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BRIEF COMMUNICATION

Agar/galactomannan gels applied to shoot regeneration from tobacco leaves

N. LUCYSZYN*, M. QUOIRIN**, M.M. HOMMA* and M.-R. SIERAKOWSKI*[1]

Laboratório de Biopolímeros, Departamento de Química and Laboratório de Micropropagação Vegetal, Departamento de Botânica**, Universidade Federal do Paraná, CEP 81531-990, Curitiba, PR, Brazil*

Abstract

This study concerns the efficacy of partial agar substitution by galactomannans as support in plant regeneration media for *Nicotiana tabacum*. The production of multiple shoots from leaf-derived callus and their rooting were evaluated. The galactomannans applied were obtained from *Cassia fastuosa* (cassia) and *Cyamopsis tetragonolobus* (guar gum – a commercial galactomannan) seeds. The results obtained on media solidified with mixtures of agar/galactomannan (3 g dm^{-3} each) gels were compared with those on media gelled with a standard concentration of agar (6 g dm^{-3}). The *in vitro* performance allowed to conclude that the use of galactomannans raised the number of shoots and improved their quality. Furthermore, the length of roots and the size of leaves were significantly higher in the media solidified with agar/guar galactomannan mixtures.

Additional key words: alternative gelling agent, *Cassia fastuosa*, *Cyamopsis tetragonolobus*, guar gum, *Nicotiana tabacum*.

Agar is the most widely used solidifying agent for plant tissue culture media, but it is relatively expensive and in many studies differences have been reported in plant response to agar brands or types (Romberger and Tabor 1971, Stolz 1971, Koda *et al.* 1988). Several explanations have been given for the variable effects of agar, including limited diffusion of medium components and water (Romberger and Tabor 1971, Stolz 1971), presence of impurities (Nairn *et al.* 1995), difference in gel strength and mineral composition (Debergh 1983, Scholten and Pierik 1998). For these reasons and considering the high price of tissue culture grade agar, attempts have been made to identify suitable alternatives. A wide range of substances, *Gelrite*® (Podwyszynska and Olszewski 1995), starch (Zimmerman *et al.* 1995, Smýkalová *et al.* 2001), sago (Naik and Sarkar 2001), gum katira (Jain and Babbar 2002) and xyloglucan (Lima-Nishimura *et al.* 2003), has been tried as substitute or partial substitute for agar.

Galactomannans (GMs) represent an important group of plant gums present as storage polysaccharides in the seeds of numerous plants, particularly in the *Leguminosae* (Reid 1985). They contain a β-D-(1→4)-linked D-mannan backbone to which are attached single α-D-galactosyl units at O-6 of certain D-mannosyl residues (Dea and Morrison 1975). In Brazil native trees have been investigated for extraction of galactomannans (Lucyszyn 1994, Petkowicz *et al.* 1998). Among these is *Cassia fastuosa*, known as cassia, *Caesalpiniaceae* family, from which was extracted one of the polysaccharides used in this work as partial substitute for agar. Two GMs are produced commercially in great amounts: guar gum from *Cyamopsis tetragonolobus* and locust bean gum from *Ceratonia siliqua* for which the mannose:galactose ratios are 1.6:1 and 3.3:1, respectively (Maier *et al.* 1993) and, usually, the first GM is less expensive.

Tobacco (*Nicotiana tabacum* L.) has long been classified as a 'model species' for *in vitro* organogenesis studies. In the present study, the effects of the partial substitution of agar by GMs on *in vitro* regeneration from tobacco leaf explants, are reported.

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Abbreviations: BAP - 6-benzylaminopurine; GM - galactomannan; MS medium - Murashige and Skoog medium; η_{ap} - apparent viscosity.

