Indian Journal of Agricultural Sciences **84** (11): 1376–81, November 2014/Article

Standardization of protocol for *in-vitro* shoot tip grafting in Kinnow mandarin (*Citrus deliciosa*)

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Received: 13 September 2013; Revised accepted: 16 September 2014

ABSTRACT

An experiment to standardize the rootstocks and PGRs for success and survival of *in-vitro* shoot tip grafting in Kinnow mandarin (*Citrus deliciosa*) was conducted during the year of 2007-08 at Department of Horticulture, Agricultural Research Station, Sriganganagar, Rajasthan. For this, *in-vitro* generated and 2-3 weeks etiolated old seedlings of cleopatra, rough lemon and carrizo were used as rootstocks. The seedlings were grafted by shoot tips measuring = 1 mm in length containing apical meristem and one or two leaf primordial which were excised from *in-vitro* generated shoots and grafted on rootstocks. Among different rootstocks used, twelve days old seedlings of Carrizo recorded the maximum success (56.60 %) and length of shoot (1.43 cm). All the concentrations of the PGRS tried to accelerate the growth of new shoot and survival per cent of STG, BAP @ 1.0 mg/l was found to be best in minimizing the time taken to bud break (12.12 days) and recorded maximum shoot length (3.10 cm). The results of the study revealed that the maximum survival (66.90 %) was observed with 2, 4-D @ 3.0 mg/l in all the PGRs tried for growth promotion of scion shoots. It is also revealed from the study that the 90 % grafted plants survival in greenhouse at after 45 days after shoot tip grafting. Based on the observations, the Carrizo as rootstocks, BAP and 2, 4-D were found to be the best protocol for shoot tip grafting in Kinnow mandarin.

Key words: Apical meristem, Kinnow mandarin, Plant growth regulators, Seedlings, Shoot tip grafting

Kinnow mandarin (*Citrus deliciosa*) is a cross between the King Sweet Orange (*C. nobilis*) and Willow Leaf Mandarin (*C. deliciosa*) made in 1915 by H B Frost at Citrus Research Centre, Riverside, California, University of California, and was released in 1935 (Frost and Kurg 1942). Kinnow fruits have a great therapeutic value owing to favourable ratio between K and Na, which makes good ionic balance in the body. Kinnow fruits are not only delicious and refreshing but also possess great nutritive value. It contains 87% moisture, 11.5-13.5% TSS, 0.048-0.05 mg/100ml calcium, 0.019-0.02 mg/100ml phosphorus, 0.09-0.1 mg/100 ml irons and 0.01-0.02 mg/100 ml vitamin C. In Kinnow, the infections by various pathogens have received attention due to decline of citrus trees and it was mainly attributed to viruses (Tristiza, Greening ring spot virus, Exocortis, Xyloporosis etc.), fungi (*Phytopthora*) and bacterial canker. Apart from these, other factors like rootstock incompatibility, poor management, malnutrition, marginal soils and quality of irrigation water also affect citrus production. Tristiza virus has wiped out the citrus industry in many countries almost to the tune of 30 million trees in Argentina and Brazil after First World War. Similar situation has also been reported from Spain, Japan, United State and India. Tristiza destroyed about a million trees in India (Fraser, 1966 and Ahlawat 1997). *In-vitro* shoot tip grafting (STG) is widely used for production of virus free plants in different countries and it offers an advantage over conventional methods of propagation ('T'-budding) for producing large number of true to type plants from healthy plant within a short period of time. Navarro (1981) had suggested that this technique can be used in citrus improvement programs for production of disease free, true to type planting material and mitigating the risk of introducing disease from one country to another. Now-a-days, many cultivated plants have been "regenerated" by shoot-meristem culture. The success of shoot tip grafting varies from 30-50 per cent or some times even higher. Several factors influence the rate of success of grafting or virus elimination, viz. type and age of root stock, culture medium, condition of root stock (etiolated or non etiolated),

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size of meristem, environmental conditions, plant growth regulators, root stock compatibility with scion and acclimatization process. Among them, plant growth regulators play an important role in per cent survival of STG. The per cent survival of *in-vitro* grafted plant was less in field condition, if they are transferred directly, without acclimatization. In present study, a complete protocol of shoot tip grafting in Kinnow mandarin have been standerdized.

MATERIALS AND METHODS

Fresh fruits of three Citrus species namely Rough Lemon (*C. jambhiri* Lush.), Carrizo Citrange (*C. Carrizo*) and Cleopatra (*C. reshnii* Tanaka) were collected from Citrus repository, Department of Horticulture, Agricultural Research Station, Sriganganagar. The fruits were washed with detergent (Tween-20 @1 ml/l) and water. The seeds were extracted and washed under running tap water for 1-2 hours. These seeds were treated with 0.2% Bavistin (a systemic fungicide of BASF, Indian Ltd., Mumbai) for 6 minutes and were washed 3-4 times with distilled water. The seed testa was removed under aseptic condition. These decoated seeds were first rinsed with 70 per cent ethanol for 30 seconds followed by sterilization under aseptic condition with 0.1 percent Mercuric Chloride ($HgCl_2$) solution (w/v) for five minutes and again rinsed 4-5 times with sterile double distilled water. These surface sterilized seeds were inoculated in culture tube (25 × 150 mm) containing 15-20 ml of basal MS medium (Murashige and Skoog 1962). The culture tubes were incubated in BOD at 27 ± 2°C in continuous darkness for 2-3 weeks. These *in-vitro* raised etiolated seedlings were used as rootstock for shoot tip grafting (Fig 1 a).

