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In Vitro Propagation of Virus Indexed Gisela-5 (*Prunus cerasus* x *Prunus canescens*) - Clonal Cherry Rootstock

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ABSTRACT

In the present investigation, a technique for *in vitro* propagation of Gisela-5 (*Prunus cerasus* x *Prunus canescens*)-cherry rootstock has been developed. Maximum *in vitro* establishment was achieved during the month of July and February. Treatment with 0.1 per cent $HgCl_2$ for 5 minutes was found to be the best for surface sterlization. Maximum *in vitro* establishment of explants (70%) was achieved on MS medium fortified with 0.5 mg/l BA and 0.5 mg/l GA_3. Highest multiplication rate of 1:5 was observed on MS medium supplemented with five different concentrations and combinations of BA, GA_3, IBA and Kin. Shoot multiplication rate and shoot length showed an increase with the increase in number of subculturing passages which increased to a maximal of 1:9 and 6 cm after third and fourth passage. Best rooting of 18.20 per cent was observed in one step procedure whereas, maximum rooting (53.33%) was observed in two step procedure of rooting. Rooted plantlets were transplanted in sterilized sand for hardening and kept in the glasshouse, where 90 per cent survival was observed after 4 weeks of transfer. *In vitro* established cultures and hardened plants were indexed for Cherry leaf roll virus, Apple chlorotic leaf spot virus and Prunus necrotic ring spot virus using DAS-ELISA procedure. All the tested samples showed negative results for the presence of these viruses, thus ensuring the production of healthy planting material.

Keywords: Gisela-5, Organogenesis, *In vitro* multiplication, Root development Acclimatization, DAS-ELISA.

INTRODUCTION

Cherry is one of the important temperate fruit crop cultivated worldwide. It belongs to the family Rosaceae. Cherries are usually grown in the coldest climates at an altitude of about 1,600 to 2,700 m above the mean sea level requiring 1,000 - 1,500 hours of chilling period during winters. Cherries are also multiplied clonally by grafting the scion cultivar on rootstock like majority of fruit crops. As compared to the demand of about 10,000 plants annually (Anonymous, 2016), the main factor that restricts cherry cultivation in India is the limited nursery plant production of seedling rootstocks. Seedling rootstocks are not uniform and show great variability in tree vigour, bearing age etc. Vigorous trees require large spaces for planting, have prolonged juvenility and induce late bearing. These difficulties can be overcome by the use of dwarfing clonal rootstocks. Gisela-5 is a very important dwarfing cherry rootstock and is a hybrid between *Prunus cerasus* and *Prunus canescens* (Clapa *et al*., 2013). It is considered as very useful and economically important dwarfing rootstock for intensive sweet cherry growing in the temperate conditions. Gisela-5 has performed very well worldwide with different soil and climatic conditions, with a great number of cultivars, various training systems and planting densities and tends to advance both flowering and fruit ripening by two to four days. Trees on Gisela-5 rootstock have shown good winter hardiness, and scion compatibility has not been an issue. It is very difficult to multiply Gisela-5 through various conventional techniques used for multiplication of planting material of fruit trees which can be overcome by the technique of micropropagation.

The aim of the present research was to develop an effective protocol for micropropagation of Gisela-5 rootstock by

in vitro establishment, multiplication and rooting of plantlets.

MATERIALS AND METHODS

Plant material

Experimental plants of Gisela-5 were selected from nursery maintained at PCDO (Progeny cum demonstration orchard) Bajaura, Kullu (HP), India. Shoot cuttings of approximately 45 cm were procured from the selected mother plants which were not virus indexed, in every month from October, 2014 to September, 2015. For surface disinfection, explants were washed 2-3 times with tap waterand then treated with 1 per cent carbendazim solution for 30 minutes, followed by 2-3 washings with sterilized distilled water. Inside the Laminar air flow chamber, the axillary and terminal bud explants were treated with different concentrations of surface sterilant solutions (NaOCl, $HgCl_2$) for different durations of time and then washed with sterilized distilled water 2-3 times to remove the traces of sterilants before inoculation.

