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Cost effective *in vitro* propagation of Gisela 5 cherry rootstock

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ABSTRACT

To decrease the cost of *in vitro* cultures of a clonal cherry rootstock Gisela 5 without any adverse effect on shoot quality, expensive medium components, *i.e.* sucrose, agar and distilled water were replaced with inexpensive substitutes i.e. table sugar, glucose, corn starch, isabgol (Psyllium husk), tapioca seeds and filtered water for the preparation of low cost mediums (LCMs). Different concentrations and combinations of these components were used for *in vitro* shoot multiplication and rooting. LCM_1 comprising of MS salts with 3% sucrose and 4% corn starch as well as LCM_5 containing MS salts with 4% table sugar and 4% corn starch were found to be the best for *in vitro* shoot multiplication and showed no significant difference in comparison to control. Highest shoot multiplication (1:5) with average shoot length 2.32, and 2.30 cm was observed on MS salts with 3% sucrose and 4% corn starch and MS salts with 4% table sugar and 4% corn starch, which did not show a decline on subsequent subcultures. For *in vitro* rooting, full strength, ½ strength and ¼ strength MS medium was used alone and with table sugar and corn starch to prepare low cost rooting media (LCRs). Highest *in vitro* rooting (100%) was obtained on half strength liquid and solid MS medium fortified with 0.5 mg L^{-1} IBA, and ½ LCR_1. *In vitro* rooted plantlets were hardened in cocopeat with maximum survival (90%) on initial drenching with jeevamrut (3%). No morphological variations among regenerants were observed. A decline of 70% in cost of individual plantlet was observed by using these alternate low-cost agents.

Key words: *Prunus cerasus* × *P. canescens*, *in vitro* shoot multiplication, *in vitro* rooting.

INTRODUCTION

Gisela 5, a very important dwarfing cherry rootstock developed in Germany, is a hybrid between *Prunus cerasus* and *P. canescens*. It performs very well for high density planting (HDP) worldwide. The demand of this rootstock is more in comparison to its production which cannot be met by traditional propagation procedures. This difficulty can be overcome by the use of an alternative propagation strategy like *in vitro* propagation, which is a widely used method for multiplication of planting material. A protocol for *in vitro* propagation of Gisela 5 has been developed in our laboratory (Thakur *et al.*, 11) but cost effectiveness in this technique is required to decrease the cost of planting material for commercial exploitation.

The present investigation was carried out in the Department of Biotechnology, Dr Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP), India in the year 2017-18 with the aim to develop a cost-effective *in vitro* propagation protocol for Gisela 5 by using alternate carbon source and gelling agents, respectively.

MATERIALS AND METHODS

Gisela-5 rootstock growing in PCDO Bajaura, Kullu (HP) were used to generate the stock cultures which served as explant source for the present study. Axillary buds were used as explants for raising stock cultures which are being multiplied on MS (Murashige and Skoog, 6) salts + BA 0.3 mg L^{-1}+ GA_3 0.2mg L^{-1}+ IBA 0.1 mg L^{-1}+ 3% sucrose + 8 g L^{-1} agar (Thakur *et al.*, 11), hereafter referred as multiplication medium (MM). Subcultures was done repeatedly after 4 week interval until sufficient cultures were available for the experiments. Four weeks old cultures were subsequently cultured on control medium i.e. medium containing agar and sucrose and on low cost media (LCMs) respectively for the experiments. The cultures were incubated at 25± 2°C with 16 h photoperiod of 40 µmol $m^{-2}s^{-1}$, provided by cool white fluorescent tubes (Philips, India).

MS medium was used as the basic medium for all the experiments. All the LCMs were prepared in filtered water obtained from water purifier (Kent, India). Carbon sources such as glucose and table sugar were added at different concentrations (20, 30 and 40 g L^{-1}) to the medium before optimizing the pH of the medium. The pH of the medium was adjusted to 5.8 with the help of 0.1N HCl or NaOH before the addition of gelling agents. Different concentrations of gelling agents i.e. corn starch, isabgol (psyllium husk) and tapioca seeds were added after warming the MM. Tapioca seeds were soaked in water a day before medium preparation and were added to the flasks containing liquid MM. Medium was sterilized by autoclaving at

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121°C and 1.06 kg cm^{-2} for 15 min. Culture flasks containing isabgol were stirred continuously to get uniform suspension before it forms a gel.

