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Effect of rootstock and age of seedling on success of *in vitro* shoot tip grafting in Kinnow mandarin

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ABSTRACT

The *in vitro* shoot tip grafting (STG) is a method employed for production of virus-free plants. The present investigation was carried out to study the effect of rootstock and age of seedling on success of *in vitro* shoot tip grafting in Kinnow mandarin. Four citrus rootstocks, *viz*., sour orange (*Citrus aurantium* L.), Carrizo citrange [*Citrus sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.], rough lemon (*Citrus jambhiri* Lush.) and Cleopatra mandarin (*Citrus reshni* Hort. ex. Tan.) were selected for present study. The maximum STG success was observed in Carrizo citrange on 12-day-old seedlings. STG on rough lemon rootstock took minimum number of days for bud sprout followed by Carrizo citrange. The highest number of leaves per graft was recorded on 12-day-old Carrizo citrange seedlings followed by rough lemon. The highest scion shoot length was observed on 12-day-old seedlings of Carrizo citrange followed by that of 16-day-old rough lemon seedlings. The maximum number of roots per graft was produced in Carrizo citrange. The highest per cent transplanting success of STG plantlets was observed on Carrizo citrange followed by rough lemon. It was observed that the Carrizo citrange was found best for most of STG parameters, *viz*. per cent STG success, number of leaves per graft, scion shoot length, number of roots per graft, stock root length and transplanting success.

Key words: Citrus rootstocks, *in vitro* shoot tip grafting, Kinnow mandarin.

INTRODUCTION

Kinnow is a hybrid mandarin (*Citrus nobilis* Loureiro × *C. deliciosa* Tenore) developed by H.B. Frost in 1915 (Hoa *et al*., 3), which is widely grown in North India. Virus and virus-like diseases are the major production impediments in Kinnow growing area. There is no way to an increase in the existing yield levels and securing the citrus industry from graft transmissible diseases which causing decline, until the planting material is made free from important virus and virus-like pathogen in the country. The viral infection cannot be managed under field conditions. In *Citrus* species, the technique of *in vitro* shoot tip grafting (STG) has been well documented for the production of healthy planting material (Navarro, 7). The standard procedure of shoot-tip grafting technique as described by Navarro *et al*. (9) was used which consisted of grafting of *in vitro* generated etiolated seedling at early stage (2-3 weeks) under aseptic conditions, with a small shoot tip (0.1-0.2 mm). However, few reports have mentioned its application in Kinnow. Taking this in account, the present investigation was conducted to find out the effect of rootstock and age of seedling on success of *in vitro* shoot tip grafting.

MATERIALS AND METHODS

The present experiment was conducted in Department of Horticulture, College of Agriculture, CCS HAU, Hisar and Center for Plant Biotechnology, Department of Science and Technology, Government of Haryana, Hisar during 2011-12. Four rootstocks, *viz*. sour orange (*Citrus aurantium* L.), Carrizo citrange [*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.], rough lemon (*C. jambhiri* Lush.) and Cleopatra mandarin (*C. reshni* Hort. ex. Tan.) were selected for experiment. Shoot tips of scion cultivar Kinnow were taken from *in vitro* proliferated cultures on Murashige & Skoog (6) medium. A stereoscopic microscope was used for *in vitro* shoot tip grafting.

Fresh fruits of different rootstocks were collected in the month of January-February. Seeds were extracted and stored in juice in refrigerator. The stored seeds were washed with 2-3 drops of Tween-20® per hundred ml of water for 10 min. followed by washing under running tap water for 30 min. to remove the effect of detergent. These washed seeds were treated with 0.2% carabendazim and 0.1 per cent streptocycline for 20 min. on a magnetic stirrer at 50°C and then washed 3-4 times with double distilled water. Thereafter, seeds were decoated and surface sterilized with 0.1% $HgCl_2$ for 5 min. followed by 3-4 times washing with autoclaved double-distilled water. The seeds were treated with ethanol 70% for 30 sec followed by 3-4 times washings with autoclaved double-distilled water. The surface-sterilized seeds were inoculated individually in culture tubes containing Murashige and

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Skoog (1962) medium supplemented with BAP 0.5 mg/l and NAA 1.0 mg/l for germination. The culture tubes were incubated at 25 ± 2°C temperature in continuous darkness for 2-4 weeks. Seedlings recovered by *in vitro* seed germination were used as rootstocks. Two-to-three week (12 to 18 days) old etiolated seedlings were removed from the test tubes under aseptic conditions and decapitated leaving about 2-4 cm of epicotyl under the laminar air-flow. Cotyledons were detached and root tips cut back to about 1-2.5 cm. An inverted-T incision was made on each epicotyl through the cortex with a sterile razor blade. Decapitated rootstock was kept moist by putting a drop of liquid media at cut surface to avoid desiccation.