* Author for correspondence; fax: (+55) 41 33613186, e-mail: mariarita.sierakowski@ufpr.br

A commercial guar from seeds of *Cyamopsis tetragonolobus* (L.) Taub. was imported from Indian Gum Industries, Jodhpur, India, and was provided by Herbarium S.A., Curitiba, Brazil. Agar was imported from Germany (*Merck*®). *Cassia fastuosa* Willd. seeds (50 g), collected in Curitiba, Paraná State, Brazil, were boiled in water for 20 min in order to inactivate the enzymes. The GM was obtained by exhaustive aqueous extraction (five times) from milled seeds and the residue was removed by centrifugation for 40 min at 5 922 *g*. The combined supernatants were evaporated to a small volume under reduced atmosphere at 40 °C. Then NaCl was added in order to obtain a concentration of 0.1 M and the polysaccharide was precipitated with commercial ethanol. The GM obtained was filtered through nylon cloth and dried at 25 °C.

Static rheological measurements were done on media solidified with several combinations of agar/guar galactomannan (6/0, 5.75/0.25, 5.5/0.5, 5/1, 4.5/1.5, 4/2, 3.5/2.5, 3/3, 2.5/3.5 and 2/4 g dm^{-3}, respectively) in order to test their gelling strength. After stabilization at 25 °C with the aid of a *Brookfield TC-500* (*Brookfield Engineering Laboratories*, Middleboro, MA, USA) circulating water bath, measurement of apparent viscosity of gels was carried out in a *Brookfield LVDV-III* rheometer, with *Helipath* spindle (T-F), at 0.2 rpm.

Tobacco seeds (*Nicotiana tabacum* L. cv. Amarelinho Maus) were surface-sterilized in 70 % ethanol for 30 s, then in 2.5 % NaOCl for 30 min, and rinsed 3 times in sterile distilled water. The sterilized seeds were placed to germinate on MS (Murashige and Skoog 1962) basal medium without growth regulator (MS_0) utilizing 6 g dm^{-3} agar as support. Leaf sections (6 to 8 mm diameter) were excised from 6-week-old seedlings and used as explants for *in vitro* experiments. For the media preparation with the agar/GM mixtures, the GM was dissolved in distilled water by stirring for 12 h at 25 °C and added to liquid medium. The pH of all media was adjusted to 5.8 with NaOH or HCl solution, the volume was checked and agar was added. The media were autoclaved for 20 min at 121 °C and 1.2 kg cm^{-2}, then cooled and kept for 4 - 7 d. All cultures were maintained in a growth chamber at 25 ± 2 °C under "daylight" fluorescent tubes providing an irradiance of 40 μmol m^{-2} s^{-1} with a 16-h photoperiod.

In the preliminary test of tobacco organogenesis, agar (6 g dm^{-3}) and three combinations of agar/guar galactomannan (5/1, 4/2 and 3/3 g dm^{-3}) indicating 15, 33, and 50 % of agar substitution, respectively, were chosen as support for culture media. The explants were inoculated into Petri dishes (90 × 20 mm) on culture medium containing MS mineral salts and organic compounds, sucrose (30 g dm^{-3}), and supplemented with 4.44 μM 6-benzylaminopurine (BAP) (MS_1). For definitive tests, the explants were inoculated into MS_1 medium solidified with agar (6 g dm^{-3}), agar/guar (3 g dm^{-3} each) or agar/cassia GM mixtures (3 g dm^{-3} each). The agar-gelled medium was the control. For the rooting phase, the shoots with a minimum length of 10 mm obtained in the control medium or in the medium with GM as partial substitute for agar were transferred into glass flasks (80 × 130 mm) containing 30 cm^3 of MS_0 medium (with and without GM) and covered with aluminum foil.

Shoot formation efficiency was evaluated after 30 d in culture by counting the shoot number and recording the mean fresh mass increase of the explants between the first and the last day of the culture. The percentage of rooted shoots, the number of roots per shoot and the mean root size were recorded after 10 d in the medium. For growth evaluation of the plantlets, a random selection of rooted plants was maintained in the rooting medium. After 30 d the number of leaves per explant and length of leaves were evaluated.

A completely randomized design was used for *in vitro* experiments. Every treatment consisted of eight experimental units (plates or flasks) with six explants per unit, and all were repeated at least twice. Data were submitted to one-way analysis of variance (*ANOVA*). Treatment means were compared by Tukey's test using *MSTAT* program (Michigan State University), version *2.11*. Significance was recorded at $P < 0.05$.