Relative matric potential of the medium was measured as described by Agrawal *et al.* (1) using filter paper disc (Whatman No. 3). The pH of the medium was adjusted to 5.8 and 20ml was poured in sterilized culture tube. The medium was allowed to solidify at 25± 2°C for 24 h. Air dried filter paper discs (20 mm dia.) were individually weighed and volume of liquid medium 2.6 times the weight of each filter paper was added to the surface of gelled medium where after the filter paper disc was placed on each medium. The moistened filter paper discs were allowed to equilibrate for 21h, after which they were removed and weighed. The relative matric potential was calculated as the fraction of liquid gained or lost from the filter paper discs relative to the amount initially added. Data on relative matric potential were recorded for ten replicates of each medium.

Microshoots with 3-4 nodes were excised from 4 weeks old control cultures maintained through periodic subculturing and cultured on the fresh medium for different experiments. Five shoots were cultured in each flask which was closed with cotton plugs wrapped in muslin cloth. Microshoots approximately 3-4 cm long were used for root induction. Shoots were cultured on full, half, ¼ strength MS medium and low-cost rooting (LCR) media supplemented with 0.5 mg L^{-1} IBA (Thakur *et al.*, 11). The cultures were incubated in dark for 48 h and then transferred under fluorescent light.

Rooted plantlets were carefully removed from the culture vessels and washed gently under running tap water for 1-2 h to remove agar adhering to it. These were then treated with fungicide solution (0.5% carbendazim) for 30 min and planted in small pots filled with sterilized cocopeat. The plants were maintained at high humidity (75-80%) in glasshouse at 25± 2°C. Survival and growth of plants were observed after 2-3 weeks of transfer. Jeevamrut was prepared by mixing 200 l water, 10 kg fresh cow dung, 5-10 l cow urine, 2 kg jaggery, 2 kg pulse flour and handful of soil from farm (Palekar, 8). The solution was allowed to ferment in shade for 2 to 7 days in a container. The solution was stirred daily and 3% of this concoction was drenched in the potting mixture, while transplanting followed by its foliar spray at 7 days interval to find its effect on plantlet survival and growth.

The data was analysed using Univariate statistical procedures. Descriptive analysis of the data was performed using SPSS 16. Each experiment was repeated three times. Analysis of variance (ANOVA) with comparative Duncan's multiple range tests at 5% was used to determine the significant difference between treatments.

RESULTS AND DISCUSSION

The proliferated shoots of Gisela 5 with 2-4 nodes when cultured onto MM and LCMs showed similar results on distilled water and filtered water containing medium (Fig. 1a & b). While testing the carbon sources, highest multiplication rate of 1:3 with healthy shoots and average shoot length of 2.0 cm was observed in the MS medium containing 40 g L^{-1} table sugar, gelled with 8 g L^{-1}agar. At all concentrations of glucose, the shoots failed to multiply (Table 1), hence it was not used in further experiments. Goel *et al.* (3) also reported better growth performance and multiplication rate in ordinary market grade sugar and in Daurala sugar cubes during micropropagation of Indian snakeroot (*Rauwolfia serpentina*).

On testing various gelling agents maximum shoot multiplication (1:5) similar to control was obtained on nutrient medium gelled with 40 g L^{-1} corn starch followed by 1:4 on 20 g L^{-1} isabgol (Table 2; Fig. 1c & d). Similarly, Henderson and Kinnersley (4) reported that growth and differentiation of plant cell cultures increased on media gelled with corn starch instead of agar.

After studying the individual effect of carbon sources and gelling agents, their combined effect on shoot multiplication was evaluated wherein the best concentration of carbon source and gelling agents were used in LCM_1 to LCM_7. Maximum multiplication rate of 1:5 with healthy shoots was obtained on LCM_1 and LCM_5, both gelled with corn starch, as compared to control (Table 3). On all other combinations of table

Table 1. Effect of different concentrations of various carbon sources on *in vitro* shoot multiplication.

