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Influence of Gelling Agent on Micropropagation Cost and *in Vitro* Conservation of Turmeric (*Curcuma longa*) Germplasm

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ABSTRACT

To examine the effect of different gelling agents on micropropagation and cost effective *in vitro* conservation of *C. longa* cv. Sona cultures, six gelling agents viz. 7 g l^{-1} Agar (Himedia PT Pure), 2.5g l^{-1} Clarigel (Himedia), 4.5g l^{-1} Clarigar (Himedia), 6g l^{-1} Gelzen (Sigma), Isabgol 3.5g l^{-1} (Baidyanath) and 2.5g l^{-1} Phytagel (Sigma) were tested. Shoot bud explants excised from *in vitro* established cultures were inoculated on basal salts of Murashige and Skoog (MS) medium supplemented with 2.5 mg l^{-1} BAP + 3% sucrose. Highest rate of shoot multiplication without hyperhydric transformation was recorded with 2.88± 0.03 shoots/ explant in the cultures grown on media solidified with Clarigar, compared to regularly used gelling agent agar (2.31± 0.38). After 12 months of conservation, highest 85% survival of cultures was also recorded in the medium solidified with Clarigar, whereas only 50% of cultures survived on agar supplemented media. Regenerated plantlets were successfully acclimatized and produced healthy rhizomes using soil: sand: Farm Yard manure (2:1:1) with 85% survival.

Key words: Turmeric, micropropagation, *in vitro* conservation, gelling agents

INTRODUCTION

Turmeric (*Curcuma longa* L.), belongs to family Zingiberaceae is an industrially important spice crop widely cultivated in India. Tissue culture technique can be utilised for the propagation and in vitro conservation of vegetatively propagated crops, in vitro plantlet regeneration and medium-term conservation has been optimised in a few Curcuma sp. (Tyagi *et al*., 2004). Agar is the most popularly used gelling agent for micropropagation and conservation of vegetatively propagated germplasm in *in vitro* (Babbar and Jain, 2006), because of its convenient gelling properties, stability & resistance to metabolism during use (Henderson and Kinnersley, 1988). Agar is extracted from red algae *Geladium*, *Gracilaria* and *Pterocladia* etc., collectively referred to as agarophytes, it is a solidify medium or a support matrix is almost indispensible for good shoots & roots regeneration. Bhattacharyya *et al.* (1994) stated that use of low-cost gelling agent & support matrices for industrial scale plant tissue culture were found satisfactory & could compare well with that of agar contain medium. In recent time, a wide range of substances, viz. carrageenan, ficoll, agarose, gelrite, gum katira, isabgol, & starches have been tried with limited success as substitute for agar (Jain and Babbar, 2002). The importance of using pure grade gelling agents in experimental tissue culture work is widely acknowledged. Lower grade gelling agents may contain various ions, sulfonated polysaccharides and long fatty acids, but their effect on plant growth *in vitro* is unknown (Puchooa *et al*., 1999). The design of cost efficient in vitro conservation protocols is a prerequisite in the adoption of the low cost tissue culture technology in developing countries. Because tissue culture grade agar is the most expensive component of plant tissue culture media, Here in, attempts have been made to identify cost effective alternative of agar for in vitro morphogenic responses and conservation. The effects of different gelling agents were assessed with respect to high shoot multiplication and *in vitro* conservation of *C. longa* cv. Sona without the occurrence of hyperhydracity of cultures.

