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In-vitro micrografting technique in sweet orange (*Citrus sinensis*) cv. Blood Red to produce virus free plants

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ABSTRACT

This study was carried out to assess the potential use and applicability of micrografting technique for the development of virus free nursery in sweet orange (*Citrus sinensis* Osbeck) cv. Blood Red Malta. The micro grafting was carried out using 11-20 days old etiolated seedlings of Rough Lemon, Sour orange, Carrizo and Karna Khatta rootstocks. An apical meristem (1-2 mm) having 3-leaf primordial (0.3-0.5 mm) were taken from the axillary proliferated nodal segments. Finely excised meristems were placed on aseptically grown rootstocks making an inverted 'T' incision *in vitro.* Micro grafts were inoculated on MS media added with different Plant Growth Regulators, *i.e.* IAA, NAA and 2,4-D each under varied concentrations (control, 0.1 ppm, 0.5 ppm, 1.0 ppm , 1.5 ppm, 2.0 and 3.0 ppm). Highest grafts success (60%) was recorded when 13 days old Carrizo seedlings were used as rootstock. Among all the treatments, scion treated with 2,4-D at 3.0 mg/l exerted maximum positive effect on the success of STG (70.00%). Overall, Sweet orange cv. Blood Red, micro grafted on Carrizo rootstock culturing on liquid MS media containing 2, 4-D at 3.0 mg/L resulted maximum success and growth of grafts. Successful micrografts were hardened under controlled conditions and further shifted in insect proof net house to develop disease free mother progeny.

Key words: Blood Red, *Citrus aurantium, C. carrizo, C. jambheri, C. karna,* Micrografting

Sweet orange (*Citrus sinensis* Osbeck) is one of the most important fruit crop known by the humans since antiquity and is a good source of vitamin "C" with high antioxidant potential. Sweet orange tree belongs to the family *Rutaceae.* In, Rajasthan, the interest of farmers in the adoption of sweet orange is increasing continuously due to suitable agro-climatic conditions, higher yield of crops and government policies. However, healthy planting material for fulfilling plantation demand is very limited. Citrus orchards and nurseries survey based on the characteristic symptoms expression and serological indexing reported that the major virus, viroid and prokaryotic diseases commonly observed were Citrus Tristeza, Citrus Variegation, Citrus Exocortis, Citrus Cachexia (Xyloporosis), Citrus Greening and Stubborn (Arif *et al.* 2005). Various methods, viz. Nuceller Line Breeding, Thermo-therapy, Meristem Culture and Shoot Tip Grafting (STG) have been employed for producing virus free citrus plants (Weathers and Calaven 1959, Roistacher 1977). The limitation of the former method is long juvenile period (7-8 years), and excessive vigour. Thermo-therapy technique is ineffective to eliminate heat resistant viruses like Exocortis and Xyloporosis and cannot be used with heat susceptible species (Roistacher 1977, Navarro 1988). To overcome these problems, some alternative techniques, viz. Meristem Culture and Shoot Tip Grafting are preferred for virus elimination in Sweet Orange. In this perspective present investigation will be taken to standardize the micropropagation technique for *in vitro* Shoot Tip Grafting for raising virus free bud woods in Sweet Orange cv Blood Red.

MATERIALS AND METHODS

The experimental work was carried out at the tissue culture laboratory, Department of Horticulture, Agricultural Research Station, Sriganganagar, SKRAU, Bikaner under the controlled conditions during the year 2012-13. Freshly harvested seeds of *Citrus* species, cleopatra (*Citrus reshnii*), sour orange *(Citrus aurantium*), karna khatta (*Citrus Karna*), carrizo (*Citrus carrizo*) and jhatti khatti (*Citrus jambheri*) were obtained from the Experimental Fruit Garden, Prior to use seeds were held in sterilised distilled water. To surface sterilize, seeds were washed under running tap water The seeds were washed with detergent (Teepol), pretreated with 0.2% (w/v) Bavistin (a systematic fungicide) for 10 minutes followed by washing with double distilled water. The seeds were placed in the vertical position in test tubes one third filled with solidified (1% Agar) MS medium fortified

