is about one-eighth that of the agar used in this study and one-hundredth that of Difco Bacto agar.

The preparation used in the present study consisted of mucilage along with the husk. The husk even after autoclaving remained suspended and formed a gradient once the medium solidified. Despite this, the media remained reasonably transparent and offered no serious problems with respect to observations or photography of roots penetrating the medium. In comparison, the media gelled with the agar used in the present study appear almost opaque. However, this problem with 'Isubgol' gel can be overcome by using purified mucilage that is devoid of husk.

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