Culturing of explants for establishment

Explants (1-2 cm) were excised from the cuttings and cultured vertically in culture tubes (25×100mm size) containing 10ml nutrient medium (Murashige, 1974) comprising of MS salts, 3% sucrose and supplemented with varying concentrations of cytokinins (BA, Kin, TDZ) and gibbrellin (GA_3). Agar (0.8%) was used for gelling. Cotton plugs were used for plugging the culture vessels. The nutrient medium was sterilized by autoclaving at 1kg cm $^{-2}$ for 15 minutes. The cultures were incubated at 25±2ºC under 16/8-h photoperiod with 3000 lux light intensity. The explants which showed vigorous bud break were selected and transferred to fresh medium of same or different composition for further shoot elongation.

In vitro shoot multiplication

Shoots were multiplied by the method of enhanced release of axillary bud (Murashige, 1974). MS medium used for multiplication of shoots consisted of different combinations and concentration of growth regulators (BA, Kin, TDZ, GA_3 and IBA) (Table-3). Best growth regulator concentration for shoot growth and development of axillary branching was found out by recording the rate of shoot multiplication, length and quality of shoots in four weeks old cultures. The serial subculturing was performed after every 4-5 weeks by separating and transferring the shoots into fresh nutrient medium.

In vitro induction of rooting

Microshoots from 4 weeks old cultures were used to make cuttings of approximately 2-3 cm long for root induction. The lower leaves were removed from micro shoots and two procedures were followed for rooting. In single step procedure, shoots were cultured on half strength MS medium supplemented with different concentration of auxins viz. IBA, IAA, NAA. In two step procedure, shoots were kept in high concentration of IBA (0.5-1.0 mg/l) enriched liquid MS medium for 24-48 hours under dark conditions and then transferred to auxin free half strength MS medium for root induction and elongation. 4 g/l agar was used for solidification of medium in all the rooting experiments for easy removal of plantlets for hardening. Rooting frequency was recorded after 4 weeks of culture in both the methods followed.

Acclimatization and hardening of rooted plantlets

For acclimatization and hardening, *in vitro* regenerated plants were removed from culture tubes and washed under running tap

water for 1 hour to remove agar sticking to the roots. Thereafter, the plants were kept dipped in 1% solution of carbendezim for 30 minutes before transferring to plastic pots containing sterilized sand. The pots were covered with glass jars and kept in glass house. Jars were removed after 15 days and survival rate of plantlets were recorded after one month of transfer to *in vivo* conditions.

Serological virus indexing of *in vitro* established cultures

The *in vitro* established cultures and hardened plants of Gisela-5 were subjected to serological indexing against ACLSV, PNRSV and CLRV using double antibody Serological sandwich enzyme linked immunosorbent assay (DAS-ELISA) as described by Clarks and Adams (1977) with modifications. Testing kit from Bioreba AG, Switzerland was used for this assay.

The experiments were repeated three times with similar trend of results using completely randomized design (Gomez and Gomez 1984). The significance of treatment effects on various parameters was determined using analysis of variance (ANOVA). If the treatments were found to be significant, then their comparative performance was tested after obtaining the critical difference ($CD_{0.05}$).

RESULTS AND DISCUSSION

Influence of different months of the year on *in vitro* establishment of explants

Explants collection and initiation during different months of the year had significant influence on *in vitro* culture establishment of Gisela-5. As evident from figure-1, maximum *in vitro* per cent establishment of buds was achieved in the month of July (70%) followed by February (51.50%), which was comparable to 50 per cent in the month of May. These results are similar to the resultsof Thakur *et al.* (2001) who cultured apical and terminal buds of *Alnus nepalensis* throughout the year but could achieve best establishment during the month of October.The importance of timing of explants collection with respect to contamination and growth has also been studied and it was reported that there is lesser *in vitro* establishment of cultures