Medium composition	Amount of carbon source used (g L^{-1})	Average shoot length (cm)	Multiplication rate
MM+ sucrose + 0.8% agar (control)	30	2.50[a]	1:4
MM+ glucose + 0.8% agar	20	1.00d[e]	1:1
	30	1.20[d]	1:1
	40	1.50[c]	1:2
MM+ table sugar + 0.8% agar	20	1.00[e]	1:1
	30	1.50[c]	1:1
	40	2.00[b]	1:3

Multiplication Medium (MM)= MS + 0.3 mg L^{-1} BA + 0.2 mg L^{-1} GA_3 + 0.1 mg L^{-1} IBA

Means followed by different letters are significantly different at P = 0.05 according to Duncan's multiple range test

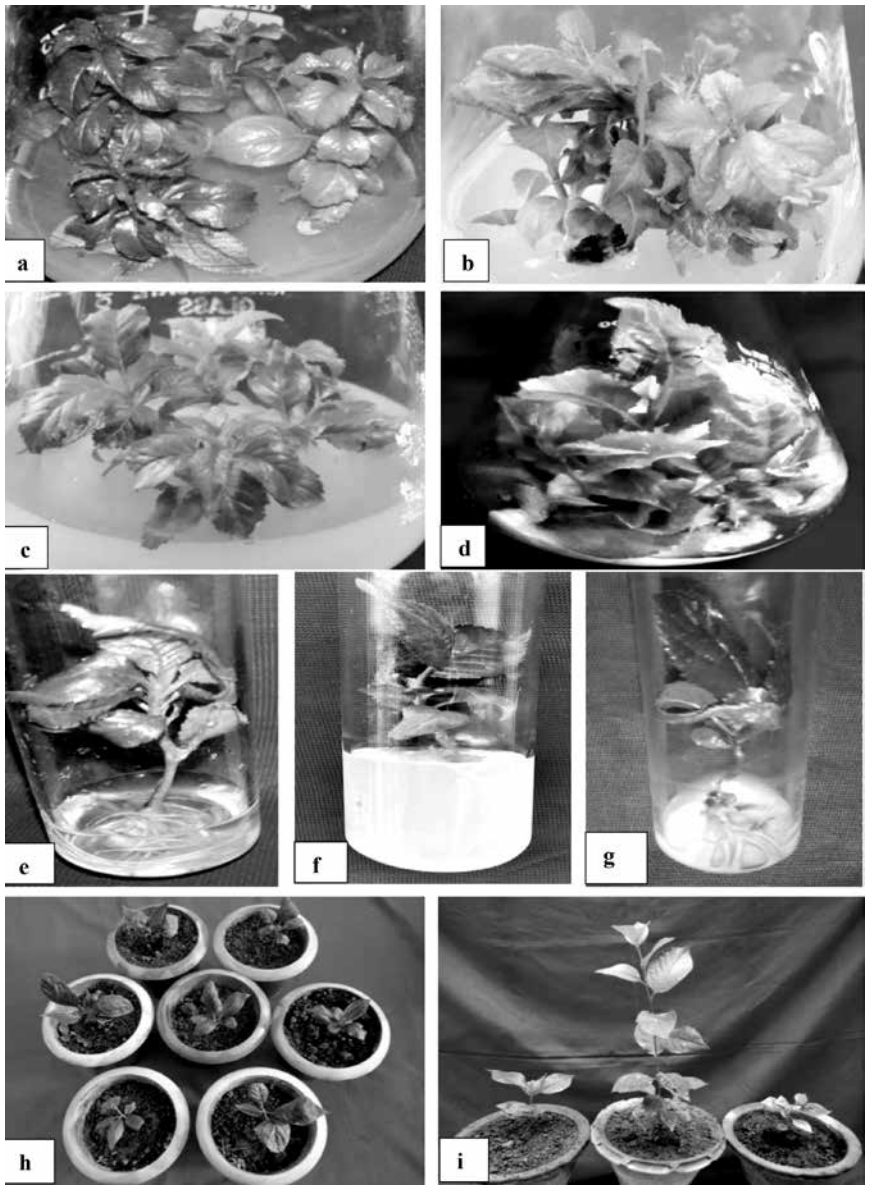

Fig 1. *In vitro* shoot multiplication on control (a) filtered water containing medium (b) LCM_1 (c) & LCM_5 (d); *in vitro* rooting in ½ liquid MS medium (e) ½ solid MS medium (f) & ½ LCR_1 (g); one month (h) & six months and (i) old hardened plants.

sugar and other gelling agents shoot multiplication was less.

Matric potential is the capacity with which water is held by the solid phase of gel and expressibility of ease with which it is expressed in response to mechanical deformation of the gel by explant (Owens and Wozniak, 7). The rate of transport of water through gel is controlled by matric potential associated with gel structure and capillarity that determines water and nutrient availability to the growing cells (Cameron, 2). The relative matric potential of LCM_1 & LCM_5 was calculated to be 1.75 and 1.60, respectively as compared to 1.56 in control (Table 3). Medium containing isabgol and tapioca seeds had lower relative matric potential than agar gelled medium indicating low availability of free water and dissolved nutrients in the matrix. This reason can be attributed to slow transport of water and other nutrients resulting in lower multiplication and growth in comparison to corn starch and agar gelled media. It has also been demonstrated by Owens and Wozniak (7) that even a small difference of matric potential can cause a noticeable effect on growth of tissues *in vitro*.