MATERIALS AND METHODS

Sona is a high yielding, high curcumin content variety of *C. longa* having bold, orange colour, without tertiary fingers rhizome released through clonal selection from the germplasm collection of Chundakuzhy in Ernakulum Kerala Agricultural University in 2006. In vitro shoot cultures were maintained in Tissue Culture repository at NBPGR, New Delhi since 2010-11 through periodic subculture of 6-8 months interval on MS basal salts supplemented with 2.5 mg l^{-1} BA + 3% sucrose (w/v) + 0.8% Agar (w/v). To obtain the sufficient stock of *in vitro* shoot tip explants (basal 1-2 cm swollen part of shoot containing shoot bud) were used for present stud. For initiating the experiment on micropropagation and conservation, repeated subculturing of stock cultures was done every 3-4 weeks interval on the above medium. For entire experiment on cost effective *in vitro* propagation and conservation, explants were excised from 4- weeks old cultures maintained on above stated medium. Leaves and roots were removed. Shoot bud explants (1-2 cm long) of *C. longa* L. cv. Sona were cultured on modified Murashige and Skoog (1962) media supplemented with 2.5 BAP mg l^{-1} and solidified with six different gelling agents viz., 7g l^{-1} Agar (Himedia PT Pure), 2.5g l^{-1} Clarigel (Himedia), 4.5g l^{-1} Clarigar (Himedia), 6g l^{-1} Gelzen (Sigma St. Louis, MO, USA), 3.5g l^{-1} Isabgol or Psyllium, the husk obtained from the seeds of Plantago ovate forsk. Family Plantaginaceae (Sat Isabgol™ Baidyanath, Sidhpur, Gujarat, India) and 2.5 g l^{-1} Phytagel

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(Sigma St. Louis, MO, USA), has been successfully used as gelling agent in plant tissue culture media (Babbar and Jain, 1998). All the chemicals used for media preparation were of AR (Analytical reagent) grade Himedia, BAP was procured from Sigma St. Louis, MO, USA.

Cost analysis

The existing cost of all the gelling agents (Agar, clarigar, Clarigel, gelzen phytagel and Isabgol) at the time of conducting the experiment were taken to calculate cost of each medium. Calculation for cost was done in Indian rupees (INR) by taking the actual amount (on weight basis) of each gelling agent for making one liter of final medium.

Establishment of in vitro derived plants/ *ex vitro* acclimatization

For hardening, plantlets from eight weeks old *in vitro* grown cultures were take out carefully from culture tubes, washed under running tap water to remove adhering gelling agent (Fig. 1C) and transferred into hardening plastic tray containing sterilized soilrite (Fig. 1D). The plants were covered with perforated transparent polyethylene bags (20 × 30 cm) to facilitate aeration and maintain humidity (70-80%). The plants were kept at 25±2°C and 10-h photoperiod for 1 week under light provided by incandescent bulbs with irradiance of 10 µ mol m^{-2} s^{-1}. Plantlets were irrigated with ¼ MS salts up to two weeks periodically. Polyethylene bags were removed slightly after 1 week and completely after 2 weeks to expose the plants to ex vitro conditions. After 3-4 weeks, the plantlets were transferred to earthen pots (12 inch diameter) filled with potted soil, sawdust and FYM mixture (2:1:1). Observations were recorded for number of plants survived out of total number of plantlets subjected to hardening for calculation percent survival of cultures.

Culture conditions

The pH of the entire tested medium was adjusted to pH 5.8 with 0.1 M NaOH or 0.1 M HCl prior to addition of the gelling agent and sterilization. For Isabgol supplemented media, isabgol was suspended in distilled water at room temperature by vigorous stirring with a glass rod. Approximately 20 ml of the medium was dispensed into each culture tube (25 × 150 mm, Borosil, Mumbai, India). The medium was autoclaved at 1.05 kg/$cm^2$and 121°C temperature and 1.06 kg cm^{-2} pressure for 20 min. The cultures were incubated at 25 ± 2°C and a light irradiance of 40 µmol m^{-2} s^{-1} provide by cool white fluorescent lamps (Philips, Mumbai, India) under 16-h light condition. Each culture tube received one explant. After raising cultures aseptically, culture tubes were enclosed with polypropylene caps (Tarsons, India) and sealed with parafilm to prevent dehydration of media and cultures.