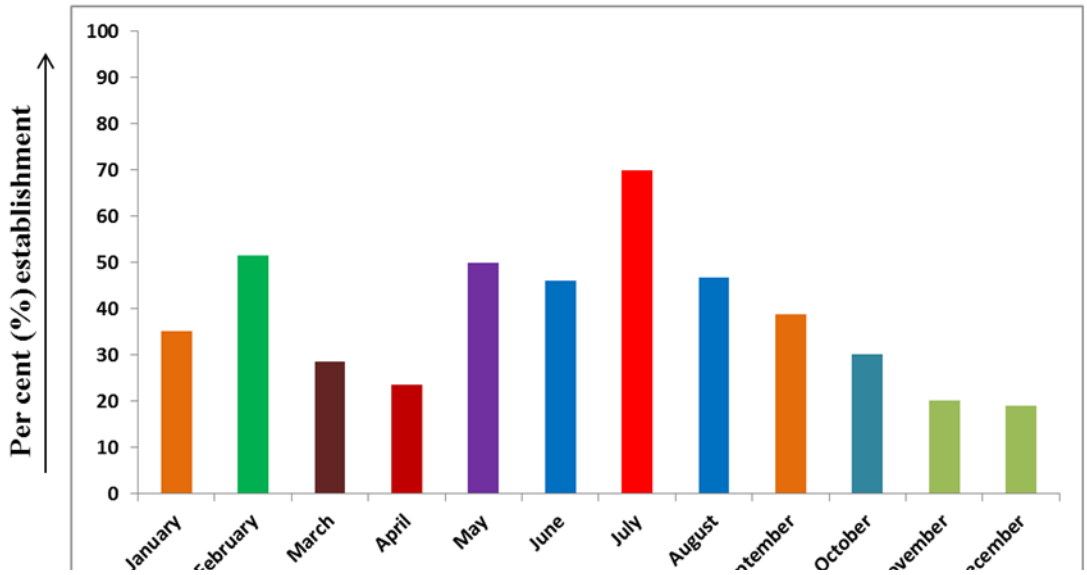

Figure 1: Effect of months of the year on percentage *in vitro* establishment of buds

during spring in clonal cherry rootstocks-Gisela-5, MaxMa 14 and TabelEdabriz due to contamination (Fidanci *et al.*2008).

Surface sterilization of explants

Surface sterilization of explants is the first and most important step in establishing *in vitro* cultures, to prevent explants from different kinds of contaminations. There are many reports in which cherry explants were successfully surface sterilized using solutionof sodiumand calcium hypochlorite (Jones and Hopgood 1979, Pevalek-Kozlina and Jelaska 1987), but in our material, sodium hypochlorite didn't prove effective in disinfection of explants, whereas $HgCl_2$ served as a good disinfectant (Table-1). The ineffectiveness of sodium hypochlorite may be due to the susceplible cuticle. . Hossini *et al.* (2010) and Muna *et al.* (1999) reported $HgCl_2$ treatment effective in controlling contamination during *in vitro* establishment of cherry rootstocks as compared to NaOCl and $Ca(OCl)_2$.

***In vitro* establishment of aseptic cultures and bud burst**

After surface sterilization, buds were cultured on MS medium supplemented with different combination and concentration of growth regulators. Maximum in vitro bud establishment of 70 per cent was achieved on MS medium fortified with 0.5 mg/l BA and 0.5 mg/l GA3 (Fig 2 and Table-2). The established buds showed shoot proliferation and elongation on the same medium.

Table 1: Effect of different duration of surface sterilants treatment on *in vitro* survival of explants

Sr. No.	Surface Sterilization Treatment	Duration of Treatment	No. Of Buds Cultured	Percent Uncontaminated Buds	Percent Bud Surviving
1.	Control	-	10	0.00(0.00)	0.00(0.00)
		5 min	13	0.00(0.00)	0.00(0.00)
2.	1.0% NaOCl	10 min	15	16.66(24.08)	-
		15 min	12	33.33(35.25)	25.00(29.98)
		20 min	10	60.00(50.74)	50.00(44.98)
		5 min	15	23.33(28.86)	50.00(44.98)
3.	1.5% NaOCl	10 min	10	60.00(50.74)	20.00(26.57)
		15 min	12	71.66(57.81)	33.33(35.24)
		20 min	14	77.14(61.41)	-
		5 min	11	18.18(25.22)	50.00(44.98)
4.	2.0% NaOCl	10 min	16	26.25(30.80)	-
		15 min	12	100.00(90.00)	-
		20 min	10	100.00(90.00)	-
		3 min	15	13.33(21.40)	50.00(44.98)
5.	0.1% $HgCl_2$	4 min	12	41.43(40.05)	40.00(39.21)
		5 min	14	64.12(53.18)	77.77(61.84)
		3 min	16	25.00(29.98)	25.00(29.98)
6.	0.2% $HgCl_2$	4 min	13	77.69(61.79)	-
		5 min	14	100.00(90.00)	-
$CD_{0.05}$				0.97(0.64)	1.093(0.682)
SE±				0.33(0.22)	0.379(0.237)

*Values in parenthesis are arc sine transformed values.
CD = Critical difference
SE = Standard error

On increasing the concentration of BA to 1.0 mg/l, per cent bud proliferation showed a decline to 65 per cent. When GA_3 is used with Kin, *in vitro* establishment per cent decreased and this growth regulator combination took maximum days for buds to sprout.