Table 1. Effect of agar and agar/guar galactomannan mixtures as support to tobacco organogenesis after 30 d of culture in MS_1 medium. Means were calculated from measurements of all shoots > 5 mm in height. Means of two independent experiments ± SD. Means followed by the same letter are not significantly different as indicated by the Tukey's test at $P < 0.05$.

Concentrations agar/guar GM [g dm^{-3}]	Apparent viscosity [mPa s]	Number of shoots [$explant^{-1}$]	Fresh mass [g]
6:0	951360	9.23 ± 1.65b	3.586 ± 0.37a
5:1	1021660	8.70 ± 1.13b	3.551 ± 0.26a
4:2	726408	10.20 ± 2.20ab	3.593 ± 0.51a
3:3	529575	12.48 ± 2.17a	3.903 ± 0.43a

The effect of several concentrations of guar galactomannan on the apparent viscosity (η_{ap}) of agar/guar mixtures was evaluated by the rheology measurements. The values of η_{ap} revealed that the best interaction between these polysaccharides occurred with 4 % of agar substitution (5.75/0.25 g dm^{-3}, agar/guar galactomannan, respectively) (data not shown). The η_{ap} value was much higher than that observed for the agar gel alone (6 g dm^{-3}), indicating that the substitution of a part of a normal gelling polysaccharide by a non gelling substance, in this case galactomannan, results in a strong physical interaction between both polysaccharides. Furthermore, 17 % of substitution (5/1 g dm^{-3}) gives an

Table 2. Effect of agar and agar/galactomannan mixtures as support to tobacco organogenesis after 30 d of culture in MS_1 medium. Means were calculated from measurements of all shoots > 5 mm in height. Means of three independent experiments ± SD. Means followed by the same letter are not significantly different as indicated by the Tukey's test at $P < 0.05$.

Concentrations [g dm^{-3}]	Number of shoots [explant^{-1}]	Fresh mass [g]
Agar (6)	8.83 ± 0.94c	2.99 ± 0.24b
Agar/guar GM (3/3)	13.30 ± 1.50a	3.98 ± 0.29a
Agar/cassia GM (3/3)	10.65 ± 0.59b	3.71 ± 0.19a

apparent viscosity similar to that of agar alone, and 50 % of substitution (3/3 g dm^{-3}) was found to be the maximum substitution which offers the minimal resistance necessary to support the explants on the medium surface.

The results of the effect of guar concentrations on tissue culture showed that a higher number of shoots per explant was obtained in the medium solidified with agar/guar mixture in the concentration of 3 g dm^{-3} each (with lower apparent viscosity), with statistically significant difference in comparison with the results obtained on the agar gelled medium at 6 g dm^{-3}. However, the fresh mass of the explants was similar on the different media (Table 1). Thus, this mixture of agar and guar was chosen for the following *in vitro* tests and the same proportion was applied to agar/cassia GM mixture. The first response of explants on these culture media (with or without agar substitution) was observed after 8 d of culture in all media. Explants became swollen and exhibited at least a 2-fold increase in diameter (data not shown). 30 d after transferring the explants to the MS_1 medium, greenish-white granular calli, with high number of shoots were produced on the three media. The regeneration capacity was not evenly distributed across the callus surface, and some explants did not show any evidence of regeneration. The number of shoots/explant and fresh mass increased significantly when agar/guar or agar/cassia GM mixtures were used in comparison with the medium solidified with agar only (Table 2). The shoots that developed on these media were transferred to the rooting medium gelled with agar (6 g dm^{-3}), agar/guar or agar/cassia mixture (3 g dm^{-3} each). In these media, all shoots rooted and grew normally. In the presence of agar/guar mixture, the root length was significantly higher than for explants cultured in medium with agar only or agar/cassia mixture (Table 3). For the number of roots and percentage of rooted shoots, no significant difference was observed between media prepared with the three gelling agents (Table 3). After 30 d, shoots elongated and developed into plantlets. It was observed that the size of leaves was greater in media where agar was partially substituted by guar GM (Table 4).