In shoot tip grafting, scion apical meristem was approximately 0.2-0.3 mm in length. The shoot tips of Kinnow mandarin were excised from *in vitro* raised shoots. These shoot tips (0.2-0.3 mm) along with 1-2 leaf primordial were dissected under stereoscopic microscope in laminar air-flow. Scions were kept moist by putting a drop of liquid medium to avoid desiccations at the time of grafting. An inverted-T cut was made on decapitated apical portion of rootstock. For preparation of inverted-T cut, a vertical cut of about 3-4 mm was made at apical 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 0.2-0.3 mm was inserted in the cortex of cut end. All operations were carried out under aseptic conditions. The STG plants were cultured in a liquid medium composed of MS (Murashige and Skoog, 6) macro-and micro-elements fortified with the vitamins medium (WM) and sucrose @ 7.5 per cent. A folded Watman No. 4 filter paper platform was placed in the culture tube. The culture tubes were capped to ensure high relative humidity inside the tube. The micro-grafts were kept at 25 ± 1°C and exposed daily to 16/8 h photoperiod (2000 lux florescent tubes).

Five weeks after grafting, the scion of successful grafts produced 2-5 expanded leaves. The STG plants were then kept inside the culture tubes, for 1 to 2 weeks, containing half-strength MS medium. These STG plants were carefully taken out from the culture tubes and washed with sterilized distilled water to remove any adhering medium. Then, the STG plants were treated with 0.2 per cent carbendazim solution for 5 min. to prevent fungal infection. The STG plants were transferred to plastic pots containing autoclaved mixture of soil, sand and vermi-compost in the ratio of 1:1:1 and kept in greenhouse by covering pots with polyethylene bags to maintain humidity, temperature. In green house, STG plants were fertigated with half-strength of MS salts for three times during acclimatization at weekly interval. After 10-12 days, polyethylene bags were removed initially for a short duration (1-2 h) daily for 4-5 days. Gradually, the daily exposure time was increased 1 h for each day. Polyethylene bags were completely removed after 20 days. The observations were recorded for number of STG survival after 15, 30 and 45 days of transplanting in pots under greenhouse.

In order to test the significance of variation in experimental results obtained from different parameters of STG were statistically analyzed by using completely randomized block design.

RESULTS AND DISCUSSION

The maximum grafting success was recorded in Carrizo citrange followed by the rough lemon. Among the age of seedlings, mean success was higher on 16-day-old seedlings. The highest STG success was observed in Carrizo citrange on 12-day-old seedlings (38.23%). The 12 to 14 day-old seedlings in Carrizo citrange, 16-days-old in rough lemon and Cleopatra mandarin and 18-day-old seedlings in sour orange were found better for STG, respectively (Table 1).

These findings are in agreement with those reported by Navarro (8). Dass *et al*. (2) reported that the overall success of *in vitro* grafts was more in Troyer citrange followed by Carrizo citrange and rough

Table 1. Effect of rootstock and age of seedling on STG success (%) in Kinnow.

Rootstock	Seedling age (days)				Mean
	12	14	16	18	
Sour orange	16.67 (24.04)*	18.33 (25.30)	23.33 (28.84)	28.33 (32.13)	21.67 (27.58)
Carrizo citrange	38.33 (38.23)	33.33 (35.24)	26.67 (31.06)	16.67 (24.04)	28.75 (32.14)
Rough lemon	26.67 (31.06)	31.67 (34.22)	36.67 (37.24)	18.33 (25.30)	28.33 (31.95)
Cleopatra mandarin	16.67 (24.04)	23.33 (28.84)	31.67 (34.22)	18.33 (25.30)	22.50 (28.10)
Mean	24.58 (29.34)	26.67 (30.90)	29.58 (32.84)	20.42 (26.69)	
CD at 5%	Rootstock (R) = 1.64		Seedling age (D) = 1.64		R × D = 3.27

*Figures in parenthesis indicate the angular transformed values.

lemon. Karunakaran *et al.* (4) found maximum per cent success of STG when Coorg mandarin was used as scion with Rangpur lime and Troyer citrange rootstock seedlings. The results of the present study are in conformity with those of Hoa *et al*. (3) and Kumar (5). The diameter of seedling is also a deciding factor in success of STG. It was also observed that the Carrizo citrange seedlings were thicker than other seedlings. According to Navarro (8), Troyer citrange seedling reached to a size of 3-5 cm with a diameter of 1.6-1.8 mm at the point of grafting within 12 days. This study indicated that the success of *in vitro* grafting depended on type of rootstocks used because of compatibility differences between rootstock and the scion. The reduction in successful grafts with older rootstock may be due to harder stem, which was difficult to cut and insert the scion on it. The lower success in younger seedlings appears to be due to precocious callus formation that may bury the scion within it.

