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RESEARCH ARTICLE

Effect of plant derived gelling agents as agar substitute in micropropagation of mulberry (*Morus indica* L. cv. S-1635)

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Abstract

The design of cost efficient tissue culture protocols is a prerequisite in the adoption of the low cost tissue culture technology in developing countries. Three plant derived gelling agents namely corn flour (*Zea mays var. amylacea*), Cassava powder (*Manihot esculenta*), arrowroot (*Maranta arundinaceae*) and their combinations with agar have been tested in search of an alternative and cost effective potential gelling agent for mulberry (*Morus indica* L. cv. S-1635) micropropagation. It was found that corn flour at 22 g l^{-1} in combination with 3.5 gl^{-1} agar produced significantly higher and healthy micro- shoots of length (4.57 ± 0.32 cm) among the treatments. The selected plant derived alternative gelling agents are easily available in the market and can be added with ease thereby, serving as inexpensive substitute of agar.

Introduction:

Mulberry is a deciduous woody perennial belonging to Genus *Morus* and family Moraceae. It is cultivated largely in Asian countries for its foliage and considered as the main food for silkworm (*Bombyx mori*). Mulberry is a heterozygous, cross-pollinated plant with relatively long juvenility due to its tree nature. Like other trees, mulberry also needs a long time (~15 years) to develop a desirable variety through conventional breeding approach (Ravindran *et al*., 1988). It is conventionally propagated through stem cutting clones and rarely graftings. Propagation via cutting is highly restricted to certain months of the year and is labour intensive. Micro propagation could provide us an alternative to the conventional vegetative propagation of mulberry with desirable traits (Thorpe, 1983).

Tissue culture methods are being used increasingly as an adjunct to traditional propagation methods for rapid regeneration and multiplication of improved mulberry cultivars. A continued research strategy is urgently needed to increase the economic feasibility and thereby the adaptability of the developed tissue culture protocols for mulberry. The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of the season and weather. However, the technology is capital, labor and energy intensive. Hence, it is necessary to have low cost options for micropropagation. The main criteria that limits the application of micropropagation of mulberry in commercial scale is the cost involved in the technique.

Agar is one of the most expensive and commonly used gelling agents in tissue culture media, contributing about 70 % of the total production cost (Prakash, 1993). Gelling agents are usually added to the culture medium to increase its viscosity as a result of which plant tissues and organs remain above the surface of the nutrient medium (Prakash *et al*., 2000). Exclusive use of agar is resulting in over exploitation of its resources (Jain and Babbar, 2005; Deb and Pongener, 2010) and makes it essential to look for other alternative and cheap sources to make tissue culture techniques economically feasible. Although agar was thought biologically inert and non-toxic, its adverse effects have been reported (Debergh, 1983). Naik and Sarkar (2001) used sago as cheaper gelling agent for potato regeneration. Gebre and Sathyanarayana (2001) studied potato regeneration, using commercial cassava and sago as gelling agents. Jain and Babbar. (2005) used isabgol at 3 per cent for both bacterial and fungal culture. Kuria., *et al* (2008) reported that the use of 10% cassava starch reduced cost by 42.5% in comparison with use of agar in

micropropagation of potato. Zimmerman *et al*. (1995) reported that medium gelled with corn starch at 50 g l-1 plus gelrite at 0.5 g l-1 was just as effective as medium gelled with agar for shoot proliferation of apple and raspberry.

The present study includes preliminary screening of gelling potential and further evaluation of the alternative low cost agar substitutes in tissue culture medium for mulberry micro-propagation. Nodal explants of mulberry (*Morus indica* L.cv S-1635) are used to evaluate three alternate cheap gelling agents namely corn flour, arrow root powder and tapioca flour to substitute the expensive agar in tissue culture media. The study envisages the possibility of reducing the expense on media in mulberry tissue culture.

The objective of this study was to evaluate the potential of three plant derived gelling agents as agar substitute in micropropagation of mulberry (*Morus indica* L. cv. S-1635) using nodal cuttings.