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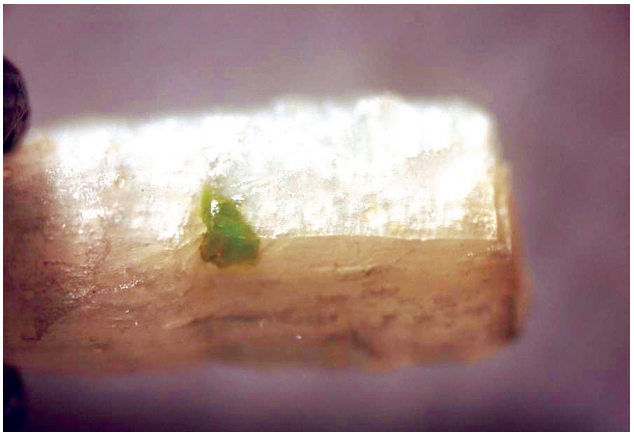

Fig 1 Meristem placed on rootstock

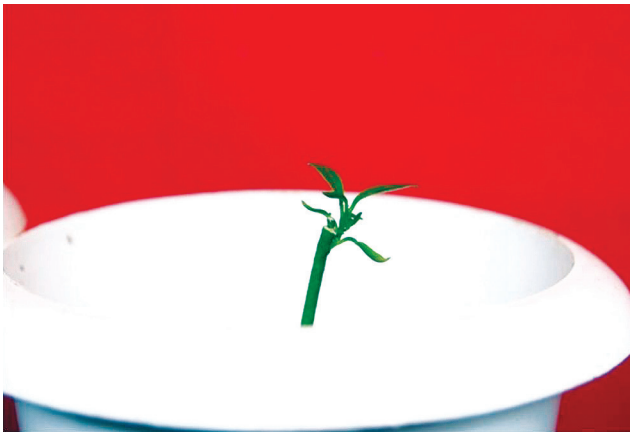

Fig 2 Sprouted meristems on rootstock

with 3% sucrose and adjusted at 5.8 pH. Test tubes were kept for incubation in BOD chamber under the controlled environment of temperature (25±2°C) and 60-70% relative humidity (RH) and 12 hr photoperiod. The rootstocks were used for decapitation was obtained at 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 days age for all species. These *in-vitro* raised etiolated seedlings were used as rootstock for micro grafting. The rootstock were trimed out after removing 5 cm root and 4 cm shoot from epicotyls. In shoot tip grafting, scion is a apical meristem approximately <0.2 mm in length were excised from *in vitro* generated shoots of nucellar seedlings of Blood Red. These shoot tips of < 0.2 mm in size along with 1-2 leaf primordia were dissected under stereo microscope in laminar air flow with help of sterilized eye surgery blade, scissor and forcep. The shoot tips were pre treated with different concentrations of auxins (NAA/ IBA/2,4-D – 0.0, 0.1, 0.5, 1.0, 1.5 and 3.0 mg/l).

In this procedure, An inverted "T" cut made on decapitated apical portion of rootstock. For preparation of inverted "T" cut, a vertical cut about 3-4 mm was made at distal end of decapitated seedling, after that a horizontal cut was made at half of the rootstock diameter at the base of vertical cut. The flaps of cut were opened and excised shoot tip of < 0.2 mm was inserted in the cortex of triangle cut. A bridge form folded Whatman no.4 filter paper platform was placed in the culture tube. The centre of the platform was perforated for insertion of the root portion for making the graft portion stand straight in the test tubes. These micrografts were kept at 25 ± 2°C in continuous dark for 24 h and after that they were exposed daily to 13:11 h's photo period. When scion of successful grafts produced 3-5 expanded leaves, the micrografts were then kept for 2 to 3 weeks inside the culture tube containing half strength of MS solution supplemented with IBA at 0.5 mg/l to ensure better rooting. These micrografts were carefully taken out from the culture tubes. These grafts embedded in tray which contained half strength MS solution for 24 h then transfer in hardening meadia containing cocopeat, vermiculite and perlite in 1:1:1 ratio. The successful micrografts were kept in green house at 90% humidity and 26±2°C temperature. After 90 days of hardening, some of successful plants shifted in insect proof net house to maintain mother progeny and remaining shifted in to field. The experiment was laid out in Completely Randomised Block Design. One treatment comprised of 10 micrografts and the experiment repeated thrice.