On replacing cytokinin from BA to TDZ in the medium, bud proliferation was very low although GA_3 was also used.The cytokinin BA promotes cell division, shoot multiplication and axillary bud formation (Sutter 1996), which may be the reason why in our studies, BA in combination with GA_3proved to be better cytokinin than others.

Figure 2: Buds sprouting after 10 days of culture

Table 2: Effect of different concentration and combination of plant growth regulators on *in vitro* establishment of explants

Sr. No.	Nutrient Medium MS basal fortified with GR (mg/l)				Days taken for bud burst	Percent bud proliferation
	BA	Kin	TDZ	GA_3		
1.	0.5	-	-	0.5	10-12	70.00(56.76)
2.	0.5	-	-	1.0	13-15	48.05(43.86)
3.	1.0	-	-	0.5	15-18	65.04(53.73)
4.	1.0	-	-	1.0	15-18	58.00(49.58)
5.	-	0.5	-	0.5	18-21	38.03(38.05)
6.	-	1.0	-	0.5	17-20	31.00(33.81)
7.	-	0.5	-	1.0	20-25	22.00(27.96)
8.	-	1.0	-	1.0	19-22	18.01(25.10)
9.	-	-	0.5	0.5	18-21	10.77(19.14)
10.	-	-	0.5	1.0	18-21	8.66(17.10)
11.	-	-	1.0	0.5	16-18	-
12.	-	-	1.0	1.0	19-21	-
$CD_{0.05}$						1.165(0.813)
SE±						0.397(0.277)

*Values in parenthesis are arc sine transformed values.
CD = Critical difference
SE = Standard error

In vitro shoot multiplication

It was observed that shoot multiplication, length of shoots and leaf size varies with the concentration of the different plant growth regulators used.

Shoot multiplication was observed in all the growth regulator combinations tried in the medium (Table-3). Highest multiplication rate of 1:6 was observed in the medium G-III (Fig 3), but the shoots were stunted followed by multiplication rate of 1:5 in

Table 3: Effect of concentration and combination of different plant growth regulators on *in vitro* shoot multiplication of Gisela-5

Sr. No.	Medium code	Medium Composition MS (Basal medium) + GR (mg/l)					Number of shoots/ explant	Average length of shoots (cm)	Quality of shoots
		BA	TDZ	GA_3	IBA	Kin			
1.	Control	-	-	-	-	-	1	1.50	Elongated shoots; no multiplication
2.	G-I	0.50	-	0.50	-	-	2	0.75	Stunted shoots with small leaves
3.	G-II	1.00	-	1.00	-	-	3	0.75	Stunted shoots with small folded leaves
4.	G-III	0.50	-	-	0.10	-	6	1.00	High shoot multiplication; stunted shoots
5.	G-IV	0.50	-	-	0.20	-	2	1.25	Low multiplication rate; big leaf size
6.	G-V	1.00	-	-	0.10	-	3	1.00	Low multiplication rate; big leaf size
7.	G-VI	1.00	-	-	0.20	-	3	1.25	Low multiplication rate; big leaf size
8.	G-VII	-	0.50	0.50	-	-	2	0.75	Vitrified shoots; translucent leaves; poor growth
9.	G-VIII	-	0.50	0.30	-	-	2	0.75	Vitrified shoots; translucent leaves; poor growth
10.	G-IX	-	1.00	1.00	-	-	4	0.75	Vitrified shoots; translucent leaves; poor growth
11.	G-X	-	0.50	-	0.10	-	2	0.75	Vitrified shoots; translucent leaves; poor growth
12.	G-XI	-	1.00	-	0.20	-	2	1.00	Vitrified shoots; translucent leaves; poor growth
13.	G-XII	0.30	-	0.20	-	-	5	2.00	Elongated stout shoots showing high multiplication rate
14.	G-XIII	0.50	-	0.50	0.10	-	5	1.50	High shoot multiplication and elongated shoots
15.	G-XIV	0.30	-	0.20	0.10	-	5	1.50	Good quality shoots with high multiplication rate
16.	G-XV	-	0.50	0.30	0.10	-	4	1.00	Vitrified shoots with unhealthy leaves
17.	G-XVI	0.50	-	0.30	0.10	-	4	2.00	Less elongated shoots with high multiplication
18.	G-XVII	0.50	-	0.30	-	-	4	1.50	Healthy and elongated shoots with good multiplication
19.	G-XVIII	0.50	-	0.20	-	-	3	1.50	Healthy and elongated shoots with good multiplication
20.	G-XIX	0.25	-	-	0.10	0.25	5	1.25	Healthy and elongated shoots with good multiplication
21.	G-XX	0.50	-	-	0.10	0.50	2	1.50	Healthy shoots with low multiplication rate
22.	G-XXI	0.30	-	0.50	0.10	-	5	2.25	More terminal shoot elongation with high multiplication rate