MATERIALS AND METHODS

Plant materials:

The study was carried out at the plant tissue culture laboratory of CSR & TI Berhampore. Nodal cuttings of mulberry variety S-1635 (*Morus indica* L.cv S-1635) at 40-45 days old shoots, after removing the leaves, the nodal region measuring about 3-4cm each containing an axillary bud from the shoot was used as explant.

Surface Sterilization:

The nodal explants were washed in running water for 2-3 times and again washed with mild detergent and rinsed thoroughly with distilled water. The explants were then surface sterilized by keeping them in 70% ethanol for 10 minutes. After washing 2-3 times with water, they were treated with 0.1% (w/v) of Bavistin for 15 minutes. They were washed with distilled water 2-3 times. Subsequently, the explants were kept in 0.1% (w/v) of $HgCl_2$ for 15 minutes. Finally the explants were serially washed 2-3 times again with distilled water before inoculation.

Preparation of MS nutrient medium:

Three plant derived gelling agents viz., corn flour (*Zea mays var. amylacea*), tapioca flour (*Manihot esculenta*) and arrow root powder(*Maranta arundinaceae*) were used instead of agar with Murashige and Skoog basal salts (1962) (MS) supplemented with 3% (w/v) sucrose and 2 mg/l 6-Benzyl amino purine. For the tissue culture medium, 0.7% agar was maintained as control, corn flour at 4.4% and a composite mixture consisting of 2.2% corn flour mixed with 0.35% agar, cassava powder at 7.2% and a composite mixture consisting of 3.6% corn flour mixed with 0.35% agar, arrow root powder at 6% and a composite mixture consisting of 3% corn flour mixed with 0.35% agar. The gelling rates were formulated based on prior preliminary investigations. Corn flour, cassava powder and arrow root powder was incorporated as thick slurry into preheated medium (pH adjusted to 5.8) prior to autoclaving. Ten millilitre of culture medium was dispensed in 20 mm test tubes and covered using cotton plugs preceding autoclaving at 1.06 kg cm^{-2} and 121^{o}C for 15 min.

Inoculation and incubation

Inoculation was carried out in a sterile laminar airflow hood chamber. Nodal cuttings were prepared to size of 2cm using sterile blade and forceps. Single nodal cuttings with an axillary bud were inoculated into a test tube containing 10 ml MS medium amended with different gelling agents. These were incubated at 25 ± 2^{0}C under a 16 h photoperiod with a photosynthetic photon flux density of 60 µmol m^{-2} s^{-1} provided by overhead cool fluorescent lamps (Philips, India 30 Watts) for 30 days.

Data collection

Collection of data on various parameters like number of days taken for bud breaking, number of multiple shoots, first formed shoot length, mean shoot length, no of nodes in first formed shoot and total no of leaves at 30 days after inoculation in the respective shooting media with gelling agent to evaluate *in vitro* plantlet performance. Further, bud breaking percentage and survival percentage in shooting media were assessed to evaluate the different gelling agents under study. The vigour of the plants were visually scored on 0-5 scale (Kuria *et al*., 2008). A cost analysis benefit of the different plant derived media gelling agents was also done.

Experimental design and data analysis

An experiment in a completely randomized block design with six treatments and a control with agar was conducted with three replicates per treatment containing ten test tubes per experimental unit. Data was subjected to analysis of variance (ANOVA) to test the significance of the differences between treatments and means were separated using the Least Significant Difference at $p < 0.05$. Bar diagrams were computed for interpretation of experimental results. The photographic illustrations were also given wherever necessary.

RESULTS

Medium clarity

Corn flour and arrow root gelled medium were opaque and dense white in appearance (Plate1d &f) while cassava starch gelled medium had poor clarity (Plate1e) compared to agar (Figure 1a,b,c). Clarity of plant tissue culture media is important for prompt detection of microbial contamination. Clarity of all the three plant derived gelling agents satisfactorily improved with addition of 0.35% agar (Plate1.g,h,i) and it also improved gel strength. Plate 1.a,b,c shows the bud initiation, bud breaking and shootlet formation in agar.