Observations and statistical analysis

In all the experiments conducted, for each treatment 4 replicated consisting of 6 cultures each were used, which was replicated twice. Therefore the data presented are as mean standard error (SE) value of 48 cultures. During the entire investigations, for regeneration of cultures observations were recorded after 15 days interval up to 60 days after inoculation. Number of shoots was counted from each explant and expressed as shoots per explant and average was worked out. The shoot length was measured between the base and tip of longest shoot in test tubes and average was worked out. Total number of leaves produced in each cultures tube was counted and divided by no. of shoots, expressed as mean no. of leaves. While subculturing, roots were removed and number of roots were counted from each culture tube and mean number of roots per explant was expressed by dividing with no. of shoots. The root length was measured from base to the apex in test tubes in centimetres. Estimation of the health of cultures was done on the basis of survival of shoots. For a given culture, the period of conservation was computed from initiation of cultures to survival of shoots when the upper leaves showed chlorosis but the lower leaves and basal shoot portion containing shoot tip remained green. The data were recorded at every 2-month interval. All data were subjected to Standard deviation (SD) calculated to determine the statistical significance of means of different treatment.

RESULTS AND DISCUSSION

Comparative studies on micro-propagation of *Curcuma longa* cv. Sona in MS medium gelled with six gelling agents revealed that the highest shoot multiplication (Table 1) and survival percentage (Table 2) of shoot bud explants in clerigar at 4.5g l^{-1} in comparison with 7.0 g l^{-1} agar. Maximum 2.88 shoots ± 0.03 shoot per explant was recorded in the medium gelled with clerigar and the minimum 1.58 ± 0.02 shoots per explant was recorded in Isabgol gelled medium in 8-week old culture (Fig. 1A).

Table 1: Effect of gelling agents on shoot multiplication of *C. longa* cv. Sona (after 8 weeks in inoculation)

Medium MS + 2.5 mg l^{-1} BAP +	No. of shoots/ explant	Shoot length (cm)	No. of leaves	No. of Roots/ shoot	Root length (cm)
0.7% Agar (Control, C)	2.41 ± 0.38	1.0 - 8.0	2.52 ± 0.43	2.46 ± 0.45	0.5 - 3.0
0.25% Clarigel (CG)	2.04 ± 0.31	1.0 - 8.0	2.66 ± 0.27	1.85 ± 0.04	0.5 - 3.0
0.45% Clarigar (CLG)	2.88 ± 0.03	2.0 - 8.0	4.99 ± 0.14	2.66 ± 0.26	1.0 - 4.0
0.6% Gelzen (GZ)	2.08 ± 0.26	1.0 - 6.0	3.25 ± 0.33	2.48 ± 0.05	0.5- 2.0
3.5% Isabgol (IS)	1.58 ± 0.02	1.5 - 4.0	2.61 ± 0.21	1.07 ± 0.32	1.0 - 3.0
0.25% Phytagel (PG)	1.79 ± 0.04	1.5 - 4.0	1.79 ± 0.16	0.04 ± 0.07	0.0 - 0.5

While the most popular used gelling agent agar produced 2.41± 0.38 shoots after 60 days of incubation with the same growth regulator composition. In this study, shoot multiplication was found to occur by development of axillary buds, which is ideal for maintaining genetic stability. In all the accessions of *Curcuma* germplasm shoot buds started sprouting within 6 to 8 days and 1-2 cm long shoots were obtained after 2-weeks of culture. Established cultures were used for further regeneration and conservation studies. 2 to 3 shoot buds were initiated from a single shoot bud explant cultured on multiplication medium, and developed into shootlets after 3-4 weeks of culture along with formation of new shoot buds. No further increase in number of shoots was observed after 4-5 weeks of culture, frequency of shoot multiplication varied significantly among accessions. Shoot length among the treatments ranged between 1.00 to 8.00 cm in 8-week-old culture. Number of leaves per explant was ranged between 4.99 to 1.79 in the tested gelling agents. Development of roots was observed after 4 weeks of inoculation in all the tested gelling agents except Phytagel. The highest 2.66 ± 0.26 roots/ explants were recorded in the culture grown in clerigar gelled medium and the lowest 0.04 ± 0.07 in the cultures inoculated on Phytagel medium. There were non-significant difference in root length per explant