medium G-XII, G-XIII, G-XIV, G-XIX and G-XXI. The addition of phytohormonesas crucial for multiple shoot formation but no significant difference in multiplication rates was found among medium supplemented with BA, GA_3 and IBA.

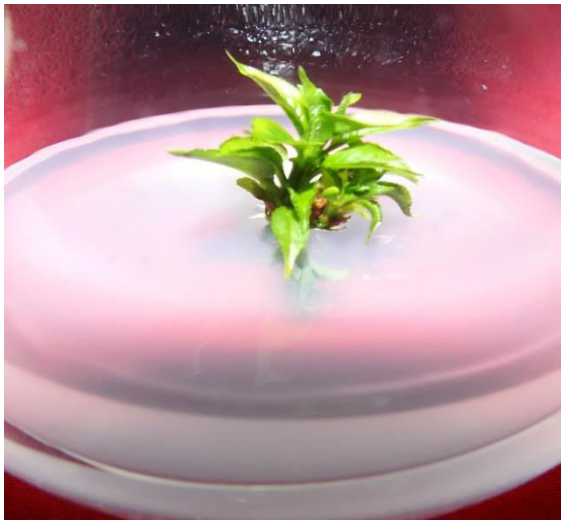

Figure 3: Stunted shoots formed on nutrient medium G-III after 4 weeks of culture

Our results are supported by the research of Buyukdemirci (2008), Filiz (2010) and Sisko (2011) who obtained best shootmultiplication of Gisela-5 on medium fortified with BA, GA_3 and IBA.The continuous presence of cytokinin in thenutrient medium is of outmost importance for the formation of new shoots (Nordstrom and Eliasson 1986). Therefore, in thepresence of BA, abundant shoot proliferation occurred. The function of continuous BA during shoot multiplication is to break the apical dominance and stimulate growth of new shoots, and complete or partial inhibition of root formation (Muna *et al*. 1999).

Effect of subculturing on *in vitro* shoot multiplication

Rate of shoot multiplication and length showed an increase with the increase in number of subculturings (Figure 4) which increased to a maximal of 1:9 and 6 cm after third passage and fourth passage, as evident from table-4. Similarly, Marjanovic *et al.* (2000) obtained three fold multiplication after third multiplication cycle during micropropagation of wild cherry. Grant and Hammatt (1999) also reported that the ability to produce roots and shoots in apple rootstock M_9 and cherry rootstock F12/1 was dependent on the total time spent in cultures which is similar to our studies.

Effect of auxins on *in vitro* root induction in one step and two step procedures

In one step procedure the micro shoots were directly cultured in half strength MS medium fortified with auxins for root induction, IBA (0.5 mg/l) proved to be the best with 18.20 per cent rooting, 3.6 cm root length and 4 number of roots per shoot (Figure 5 and Table-5). Our observation is supported by findings of many workers who used different concentrations of IBA for rooting of *in vitro* micro shoots during micropropagation of various cherry rootstocks (Canli and Demir 2014, Sarropoulou *et al.* 2014, Xu *et al.* 2015, Zamanipou r*et al.* 2015).