In the present study, explants grown on agar/guar or agar/cassia GM showed significant differences at the number of shoots, fresh mass, root length, and overall growth of leaves in comparison with that obtained on the control medium. The results obtained for all *in vitro* experiments can be related to the physical gel parameters, as a more viscoelastic medium was obtained when agar was combined with GM. The formation of a soft gel helps and/or facilitates the water and nutrients diffusion in the medium and, consequently, the growth of cells and shoots. This response is in agreement with the results related below.

Table 3. Effect of agar and agar/galactomannan mixtures as support to rooting of tobacco shoots after 10 d of culture in MS_0 medium. Means were calculated from measurements of all roots >10 mm in height. Means of two independent experiments ± SD. Means followed by the same letter are not significantly different as indicated by the Tukey's test at $P < 0.05$.

Concentrations [g dm^{-3}]	Rooted shoots [%]	Number of roots [explant^{-1}]	Length of roots [cm]
Agar (6)	76.63 ± 3.89a	4.70 ± 1.37a	3.28 ± 0.16b
Agar/guar GM (3/3)	82.50 ± 6.82a	3.90 ± 0.44a	3.80 ± 0.33a
Agar/cassia GM (3/3)	77.00 ± 9.06a	4.03 ± 0.92a	3.37 ± 0.27b

Table 4. Effect of agar and agar/galactomannan mixtures as support to growth of plants of tobacco after 30 days of culture in MS_0 medium. Means ± SD. Means followed by the same letter are not significantly different as indicated by the Tukey's test at $P < 0.05$.

Concentrations [g dm^{-3}]	Number of leaves [explant^{-1}]	Length of leaves [cm]
Agar (6)	7.75 ± 0.60a	3.12 ± 0.51b
Agar/guar GM (3/3)	7.75 ± 0.89a	3.91 ± 0.58a
Agar/cassia GM (3/3)	8.59 ± 0.83a	3.37 ± 0.36ab

Brown *et al.* (1979) observed an inhibitory effect in the formation of tobacco shoots when high levels of agar were used, and a concentration of 4 g dm^{-3} totally inhibited the emergence of shoots. According to the authors, a high concentration of agar in the medium could decrease pressure potential in the plant below threshold value for cell expansion. This stress condition would inhibit both callus growth and shoot formation. Owens and Wozniak (1991), analyzing the relationship between gel properties and the growth and regeneration of sugar beet leaf discs, identified two properties, matric potential and expressibility of the gel, that together appear to largely determine the water availability of gels. Matric potential refers to the tenacity with which water is held in the solid gel phase and expressibility to the ease with which water is expressed in response to mechanical deformation of the gel by the explant expansion and contortion during growth. These two physical properties can be related to the gel type and gelling agent concentration used in the culture medium.

Furthermore, galactomannans are stable to degradation during the plant culture period, which is favourable to use in micropropagation. Other wise starch, a relatively cheap polysaccharide, can be easily metabolized by the α-amylases presented in the medium, resulting in a gradual decrease in the consistency of the medium during the course of culture period. This can cause hyperhydricity and the consequent death of the explants (Zimmerman *et al.* 1995). So, this is the main reason for the limited use of starch as a gelling agent in tissue culture (Naik and Sarkar 2001).

The results from this work suggest that using agar/galactomannan blends as support, an efficient system for organogenic regeneration of tobacco plants from leaf-derived-callus can be established. Moreover, GMs are much less expensive than agar. In Brazil, guar gum costs approx. US$ 3 kg^{-1}, while agar varies between US$ 100-200 kg^{-1}. Since 50 % of the agar could be substituted in the propagation media, the cost would be 17 to 34 times lower. The combination of these polysaccharides in the semisolid medium offers a firm support for plant tissues and could be used for micropropagation of economically important species, when high levels of agar have inhibitory effects. The advantages of GM use, beside cost reduction, are the easy and high yield extraction from seeds of many native species.

References

Brown, D.C.W., Leung, D.W.M., Thorpe, T.A.: Osmotic requirement for shoot formation in tobacco callus. - Physiol. Plant. **46**: 36-41, 1979.