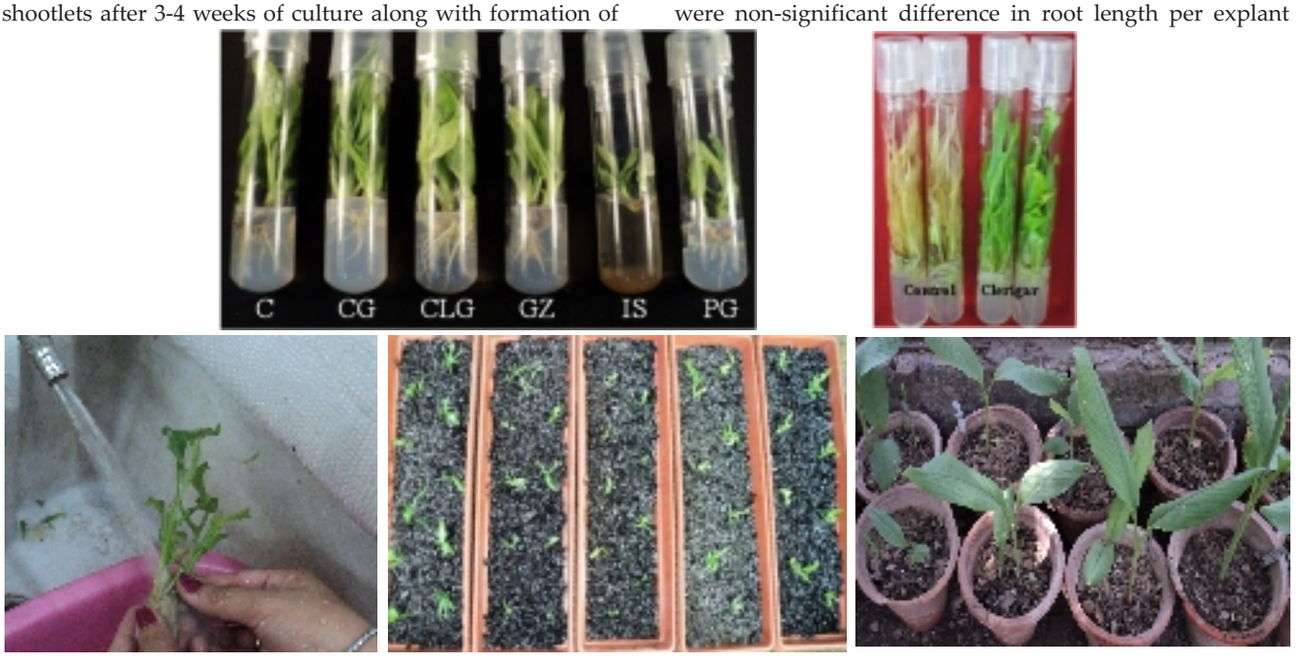

Fig: 1 (A) Representative Cultures in different gelling agents (B) 8-months old *in vitro* conserved cultures in Agar and Clerigar gelled medium (C) Removal of gelling agent for hardening (D) Cultures in soil rite during hardening process (E) Hardened plantlets

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 clerigar gelling media. However media gelled with clerigel and gelzen developed significantly lower plantlets as compared to clerigar and agar gelled media. Highest in vitro conservation period was observed in cultures on clerigar gelled media compared to cultures in agar.