In vitro shoot growth parameters

Analysis of variance revealed that there was no significant difference in the number of days taken for bud breaking among the treatments and control (Table 1). The bud breaking and survival percentage of *in vitro* plantlets was not significantly different ($p < 0.05$) in media gelled with corn flour, corn flour mixed with agar and that gelled with agar. *In vitro* plantlets cultured on either corn flour or corn flour mixed with agar were not significantly different ($p < 0.05$) in terms of number of multiple shoots per explant, primary shoot length, mean shoot length, no of nodes and leaves with respect to the plants cultured on agar alone. Results revealed that corn flour at 22 g l^{-1} in combination with 3.5 gl^{-1} agar produced significantly higher micro- shoots of length (4.57 ± 0.32 cm) among the treatments. *In vitro* plantlet vigour of the corn flour based treatments were statistically on par with agar. The plantlets produced in media gelled with corn flour and agar combination were vigorous attaining a mean score of more than 4 on a "subjective" scale (Fig 2).

Observations on frequency of shoots, nodes and leaves produced on various treatments also revealed the better performance of corn flour gelled media. The results revealed that both the treatments of corn flour (T1 and T2) are equally as good as that of agar with respect to *in vitro* shoot growth parameters. The survival percentage of the micro-shoots of the said treatments recorded at 30 days after inoculation is represented graphically in figure 1. The maximum survival of *in vitro* shootlets were observed in T2 (83.33%) among the treatments and found statistically on par with T0 (86.67%). The healthy plantlets obtained were sub cultured in standardized *in vitro* rooting media fortified with 2 mg/l of IBA and rooted plants were acclimatized and established.

Healthy growth of *in vitro* plantlets on corn flour gelled medium is an indication of incorporating corn flour as potential agar substitute in mulberry plant tissue culture.

TABLE 1: EFFECT OF DIFFERENT AGAR ALTERNATIVES ON *IN VITRO* SHOOTING OF MULBERRY VARIETY S1635.

Treatments (Gelling agents- w/v %)	No of days taken for bud breaking (days)#	Shoot growth parameters (per explant)							
		No of multiple shoots (no)¤	Length of the first formed shoot(cm) *	No of nodes on the first formed shoot (no) *	Vigour of plantlets (visual scoring 0-5) ¤	Mean shoot length (cm)¤	Leaves (no) ¤	Characteristics	
								Shoot	Leaves
Agar-0.7 % (T0)	7.52 ± 0.48a (3-12)	2.83 ± 0.23a	5.79 ± 0.42a	4.69 ± 0.28a	4.03 ± 0.13a	4.29 ± 0.34a	7.79 ± 0.42a	Greenish healthy	Normal broad
CF- 4.4 % (T1)	7.85 ± 0.67a (3-13)	1.96 ± 0.19a	4.48 ± 0.43a	3.88 ± 0.31a	3.65 ± 0.17a	3.53 ± 0.23a	6.12 ± 0.26a	Pale green healthy	Crinkled narrow
CF -2.2% + Agar- 0.35%(T2)	**6.79 ± 0.44a (4-10)**	**2.75 ± 0.22a**	**4.57 ± 0.32a**	**4.68 ± 0.25a**	**4.11 ± 0.13a**	**3.28 ± 0.22a**	**6.68 ± 0.34a**	**Greenish healthy**	**Normal broad**
TF -7.2% (T3)	7.90 ± 0.87a (4-18)	1.55 ± 0.14b	2.10 ± 0.17b	2.20 ± 0.13b	2.45 ± 0.10b	1.93 ± 0.07b	3.40 ± 0.19b	Pale green stunted	Normal small
TF 3.6% + Agar-0.35%(T4)	6.96 ± 0.59a (5-9)	1.61 ± 0.17a	2.17 ± 0.19b	2.30 ± 0.10b	2.43 ± 0.11b	1.94 ± 0.09b	3.43 ± 0.16b	Pale green stunted	Normal broad
AR- 6.0% (T5)	5.32 ± 0.49a (4-9)	1.68 ± 0.16a	1.92 ± 0.17b	1.77 ± 0.13b	2.32 ±0.10b	1.68 ± 0.08b	3.09 ± 0.22b	Greenish stunted	Crinkled narrow
AR -3.0% + Agar 0.35% (T6)	5.33 ± 0.54a (2-9)	1.52 ± 0.15b	2.21 ± 0.12b	1.18 ± 0.20b	2.62 ±0.12b	2.02 ± 0.12b	3.90 ± 0.22b	Greenish stunted	Normal broad
$LSD_{(p=0.05)}$	3.84	1.23	1.96	1.42	0.86	1.30	1.87		

#Data given in parenthesis is range of duration for bud breaking.