Table 2. Effect of different concentrations of various gelling agents on *in vitro* shoot multiplication.

Medium composition	Amount of gelling agent used (g L^{-1})	Average shoot length (cm)	Multiplication rate	Relative matric potential
MM + sucrose (30 g/l) + agar (control)	8	2.50[b]	1:4	1.56
MM + sucrose (30 g/l) + corn starch	30	2.40[b]	1:4	1.49
	40	2.50[b]	1:5	1.75
	50	2.50[b]	1:3	2.00
MM + sucrose (30 g/l) + isabgol	15	2.40[b]	1:3	1.31
	20	2.80[a]	1:4	1.54
	25	2.00[d]	1:2	1.65
	30	1.80[e]	1:1	1.66
MM + sucrose (30 g/l) + tapioca seeds	100	2.20[c]	1:3	1.79
	125	1.80[e]	1:3	1.91
	160	2.00[d]	1:2	2.07

Multiplication Medium (MM)= MS + 0.3 mg L^{-1} BA + 0.2 mg L^{-1} GA_3 + 0.1 mg L^{-1} IBA
Means followed by different letters are significantly different at P = 0.05 according to Duncan's multiple range test

Table 3. Combined effect of standardized concentrations of gelling agent and carbon source on *in vitro* shoot multiplication.

Medium code	Medium composition	Passage No.	Av. shoot length (cm)	Multiplication rate	Relative matric potential
Control	MM + sucrose (30 g L^{-1}) + agar (8 g L^{-1})	I	2.60[a]	1:4	1.56
		II	2.00[fg]	1:3	
		III	2.40[bc]	1:4	
		IV	2.50[ab]	1:4	
LCM_1	MM + sucrose (30g L^{-1}) + corn starch (40 g L^{-1})	I	2.30[cd]	1:5	1.75
		II	2.25[cde]	1:4	
		III	2.30[cd]	1:4	
		IV	2.25[cde]	1:4	
LCM_2	MM + sucrose (30 g L^{-1}) + isabgol (20 g L^{-1})	I	2.30[cd]	1:2	1.45
		II	2.10[ef]	1:2	
		III	2.30[cd]	1:2	
		IV	1.80[hi]	1:2	
LCM_3	MM + sucrose (30 g/l) + tapioca seeds (100 g/l)	I	2.20[de]	1:3	1.39
		II	2.00[fg]	1:3	
		III	2.00[fg]	1:3	
		IV	1.60[jk]	1:2	
LCM_4	MM+ table sugar (40 g L^{-1}) + agar (8 g L^{-1})	I	2.00[fg]	1:3	1.50
		II	1.70[ij]	1:2	
		III	1.50[k]	1:3	
		IV	1.50[k]	1:2	
LCM_5	MM+ table sugar (40g L^{-1}) + corn starch (40 g L^{-1})	I	2.30[cd]	1:5	1.60
		II	2.20[de]	1:4	
		III	2.25[cde]	1:5	
		IV	2.30[cd]	1:4	

Medium code	Medium composition	Passage No.	Av. shoot length (cm)	Multiplication rate	Relative matric potential
LCM_6	MM + table sugar (40 g L^{-1}) + isabgol (20 g L^{-1})	I	2.30^{cd}	1:2	1.48
		II	2.00^{fg}	1:1	
		III	1.90^{gh}	1:1	
		IV	2.20^{de}	1:1	
LCM_7	MM + table sugar (40 g L^{-1}) + tapioca seeds (100 g L^{-1})	I	2.10^{ef}	1:3	1.45
		II	2.00^{fg}	1:2	
		III	1.80^{hi}	1:2	
		IV	1.50^{k}	1:2	

Multiplication Medium (MM)= MS + 0.3 mg L^{-1} BA + 0.2 mg L^{-1} GA_3 + 0.1 mg L^{-1} IBA
Means followed by different letters are significantly different at P = 0.05 according to Duncan's multiple range test.

Shoot multiplication rate and length of shoots remained constant till 4th passage. The results on LCM_1 and LCM_5 were at par with the control and resulted in healthy elongated shoots (Table 3). No visible morphological variations were observed in the shoots in successive subcultures however, Vujovic *et al.* (13) reported a decline in shoot number after second subculture during *in vitro* multiplication of Gisela 5.