Gelling agents are usually added to the culture medium to increase its viscosity as a result of which plant tissues remain above the surface of the nutrient medium. Among the various brands of gelling agent available commercially under different trade names viz., Agarose, Gellan gum, Gel-Gro, Gelrite, Isabgol and Phytagel being used for preparation of plant tissue culture media, agar is the most commonly used one. The growth of cultures and production of shoots or roots is strongly influenced by the physical consistency of the culture medium. Many agars are extracted from the seaweed (*Gracilaria* algae) by treating it with a strong alkali solution (NaOH). 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, 1983).

Table 2: Conservation period and percent survival of cultures

Medium MS + 2.5 mg l−1 BAP +	Conservation Period (Months)	*Survival (%)
0.7% Agar (C)	8	50
0.25% Clarigel (CG)	6	70
0.45% Clarigar (CLG)	12	85
0.6% Gelzen (GZ)	7	75
3.5% Isabgol (IS)	6	70
0.25% Phytagel (PG)	6	65

**After 12 months of Inoculation*

After 12 months of in vitro conservation, about 50 - 85% (Fig. 1B) of the plantlets survived in the medium gelled with different gelling agents. The in vitro derived clumps of plantlets in sterile soilrite mixture showed 95% survival rate after 4 weeks of hardening. New laves were also developed from in vitro shoots after 35 - 40 days of transfer in 2:1:1 ratio of soil: FYM: sand (Fig. 1E).

Table 3: Cost analysis of gelling agent

Gelling agent	Amount (gL -1)	Cost/ litre (Rs.)
0.7% Agar (C)	7.00	32.60
0.25% Clarigel (CG)	2.5	42.12
0.45% Clarigar (CLG)	4.5	28.46
0.6% Gelzen (GZ)	6.0	239.68
3.5% Isabgol (IS)	3.5	2.10
0.25% Phytagel (PG)	2.5	81.73

It contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers (Debergh, 1983). Cheaper alternatives to agar include various types of starches and plant gums (Pierik, 1989, Nagamori and Kobayashi, 2001). Gelrite can be replaced with starch-Gelrite mixture (Kodym and Zapata, 2004). Some gelling agents contain inhibitory substances that hinder morphogenesis (Powell and Uhrig, 1987), and reduce the growth rate of cultures. Moreover, toxic exudates from the cultured explants may take a longer time to diffuse. Media solidified with gelling agents increase the time to clean the culture containers.

For micropropagation of fruit trees, such as apple, pear and raspberry, banana, and sugarcane, ginger and turmeric, corn starch @ 50.0 g/l has been used as a gelling agent along with low concentration of 'Gelrite'@ 0.5g/l (Zimmerman, 1995; Stanley, 1995). The number of shoots and leaves, and root length of chrysanthemum were significantly higher on 13% sago in MS medium than on agar (Bhattacharya *et al.*, 1994). 'Isubgol', a colloidal mucilaginous husk (chiefly composed of pentosans) derived from the seeds of *Plantago ovata*), has a good gelling activity, and has reasonable clarity in gelled form. 'Isubgol' at 3% in MS medium has been used for the propagation of chrysanthemum (Babbar and Jain, 1998; Bhattacharya *et al.*, 1994). However, it became difficult to detect the bacterial and/ or fungal contamination in cultures because the medium contained Isabgol is opaque and Creamish in colour.

Media chemicals cost less than 15% of micro-plant production. In some cases the cost may be as low as 5%. Of the medium components (salts, sugar and growth regulators) have minimal influence on production cost; the gelling agents contribute 70% of the total cost (Prakash, 1993). However, low cost options are available to replace expensive gelling agents, sugars and reduce the cost of water. Obsuwan and Tharapan (2016) used three gelling agents and Phytagel, Agar and Clerigar and liquid media and suggested possibility of using liquid medium to reduce production costs for growth and development of *Dendrobium* orchid.

The results of this study suggest that using clerigar instead of agar as solidifying agent is efficient for micropropagation and *in vitro* conservation of curcuma germplasm from single shoot bud explant. A significant cost reduction of approximate 15% along with enhancement in conservation period from 8 to 12 months is possible by replacing agar with clerigar as experimented.

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