The sprouting in successful grafts was faster in Rough lemon followed by Carrizo citrange. It was least on 16-day-old seedlings. However, as per interaction (Table 2), STG on rough lemon seedling of 14-day age sprouted earliest. This might be due to graft compatibility differences between species. These findings are in line with those reported by Singh *et al.* (10) in *Desi* mandarin on rough lemon. Similar results were also reported by Kumar (5) in Kinnow.

The number of leaves per graft on different rootstocks varied after 45 days of grafting. The maximum number of leaves per graft was observed on Carrizo citrange followed by rough lemon. Among age of seedlings, maximum number of leaves developed per graft, when 16-day-old seedlings were used for grafting. The interaction between rootstock and age of seedling was found significant. Highest number of leaves per graft (3.44) was recorded on 12-day-old Carrizo citrange seedlings (Table 3). These differences for number of leaves per graft in different rootstocks might be due to the growth habit of rootstocks, nutrient uptake, graft compatibility and time taken to establish connection between the stock and scion. Similar results were reported by Ali *et al.* (1), while grafting of Kinnow on sour orange rootstock. The above results are in concurrence with those of Vijayakumari *et al.* (11), who also found varied response in Nagpur mandarin on rough lemon rootstock.

The length of new shoot on different rootstocks varied after 45 days of STG. The maximum length of shoot was observed on Carrizo citrange followed by that on rough lemon. Among the age of seedling, the scion shoot length was maximum on 16-day-old seedlings. The interaction was found significant. Longest shoots (1.48 cm) were observed on 12-day-old seedlings of Carrizo citrange. In rough lemon, the maximum shoot length (1.27 cm) was recorded on 14-day-old seedlings and in Cleopatra mandarin, the maximum shoot length (1.08 cm) was noted on 16-day-old seedlings. In sour orange, maximum scion shoot length (1.05 cm) was recorded on 18-day-old seedlings (Table 4). These differences for length of scion shoot in different rootstocks might be due

Table 2. Effect of rootstock and age of seedling on days taken for bud sprouting after STG in Kinnow.

Rootstock	Seedling age (days)				Mean
	12	14	16	18	
Sour orange	24.22	22.89	20.78	20.44	22.08
Carrizo citrange	19.56	19.89	20.89	22.22	20.64
Rough lemon	20.33	18.11	19.11	21.33	19.72
Cleopatra mandarin	21.44	20.78	20.11	24.56	21.72
Mean	21.39	20.42	20.22	22.14	
CD at 5% Rootstock (R) = 0.25	Seedling age (D) = 0.25			R × D = 0.49	

Table 3. Effect of rootstock and age of seedling on number of leaves per graft* in Kinnow.

Rootstock	Seedling age (days)				Mean
	12	14	16	18	
Sour orange	1.56	1.89	2.44	3.00	2.22
Carrizo citrange	3.44	3.11	2.78	2.44	2.94
Rough lemon	2.11	3.22	2.89	2.22	2.61
Cleopatra mandarin	1.78	2.44	2.78	2.56	2.39
Mean	2.22	2.67	2.72	2.56	
CD at 5% Rootstock (R) = 0.18	Seedling age (D) = 0.18			R × D = 0.37	

*Observations were recorded after 45 days of STG

Table 4. Effect of rootstock and age of seedling on scion shoot length* (cm) after STG in Kinnow.

Rootstock	Seedling age (days)				Mean
	12	14	16	18	
Sour orange	0.53	0.70	0.80	1.05	0.77
Carrizo citrange	1.48	1.27	1.17	1.00	1.23
Rough lemon	0.72	1.27	0.92	0.89	0.95
Cleopatra mandarin	0.58	0.64	1.08	0.79	0.77
Mean	0.83	0.97	0.99	0.93	
CD at 5% Rootstock (R) = 0.03	Seedling age (D) = 0.03			R × D = 0.06	

*Observations were recorded after 45 days of STG

to the growth habit of rootstock, nutrient uptake, graft compatibility and time taken in establishing connection between the stock and scion. These findings are in line with those reported by Kumar (5). Similar results were also reported by Ali *et al*. (1) on sour orange rootstock.