On the other hand, in two step procedure for rooting, maximum rooting per cent (53.33) was observed when micro shoots were cultured in half strengthliquid MS medium fortified with 0.5 mg/l IBA for 24 hours in the dark and then further cultured on half strength basal MS medium and incubated under florescent light (Fig 6 and Table-6). Two step rooting procedure was successfully followed for rooting during micropropagation of apple rootstock MM 111(Kaushal *et al*. 2005). On increasing the time period of dark incubation, a decline in rooting per cent was observed. Very few shoots rooted (34.64%, 15.56% and 4.78%) on increasing the concentration of IBA in the liquid medium to 1.0 mg/l and dark incubation from 24 to 72 hours.

Thus, two step rooting was found better than one step rooting in our experiments. The reason was that root emergence and further growth was inhibited, if auxin was present throughout the rooting period.

Table 4: Effect of subculturing on shoot multiplication and per cent rooting

Sr. No.	Passage	Rate of multiplication	Shoot length (cm)	Average no. of leaves	Shoot type	Percent rooting
1.	Establishment	1:1	2	3	Well formed shoot with thick stem	-
2.	Istsubculturing	1:4	4	8	Well formed shoot with thin stout stem	No rooting
3.	IIndsubculturing	1:7	4.5	10	Well formed shoot with thin stout stem	10 % rooting
4.	IIIrdsubculturing	1:9	6	15	Well formed shoot with thin stout stem	55% rooting
5.	IVthsubculturing	1:9	5.5	16	Well formed shoot with thin stout stem	60% rooting

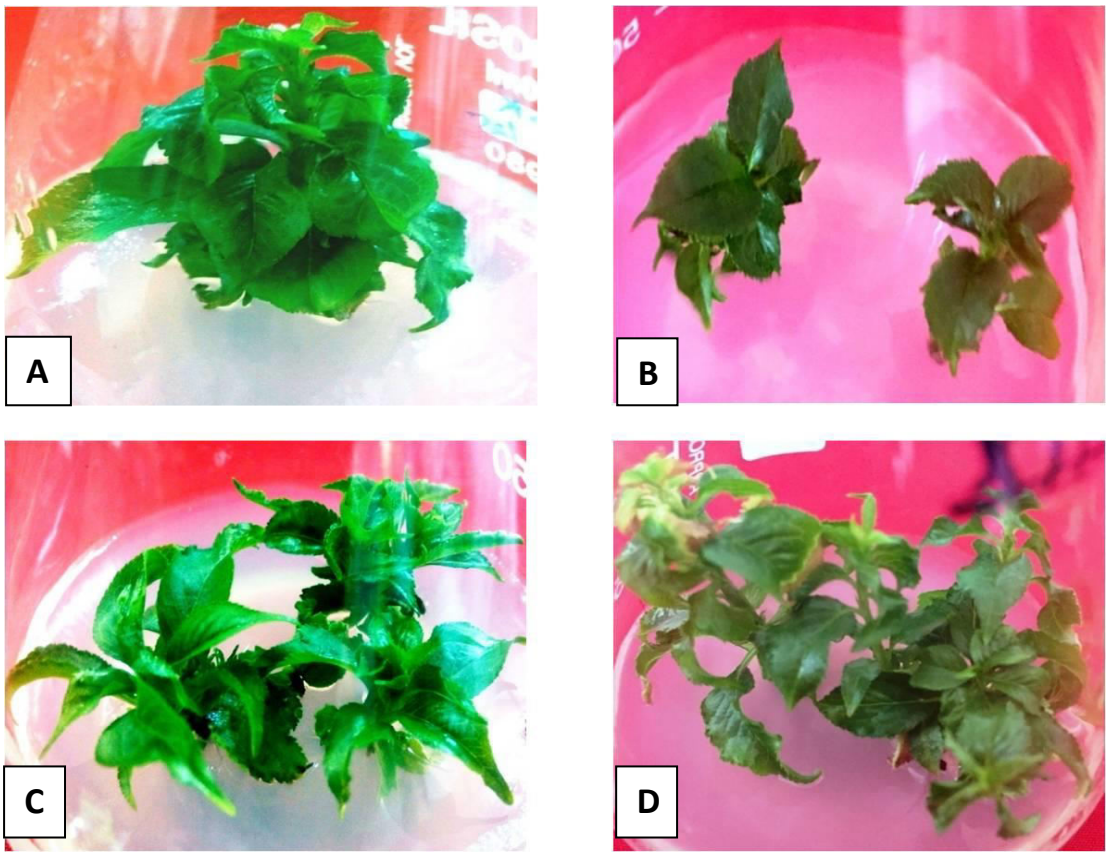

Figure 4: *In vitro* shoot multiplication after first subculture (A), second subculture (B), third subculture (C) and fourth subculture (D) on nutrient medium G-XIV