*Observation taken after 14 days of inoculation in respective treatment (media).

¤ Observation taken after 30 days of inoculation in respective treatment (media).

Means followed by the same letter within the column are not significantly different at $P < 0.05$ using least significant difference.

CF – Corn flour; TF – Tapioca flour; AR- Arrow root powder.

PLATE 1. *In vitro* shooting response of nodal explants of mulberry S1635 in MS media gelled with agar and alternative sources of agar

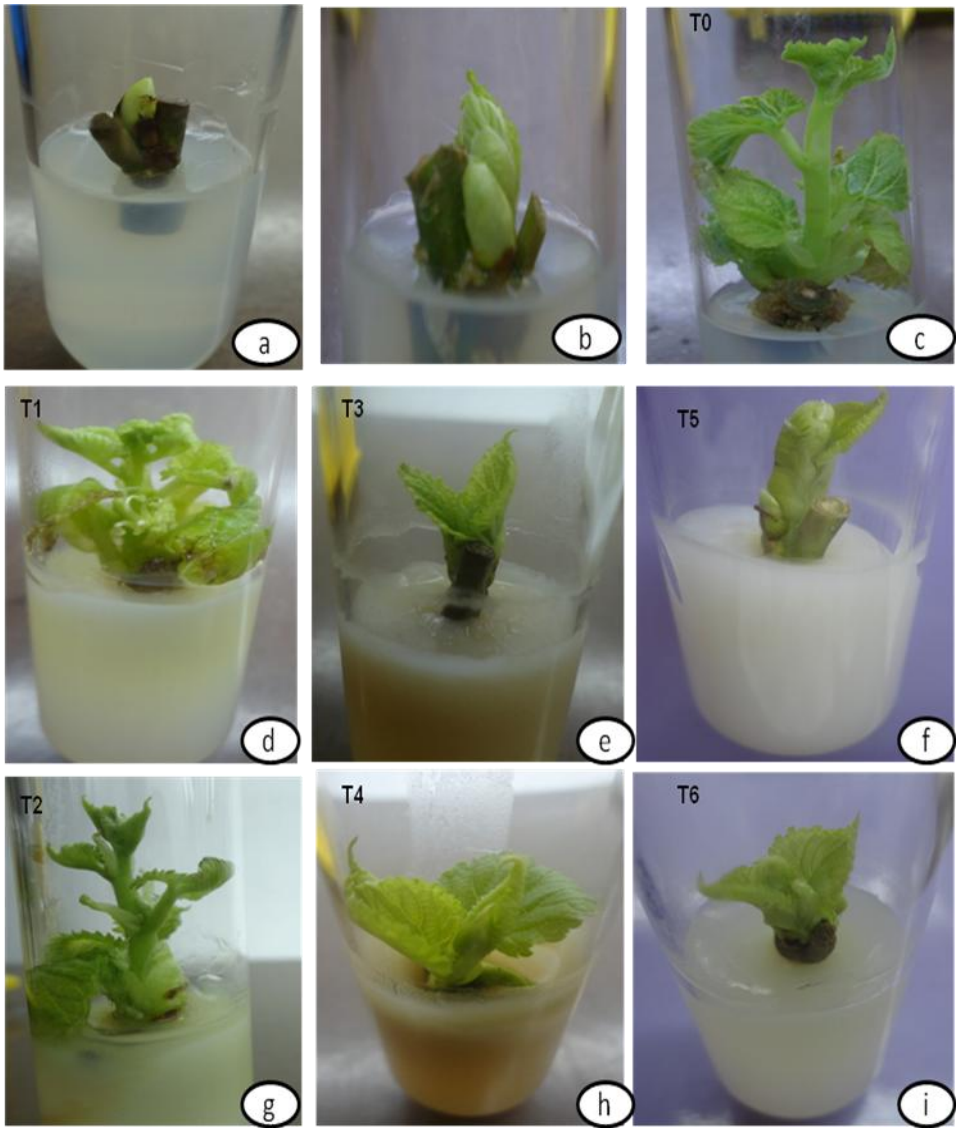


Figure 1: Effect of different gelling agents on bud breaking and survival percentage of *in vitro* culture of mulberry S1635