Dea, I.C.M., Morrison, A.: Chemistry and interactions on seed galactomannans. - Adv. Carbohydr. Chem. Biochem. **31**: 241-312, 1975.

Debergh, P.C.: Effects of agar brand and concentration on the tissue culture medium. - Physiol. Plant. **59**: 270-276, 1983.

Jain, N., Babbar, S.B.: Gum katira – a cheap gelling agent for plant tissue culture media. - Plant Cell Tissue Organ Cult. **71**: 223-229, 2002.

Koda, T., Ichi, T. Yamagishi, H., Yokshikawa, H.: Effects of phytohormones and gelling agents on plant regeneration from protoplasts of red cabbage. - Agr. Biol. Chem. **52**: 2337-2340, 1988.

Lima-Nishimura, N., Quoirin, M., Naddaf, Y.G., Wilhelm, H.M., Ribas, L.L.F., Sierakowski, M.-R.: A xyloglucan from seeds of the native Brazilian species *Hymenaea courbaril* for micropropagation of Marubakaido and Jonagored apples. - Plant Cell Rep. **21**: 402-407, 2003.

Lucyszyn, N.: Galactomananas: novas fontes do biopolímero e aplicações na indústria de alimentos. [Galactomannans: new sources of the biopolymer and applications in the food industry.] - Master Thesis. Federal University of Parana, Curitiba 1994. [In Port.]

Maier, H., Anderson, M., Karl, C., Maqnuson, K., Whistler, R.L.: Guar, locust bean, tara and fenugreek gums. - In: Whistler, R.L., Bemiller, J.N. (ed.): Industrial Gums: Polysaccharides and Their Derivatives. 3rd Ed. Pp. 215-218. Academic Press, San Diego - New York - Boston - London - Sydney - Tokyo - Toronto 1993.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Naik, P.S., Sarkar, D.: Sago: an alternative cheap gelling agent for potato *in vitro* culture. - Biol. Plant. **44**: 293-296, 2001.

Nairn, B.J., Furneaux, R.H., Stevenson, T.T.: Identification of an agar constituent responsible for hydric control in micropropagation of radiata pine. - Plant Cell Tissue Organ Cult. **43**: 1-11, 1995.

Owens, L.D., Wozniak, C.A.: Measurement and effects of gel matric potential and expressibility on production of morphogenic callus by cultured sugarbeet leaf discs. - Plant Cell Tissue Organ Cult. **26**: 127-133, 1991.

Petkowicz, C.L.O., Ganter, J.L.M.S., Sierakowski, M.-R., Reicher, F.: Galactomannans and arabinans from seeds of *Caesalpiniaceae*. - Phytochemistry **49**: 737-743, 1998.

Podwyszynska, M., Olszewski, T.: Influence of gelling agents on shoot multiplication and uptake of macroelements by *in vitro* culture of rose, cordyline and homalomena. - Scientia Hort. **64**: 77-84, 1995.

Reid, G.J.S.: Galactomannans. - In: Dey, P.M., Dixon, R.A. (ed.): Biochemistry of Storage Carbohydrates. Pp. 265-286. Academic Press, San Diego - New York - Boston - London - Sydney - Tokyo - Toronto 1985.

Romberger, J.A., Tabor, C.A.: The *Picea abies* shoot meristem in culture I. Agar and autoclaving effects. - Amer. J. Bot. **58**: 131-140, 1971.

Scholten, H.J., Pierik, R.L.M.: Agar as a gelling agent: chemical and physical analysis. - Plant Cell Rep. **17**: 230-235, 1998.

Smýkalová, I., Ortová, M., Lipavská, H., Patzak, J.: Efficient *in vitro* micropropagation and regeneration of *Humulus lupulus* on low sugar, starch-gelrite media. - Biol. Plant. **44**: 7-12, 2001.

Stolz, L.P.: Agar restriction of the growth of excised mature *Iris* embryos. - J. amer. Soc. hort. Sci. **96**: 618-684, 1971.

Zimmerman, R.H., Bhardwaj, S.V., Fordham, I.M.: Use of starch-gelled medium for tissue of some fruit crops. - Plant Cell Tissue Organ Cult. **43**: 207-213, 1995.