Table 5: *In vitro* rooting on different concentration of auxins in one step procedure

Sr. No.	Type of Auxin	Concentrations (mg/l)	Percent rooting	Root length (cm)	No. of roots per shoot
1.	IBA	0.5	18.20(4.38)	3.6	4
2.	IBA	1.0	9.45(3.23)	4.5	3
3.	IAA	0.5	2.35(1.82)	4.2	4
4.	IAA	1.0	4.00(2.22)	3.6	5
5.	NAA	1.0	11.55(3.54)	4.3	4
$CD_{0.05}$					1.27(0.23)
SE±					0.39(0.07)

*Values in parenthesis are arc sine transformed values.
CD = Critical difference
SE = Standard error

Table 6: *In vitro* rooting on different concentration of auxins in two step procedure

Sr. No.	Auxin	Concentration (mg/l)	Hours of dark treatment	Percent rooting	Root length (cm)	No. of roots per shoot
1.	IBA	0.5	24	53.33(46.89)	5.6	5
			48	26.76(31.14)	4.2	4
			72	8.23(16.66)	4.9	5
2.	IBA	1.0	24	34.64(36.04)	5.0	5
			48	15.56(23.22)	5.5	4
			72	4.78(12.62)	4.8	3
$CD_{0.05}$						1.06(0.81)
SE±						0.34(0.26)

*Values in parenthesis are arc sine transformed values.
CD = Critical difference
SE = Standard error

Figure 5: Roots development in one step procedure

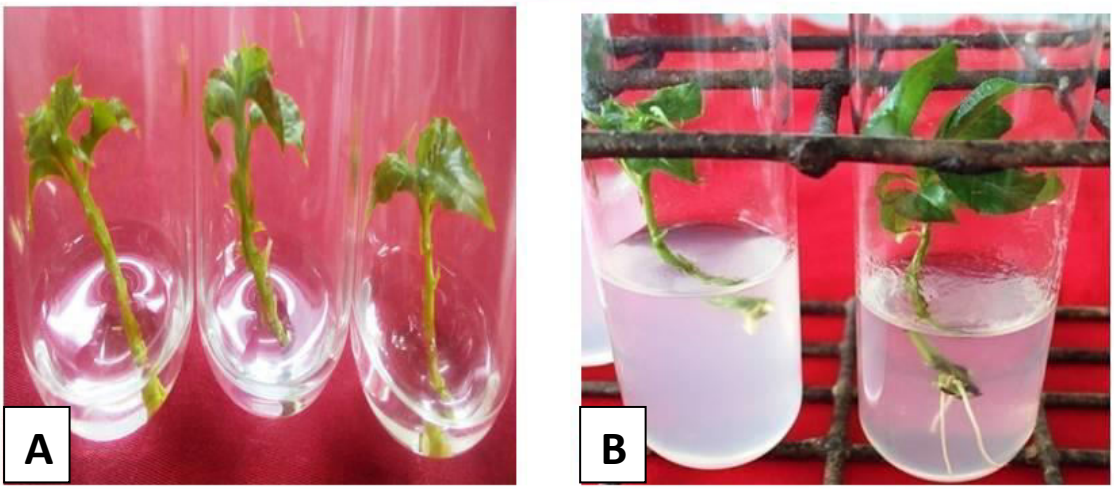

Figure 6: Microshoots kept in half strength liquid MS medium (A) and showing root formation on transfer to half strength semisolid basal MS medium (B) in two step procedure

Figure 7: *In vitro* rooted plantlets

Figure 8: Completely hardened plants after 4 weeks of transfer

Acclimatization of *in vitro* rooted plantlets

After 3 weeks, the rooted plantlets obtained following both the rooting procedures, were removed from agar gelled medium (Fig. 7) and transplanted in sterilized sand for hardening. These plants were kept in the glasshouse maintained at 25ºC and 90% relative humidity. 90 per cent survival was observed after 20 days of transfer (Fig. 8). It was observed that better root and shoot development prior to hardening determined the survival of *in vitro* raised plant (Minaev *et al.* 2003).