j. Callus formation observed in nodal explants inoculated in corn flour gelled media

k. *In vitro* shooting in 2.2% (w/v) corn flour and 0.35% (w/v) agar gelled media in comparison with the culture in 0.7%(w/v) agar gelled media.

l. *In vitro* flowering response of S1635 in 4.4% (w/v) corn flour gelled media

Figure 1: Effect of different gelling agents on bud breaking and survival percentage of *in vitro* culture of mulberry S1635

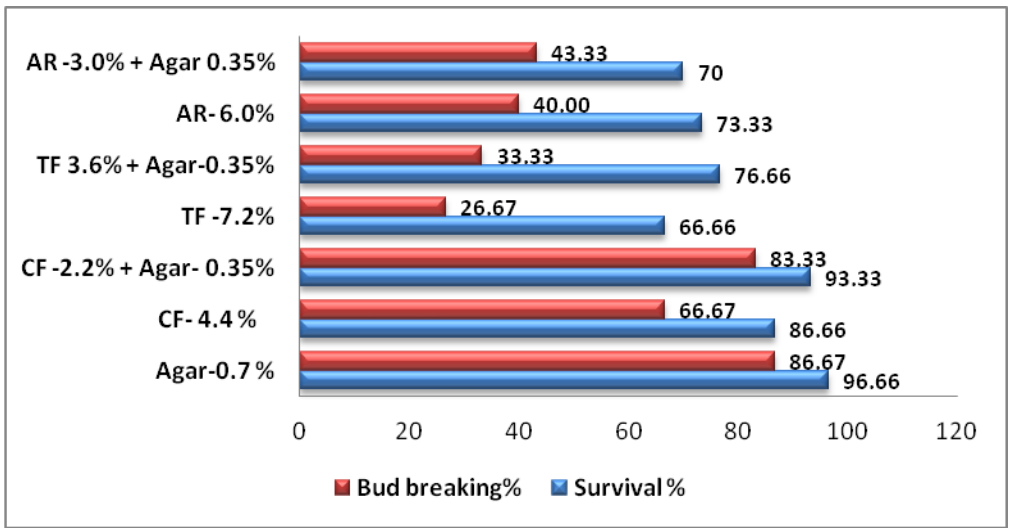
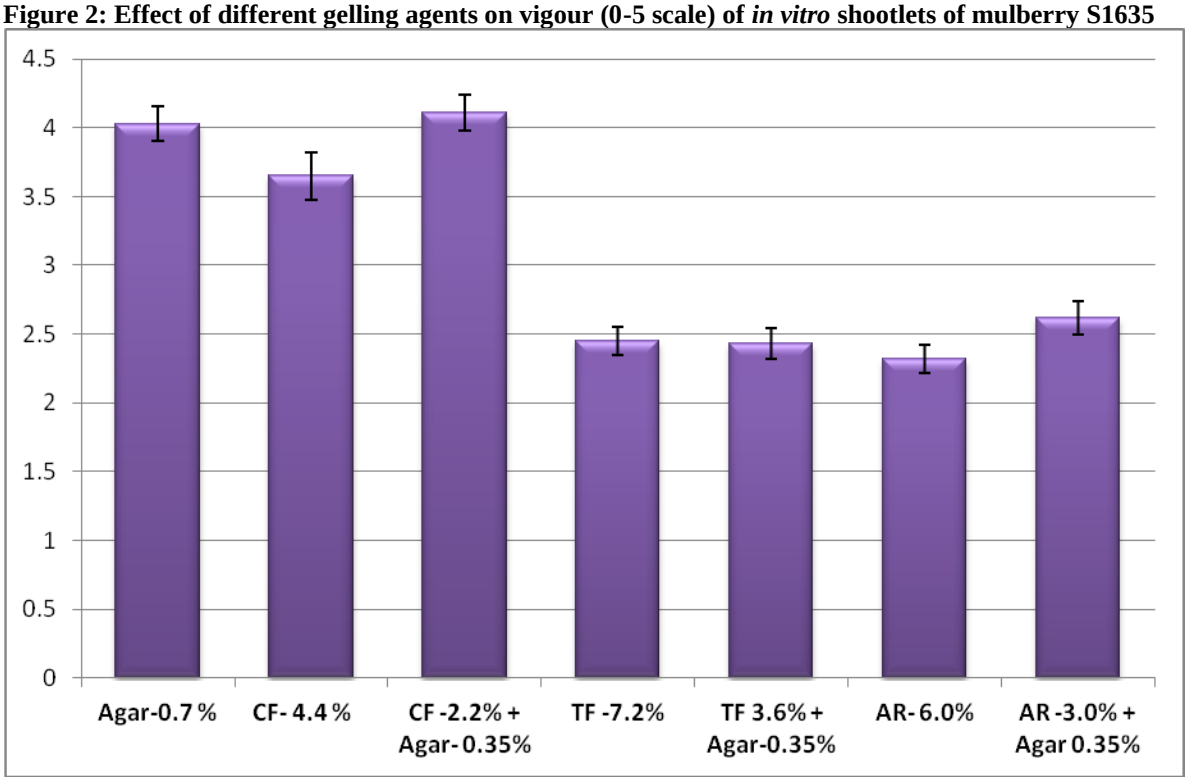