RESULTS AND DISCUSSION

Effect of age and type of rootstock on success of shoot tip grafting

Among four root stocks namely Rough lemon (*Citrus jambhiri* Lush), Carrizo citrange (*C. Carrizo*), Karna Khatta (*K. Karna*) and Sour orange (*C. aurantium*) the maximum success 62% (at 15 days old), 66.60% (at 13 days old), 54.00% (at 14 days old) and in Sour Orange 52% (at 14 days old) were observed respectively (Table 1). This study indicates that the success of *invitro* grafting depends on type of rootstock used because of compatibility difference between stock and scion. These findings are in agreement with those reported by Navarro (1988), who had used Troyer, Carrizo, Rough lemon, Sweet orange, Trifoliate orange, Etrog citron, Sour orange, Rangpur lime, Cleopatra mandarin and Sacton citrumelo rootstocks for micro grafting. Differences in grafting success were observed among these rootstocks. Dass *et al.* (1997) reported that the overall success of *in vitro* grafts was more in Troyer citrange (38.25%) followed by Carrizo citrange (29.6%) and rough lemon 25.78%. Karunakaran *et al.* (2006) reported that the maximum 30.15% success of STG was recorded when Coorg mandarin was used as scion with Rangpur lime and Troyer citrange seedlings were used as rootstock.

Age of rootstock has not shown any definite trend on success of grafting. In Carrizo, success was more at the age of 12-13 days, in Rough lemon it is 15-16 days old, in Sour orange and in Karna Khatta it was at 14-15 days (Table 1). Generally age is only the indicative of seedling growth which may be dependent on temperature, seed batch, concentration of disinfecting solution used, etc. (Navarro *et al.*, 1975). The diameter of seedling is also deciding factor in success of STG. It was also observed that the Carrizo citrange seedlings were thicker than other seedlings. The

sprouting in successful grafts was faster in Rough lemon 16.4 days followed by Carrizo citrange 17.8 days, Sour Orange 20.4 days and Karna Khatta 20 days (Table 1). This may be due to compatibility difference between species to species. These findings are in line with those reported by Jonard (1986) who reported that 15 days are required to combine connation between the scion and rootstock. The length of new shoot on different rootstock varied after 45 days of grafting. The maximum length of shoot was observed 1.88 cm (on 13 days old seedling in Carrizo and 15 days old Seedling in Rough Lemon) followed by Sour orange 1.34 cm (on 14 days old seedling) and Karna Khatta 0.98 cm (on 14 days old seedling) (Table 1 and Fig 2). This difference for length of shoot in different root stock may be due to the growth habit of root stock, graft compatibility and time taken combine connection between the stock and scion. These findings are in accordance with Lahoty *et al.* (2013) and Juarez *et al.* (2015).

The age of seedling played a significant role in success of STG. The highest per cent of successful STG was obtained when 2 week old seedling were used as rootstock. Younger seedlings as well as older (more than 15–18 days) were inferior (Table 1). The unsuccessful grafts on older seedlings showed greater proportion of scion shoot tip that had shrivelled, turned brown, and dried. Whereas, those of younger age seedlings with scion shoot tip had became quiescent and buried in callus produced by the root stock tissue. The higher incidence of browning and drying of shoot tips indicates that perhaps the grafts failure with older seedlings were related to moisture inadequacy, failure on the younger seedlings on the other hand seemed to be associated with precocious callus formation. The similar finding are also in line with Kumar *et al.* (2010)