Serological virus indexing of*in vitro* established shoot cultures and hardened plants

DAS-ELISA was performed using specific antisera against CLRV, ACLSV and PNRSV to index the *in vitro* established cultures and hardened plants of Gisela-5.The data on the mean OD value at 405 nm and serological reaction clearly indicated the absence of viruses in the *in vitro* shoots of Gisela-5. The OD values and the visual assessment on the basis ofcolour showed that these viruses (CLRV, ACLSV and PNRSV) were not present in the mother cultures of Gisela-5 (Fig. 9). The OD values of the samples recorded in the spectrophotometer were almost equal to the OD value of negative control and the values of positive control were at least more than double the values of samples and negative control. Yellow colour appeared in the wells containing positive control whereas, no colour appeared in the wells coated with negative control, which

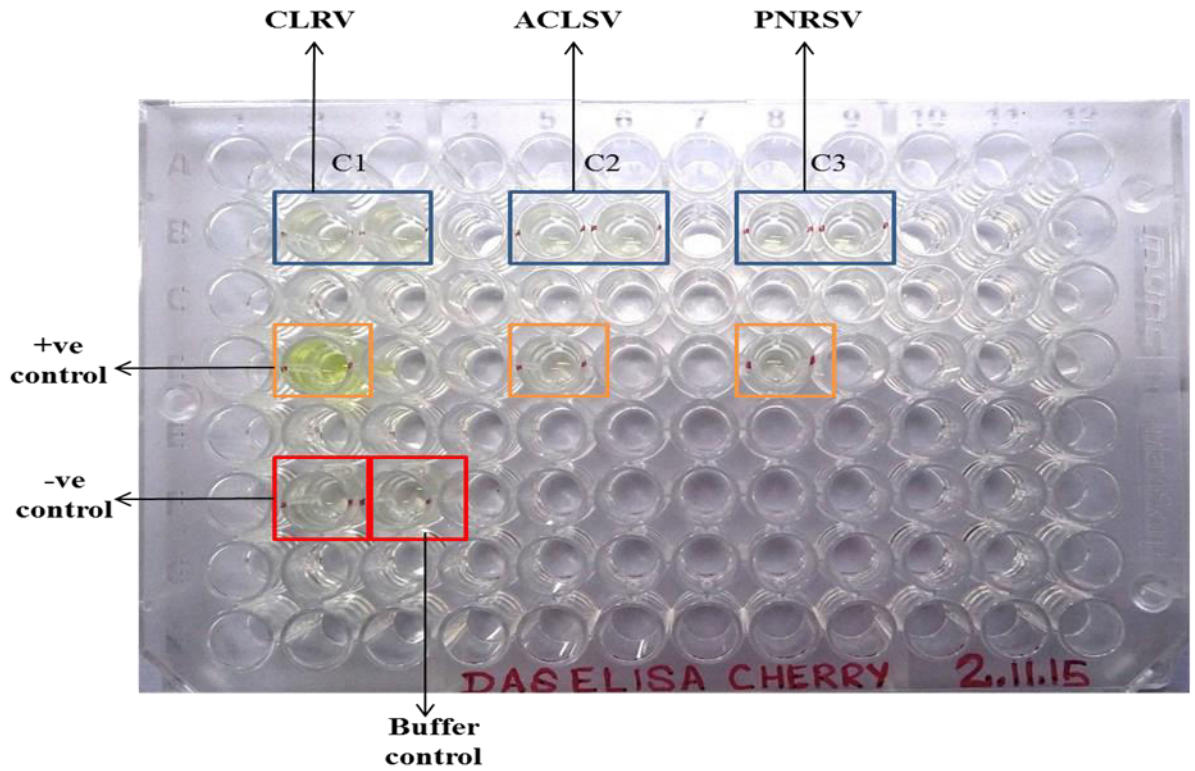

Figure 14: ELISA plate showing negative serological reaction for CLRV, ACLSV and PNRSV in mother cultures of Gisela-5

signifies that all the in vitro shoots did not contain these viruses and can be used further for the production of healthy planting material.

CONCLUSION

Gisela-5 rootstock can be propagated successfully using micropropagation technique. BA in combination with GA3 and IBA gave maximum multiplication as reported previously by other workers. However, in our experiments, it was found necessary to have short root induction period of few hours in IBA containing liquid medium followed by transfer to hormone free medium to achieve maximum rooting.

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