Figure 2: Effect of different gelling agents on vigour (0-5 scale) of *in vitro* shootlets of mulberry S1635



DISCUSSION

Comparative studies of mulberry micro-propagation in MS medium gelled with three plant derived gelling agents revealed high bud breaking and survival percentage (figure1) of nodal sections in corn flour at 22 g l^{-1} in combination with 3.5 gl^{-1} agar. The higher mortality of plantlets in other treatments was attributable to physiological anomalies evidenced by pale green, narrow wrinkled leaves and stunting of ramlets often referred as hyperhydricity. Rani and Singh (1999) reported several factors associated with hyperhydricity such as the level of cytokinin, low light, type of culture vessels, length and number of sub-cultures. Various remedies to hyperhydricity have been reported including use of solidified media with high concentration of gelling agent or a gelling agent with higher gel strength (Debergh *et al*., 1992).

The *in vitro* shoot growth parameters of nodal explants cultured in corn flour with agar gelled media showed statistically insignificant difference between the cultures grown on agar gelled media (Table 1). Healthy and vigourous plantlets with broad green leaves formed in MS media gelled with corn flour and agar was found to be the best one among the treatments under consideration (Plate2j). The results are in agreement with earlier findings by Mohamed *et al*. (2010) in micropropagation of potato. Vigorous growth was observed in cultures of corn four with agar gelled media (Fig.2) as that of cultures in agar alone. Vigorous growth also was an indication of production of high quality plantlets through low cost tissue culture methods. Growth and multiplication of shoot is not hindered in corn flour based gelling combinations of media. However media gelled with arrow root and cassava flour developed significantly lower plantlets as compared to corn flour and agar gelled media.

The results of this study suggest that using corn flour instead of agar as solidifying agent is efficient for mulberry micropropagation from single node. The combination of low concentration of agar 0.35% (w/v) with corn flour 2.2% (w/v) could offer a good supporting surface for mulberry micropropagation. A significant cost reduction of 42.95% is possible by replacing agar with corn flour and agar combination as experimented.

The selected plant derived alternative gelling agent is easily available in the market and can be added with ease, as inexpensive substitute of agar. Tissue culture technology offers an alternative for enhanced rates of multiplication. The technology is, however costly resulting in low adoption rates in developing countries. The design of cost efficient tissue culture protocols is a prerequisite in the adoption of the low cost tissue culture technology in developing countries.

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