Effect of auxins (NAA, IAA and 2,4-D) on different parameters of STG

While studying the effect of type of auxin, i.e. NAA, IAA and 2,4-D the maximum success of grafts 62.00% was recorded in Rough Lemon when apical meristem and decapitated seedlings were pre-treated with 2,4-D 3.0 mg/l followed by IAA 1.0 mg/l 60.00% and NAA 0.5 mg/l 56.00%. Although, in Carrizo, the maximum Percent survival 56.00 obtained with 2,4-D 3.0 mg/l followed by 52.00% at 0.1 mg/l IAA and 54.00% at 0.5 mg/l[1] of NAA (Table 2 and Fig 1). The higher success in 2,4-D must be due to prevention of desiccation of shoot tip and induction of early callusing. These findings are similar to those reported by Vijayakumari and Singh (2000) in Nagpur mandarin,

Table 1 Effect of age and type of rootstocks seedling on success of shoot tip grafting in Blood Red

Age of seedling (Days)	Rough lemon			Carrizo			Sour orange			Karna Khatta		
	% success	Number of days taken to bud sprout	Length of shoot	% success	Number of days taken to bud sprout	Length of shoot	% success	Number of days taken to bud sprout	Length of shoot	% success	Number of days taken to bud sprout	Length of shoot
11												
12				42 (40.32)	17.8	1.74						
13	36 (36.81)	16.4	1.32	66 (54.36)	18.4	1.88						
14	44 (41.52)	16.8	1.70	62 (52.00)	18.4	1.62	54 (47.29)	20.4	1.34	52 (46.14)	20.0	0.98
15	62 (51.95)	17.6	1.88	52 (46.14)	19.4	1.50	50 (44.98)	20.6	1.14	48 (43.83)	20.4	0.86
16	54 (47.29)	19.0	1.46	48 (43.83)	20.4	1.22	48 (43.83)	21.4	0.92	44 (41.52)	20.6	0.72
17	46 (42.68)	19.6	1.06	46 (42.68)	21.0	1.02	44 (41.52)	21.6	0.84	42 (40.32)	22.0	0.66
18	40 (39.17)	20.6	0.76	44 (41.52)	21.4	0.80	42 (40.37)	22.2	0.66			
19	36 (36.81)	21.6	0.84	40 (39.17)	22.4	0.78						
20	34 (35.60)	22.0	0.74	28 (31.87)	22.6	0.72						
SEm±	1.44	0.21	0.07	1.64	0.21	0.04	1.15	0.19	0.02	0.97	0.11	0.02
CD (P=0.05)	4.13	0.59	0.20	4.70	0.61	0.12	3.29	0.55	0.06	2.78	0.31	0.05

*Figures given in parenthesis is angular transformed values.

Table 2 Effect of NAA, IAA and 2,4-D on different parameters of STG on Carrizo and Rough lemon

Growth regulator (mg/l)	Carrizo									Rough Lemon								
	Percent success (%)			Number of days taken to sprout			Length of shoot (cm)			Percent Success (%)			Number of days taken to sprout			Length of shoot (cm)		
	NAA	IAA	2,4-D	NAA	IAA	2,4-D	NAA	IAA	2,4-D	NAA	IAA	2,4-D	NAA	IAA	2,4-D	NAA	IAA	2,4-D
Control	34 (40.37)	34 (32.53)	40 (39.11)	21.4	19.4	19	1.74	1.72	1.72	36 (42.67)	44 (47.28)	46 (42.68)	20.4	19.8	19	1.70	1.70	1.68
0.1	44 (41.52)	50 (44.98)	42 (40.32)	19.6	19.8	17.4	1.90	1.78	1.98	40 (39.17)	56 (48.44)	46 (42.98)	19.2	19.2	16.8	1.78	1.74	1.84
0.5	54 (47.29)	52 (46.14)	42 (40.32)	17.8	17.6	16.6	2.60	1.88	2.34	56 (48.44)	60 (50.80)	50 (44.98)	17.8	17.8	16	2.48	1.84	2.08
1.0	52 (46.14)	50 (44.98)	44 (41.52)	20.6	18.8	15.4	1.60	2.38	2.6	52 (46.14)	58 (49.65)	52 (46.14)	16.4	18.2	14.6	1.54	2.26	2.32
1.5		48 (43.83)	46 (42.68)		19.4	14		1.66	2.64	48 (43.83)	56 (48.44)	54 (47.29)	20.6	18.6	13.6	1.06	1.78	2.48
2.0			48 (43.83)			13.4			2.92			58 (49.65)			13			2.8
3.0			56 (48.44)			13			2.94			62 (51.95)			12.6			2.9
SEm±	0.87	1.47	1.70	0.21	0.28	0.18	0.04	0.07	0.03	1.38	1.24	1.64	0.25	0.29	0.27	0.03	0.06	0.06
CD (P=0.05)	2.53	4.29	4.97	0.61	0.81	0.53	0.13	0.22	0.10	4.03	3.63	4.74	0.73		0.78	0.10	0.19	0.19