Maximum *in vitro* rooting (100%) was achieved on half strength liquid as well as solid MS medium, and ½ LCR1 each containing 0.5mg L^{-1} IBA (Table 4; Fig. 1 e-g). This was followed by 80% rooting on ½ LCR_2, ¼ strength liquid and solid MS medium. The roots formed were thin, long and branched without callus in the above experiments whereas, the rooting response in all other combinations tried was low.

Table 4. Effect of different strengths of MS medium with standardized concentration of different gelling agents and carbon source on *in vitro* rooting.

Medium code	Medium composition	Days taken for root initiation	Days taken for complete rooting	Per cent rooting	Average root length (cm)	Average number of roots/ shoot
Liquid control	RM + sucrose (30 g L^{-1})	26	32	40.00 (39.21)	5.00^{h}	4
Solid control	RM + sucrose (30 g L^{-1}) + agar (4g L^{-1})	28	35	40.00 (39.21)	5.20^{g}	3
LCR_1	RM + sucrose(30 g L^{-1}) + corn starch (20 g L^{-1})	30	40	30.00 (33.19)	5.00^{h}	2
LCR_2	RM + table sugar (40 g L^{-1}) + corn starch (20 g L^{-1})	28	45	30.00 (33.19)	5.00^{h}	2
½ liquid control	½ RM + sucrose (30 g/l)	22	36	100.00 (90.00)	6.50^{b}	5
½ solid control	½ RM + sucrose (30 g L^{-1}) + agar (4 g/l)	24	35	100.00 (90.00)	7.00^{a}	4
½ LCR_1	½ RM + sucrose (30 g L^{-1}) + corn starch (20 g L^{-1})	20	32	100.00 (90.00)	5.50^{f}	5
½ LCR_2	½ RM + table sugar (40 g L^{-1}) + corn starch (20 g L^{-1})	29	41	80.00 (63.53)	5.20^{g}	4
¼ liquid control	¼ RM + sucrose (30 g L^{-1})	8	20	80.00 (63.53)	5.80^{e}	7
¼ solid control	¼ RM + sucrose (30 g/l) + agar (4 g L^{-1})	10	22	80.00 (63.53)	6.20^{c}	4
¼ LCR_1	¼ RM + sucrose (30 g L^{-1}) + corn starch (20 g L^{-1})	18	30	60.00 (50.74)	6.00^{d}	3
¼ LCR_2	¼ RM + table sugar (40 g L^{-1}) + corn starch (20 g L^{-1})	20	35	60.00 (50.74)	5.70^{e}	5
	SE±			0.51 (0.35)		

RM (Rooting Medium): MS + 0.5mg L^{-1} IBA
Values in parentheses are arc sine transformed values. Means followed by different letters are significantly different at P = 0.05 according to Duncan's multiple range test.

The beneficial effect of reducing MS basal medium concentration on the *in vitro* rooting ability has been demonstrated in *Quercus sobur* L. (Manzanera and Parados, 5), *Wrightia tomentosa* (Purohit *et al.*, 9) and apple rootstocks (Sharma *et al.*, 10).

The rooted plantlets obtained were removed from agar gelled medium after 4 weeks and transplanted in cocopeat for hardening. When jeevamrut (3%) was drenched in cocopeat followed by foliar spray at weekly interval maximum survival of 90% was observed (Fig. 1 h & i). However, contrary to our findings, Vujovic *et al.* (13) observed lower acclimatization potential in Gisela 5. Although these manures may not provide enough nutrients in the area of application but they help in quick building-up of fertility through enhanced activities of soil microflora and fauna (Yadav and Mowade, 14). It also contains enormous amounts of microbial load, which multiply and act as soil tonic (Palekar, 8).

Cost is the major concern during *in vitro* propagation of any plant speci. Major recurring cost during micropropagation is due to carbon source and gelling agents used in the medium. By replacing sucrose with table sugar and agar with corn starch 70% reduction in production cost for the individual plantlet was achieved. Similar studies in *Curcuma longa* (Tyagi *et al.*, 12) and banana (Agrawal *et al.,* 1) resulted in 73 and 59% reduction in cost by using isabgol as gelling agent and market sugar as carbon source.

The results presented in this paper provide an efficient, cost effective protocol for *in vitro* multiplication of Gisela 5 rootstock.

DECLARATION

The authors declare no conflict of interest.

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