The maximum number of roots per graft was noted in Carrizo citrange followed by rough lemon. These differences in number of roots per graft may be due to the growth habit of rootstocks. Among age of seedlings, there was an increasing trend regarding number of roots per graft with age of seedling, which was maximum on 18-day-old seedlings (Table 5). Root length at the time of transplanting on different rootstocks also varied. The maximum root length was observed in Carrizo citrange followed by rough lemon. Among age of seedlings, there was an increasing trend regarding length of stock roots with age of seedling, which was maximum on 18-day-old seedlings (Table 6). These differences in root length may also be due to variation in growth habit of rootstocks.

The maximum percentage of transplanting success of STG plants was observed when Kinnow shoot tip was grafted on Carrizo citrange followed by 63.07 per cent on rough lemon at 15th day after transfer in sterilized soil mixture (sand: soil: vermi-compost :: 1:1:1) under greenhouse conditions (Table 7). The higher survival of grafts on Carrizo citrange and rough lemon might be due to comparatively better graft union and compatibility between stock and scion. Similar results were also reported by Karunakaran *et al.* (4) in Coorg mandarin and Kumar (5) in STG plants of Kinnow on Carrizo citrange rootstock.

Among different rootstocks, Carrizo citrange and rough lemon gave better success of *in vitro* shoot tip grafting in Kinnow mandarin (Fig. 1). It was observed that the Carrizo citrange was best for most of STG parameters, *viz.*, STG success, number of leaves per graft, scion shoot length, number of roots per graft, stock root length and transplanting success.

Table 5. Effect of rootstock and age of seedling on number of roots per graft* in Kinnow.

Rootstock	Seedling age (days)				Mean
	12	14	16	18	
Sour orange	1.44	1.78	2.44	3.00	2.17
Carrizo citrange	2.44	2.33	3.22	3.56	2.89
Rough lemon	1.56	2.11	2.44	2.78	2.22
Cleopatra mandarin	1.56	1.89	2.22	2.67	2.08
Mean	1.75	2.03	2.58	3.00	
CD at 5%	Seedling age (D) = 0.19			R × D = NS	
Rootstock (R) = 0.19					

*Observations were recorded at the time of transplanting

Table 6. Effect of rootstock and age of seedling on stock root length* (cm) after STG in Kinnow.

Rootstock	Seedling age (days)				Mean
	12	14	16	18	
Sour orange	3.73	3.98	4.20	4.43	4.09
Carrizo citrange	5.68	5.88	5.99	6.16	5.93
Rough lemon	4.87	5.21	5.48	5.73	5.32
Cleopatra mandarin	4.20	4.50	4.81	5.10	4.65
Mean	4.62	4.89	5.12	5.36	
CD at 5% Rootstock (R) = 0.05	Seedling age (D) = 0.05			R × D = 0.09	

*Observations were recorded at the time of transplanting

Table 7. Transplanting success (%) of STG plants on different rootstocks under greenhouse conditions.

Rootstock	Days after transfer in soil mixture		
	15	30	45
Sour orange	58.33 (49.81)	45.83 (42.57)	45.83 (42.57)
Carrizo citrange	83.33 (66.17)	70.83 (57.39)	70.83 (57.39)
Rough lemon	79.17 (63.07)	66.67 (54.80)	66.67 (54.80)
Cleopatra mandarin	62.50 (52.39)	54.17 (47.39)	54.17 (47.39)
CD at 5%	10.95	8.21	8.21

*Figures in parenthesis indicate the angular transformed values.

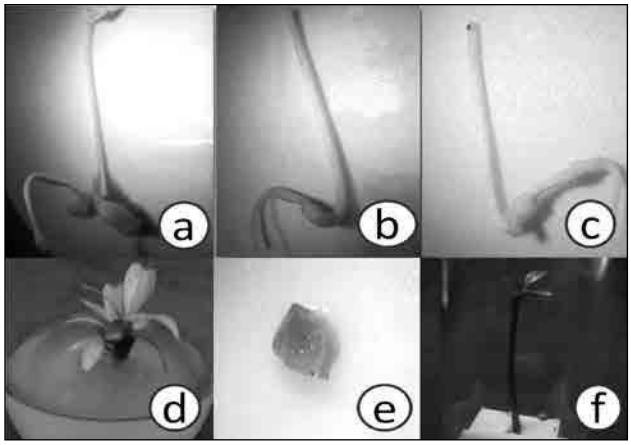

Fig. 1. Stages of *in vitro* shoot tip grafting in Kinnow mandarin; a & b = rootstock preparation; c = newly grafted plant, d & e = scion preparation; f = STG plant after 45 days of grafting.

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