*Figures given in parenthesis is angular transformed values.

Fig 3 Hardening of STG plants

Table 3 Percent Survival of grafted plant on different rootstocks in polyhouse

Percentage of plantlets survival after (in days)	Name of citrus species			
	Rough Lemon	Carrizo	Sour orange	Karna Khatta
15	95 (77.05)	92 (73.54)	88 (69.70)	82 (64.87)
30	92 (73.54)	90 (71.54)	82 (64.87)	74 (59.32)
60	90 (71.54)	87 (68.84)	75 (59.98)	70 (56.77)

*Figures given in parentheses are angular transformed values

They reported 66.66% success when the apical meristem and decapitated seedling were dipped in 2,4-D (10 mg/l). Similar results are also reported by Starrantino *et al.* (1986) and Mishra and Yadav (1999) who demonstrated increase in success per cent of grafts by application of one drop of 2,4-D (10 mg/l) at the time of graft union. These results are in accordance with Singh *et al* (2011).

Among auxin treatments, 2,4-D @ 3.0 mg/l, significantly decreased time taken to sprouting (12.6 days in Rough Lemon and 13.0 days in Carrizo) as compared to control. Similar results have also been reported by Vijayakumari and Singh (2000) in Nagpur mandarin, minimum 12.22 days were required to sprouting in apical meristem when 2.0 mgl^{-1} 2,4-D was applied for proliferation of scion and stock tip for 10 minutes. The maximum length of new shoot 2.94 cm in Carrizo and 2.90 cm in Rough Lemon were recorded with 2,4-D 3.0 mg/l. The results of the present investigation are in close agreement to those reported by Vijayakumari and Singh (2000) in Nagpur mandarin. Accordingly to them, the maximum 3.93 cm length of new shoot was recorded when the shoot tip (scion) and decapitated seedling treated with 2,4-D (10 mg/l).

Transfer of in vitro grafts to pots

In vitro plants were acclimatized by tranferring them in to pots containing hardening media cocopeat, vermiculite and perlite in 1:1:1 ratio. This supported high porosity, CEC and water holding capacity forming better rooting and overall survival of plants.

After 45 days of shifting, the maximum 95% of survival were observed when Blood Red shoot tip was grafted on Rough Lemon followed by 92.00% on Carrizo, 88.00% on Sour Orange and 82% on Karna Khatta rootstocks (Table 3). The higher survival of grafts on Rough lemon and Carrizo is due to comparative grafts compatibility between stock and scion. Similar results report by Navarro (1988), who reported 95% survival of grafts in green house. The findings are also recorded by Karunakaran *et al.* (2006) in Coorg mandarin and Sanabam *et al.* (2015).

Conclusion

In *in vitro* shoot tip grafting, Blood Red/Rough Lemon (13 days old) seedling was found better than other rootstocks. Among PGRs, 2,4-D (3.0 mg/l) was found better pre treatment for shoot tip to increase success of STG. In green house, maximum survival of plantlets and grafted plants were recorded in and Blood Red/Rough Lemon, respectively

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