In-vitro raised, 15-20 days old etiolated seedling, was taken as root stock. Upper portion including primary leaves was cut leaving only 2-3 cm long stem. Similarly roots were also trimmed to facilitate its entry in culture medium. A reverse 'T' incision was made on upper part of seedling with intact cambium. Isolated shoot tip must be placed on horizontal incision as shown in Fig 1d.

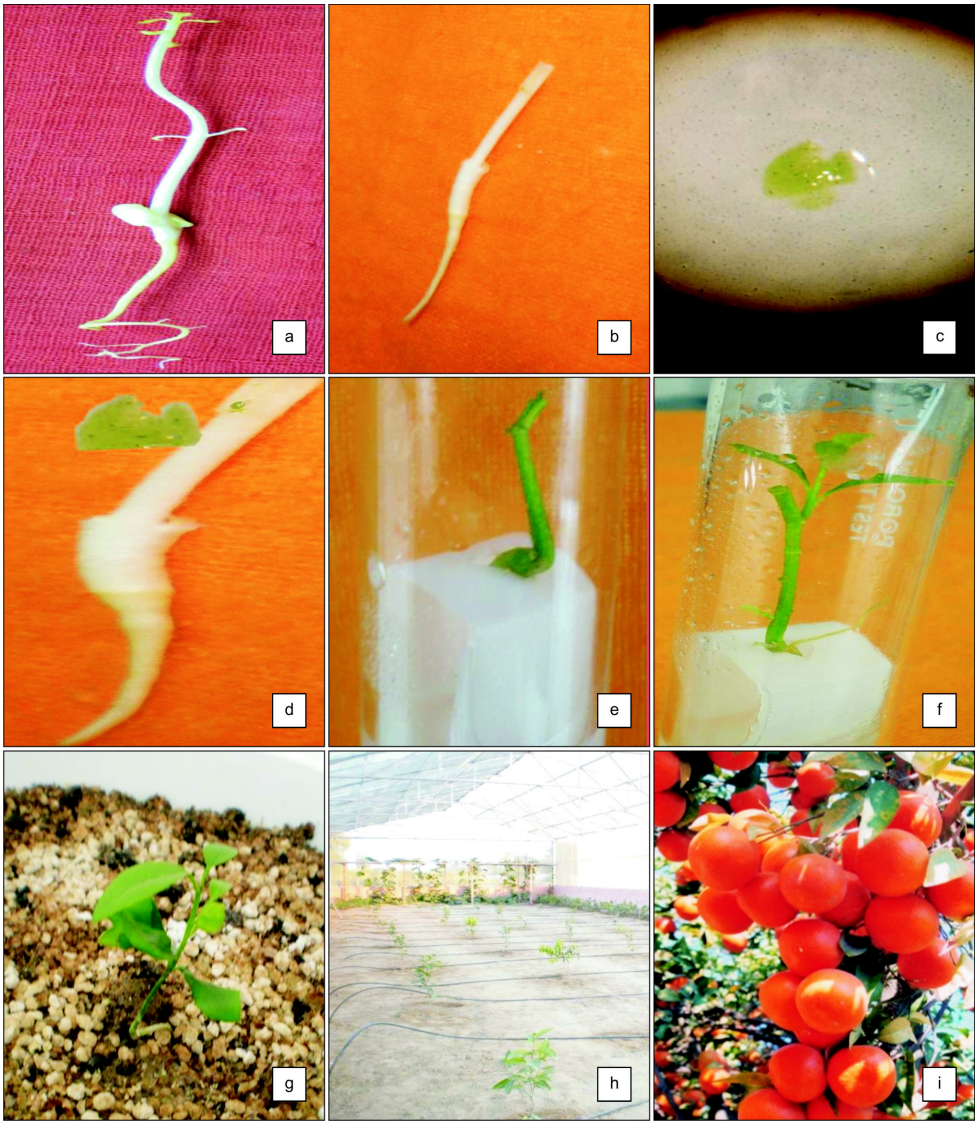

Fig 1 a. seedling of Carrizo, b. prepartaion of root stock, c. isolation of shoot tips, d. inserting of shoot tip, e. sprouting of shoot tip, f. growth of grafted plants, g. transfer of grafted plant in pot, h. grafted plant in screen proof net house, i. healthy Kinnow plant in bearing.

To obtain scion, one season old shoot (10-12 cm) of field grown plants were collected from healthy plants (Fig 1 i). The shoots were washed under running tap water for 1-2 hours. These shoot were treated with 0.2% Bavistin (a systemic fungicide of BASF, Indian Ltd, Mumbai) for 6 minutes and washed 3-4 times with distilled water. These shoots were first rinsed with 70 per cent ethanol for 30 seconds followed by sterilization under aseptic condition with 0.1 percent mercuric chloride ($HgCl_2$) solution (w/v) for seven minutes and were rinsed 4-5 times with sterile double distilled water. These surface sterilized shoots were inoculated in culture tube (25 mm × 150 mm) containing 15-20 ml of basal MS medium. The culture tubes were incubated in culture room at 27 ± 2°C light (40 m mol/m^2 /s) with 12/12 hrs photoperiod. After 20-25 days of incubation the shoots were ready to take scion shoots.

Shoot tips were excised from *in-vitro* generated shoots with the help of eye surgery blade, scissor and forceps. The dissection process was carried out under a stereoscopic microscope kept in laminar air flow bench. These shoot tips of < 1.0 mm in size along with 1-2 leaf primordia were dissected and quickly placed on root stock seedling which were already prepared by trimming of root and shoots (Fig 1c).

The micrografts were treated with different Plant Growth Regulators (PGRs) in various concentrations, i.e. BAP and Kinetin at 0.5, 1.0 and 2.0/mg while at NAA and IAA at 0.1, 0.5 and 1.0, 2.0/mg and 2, 4-D at 1.0, 2.0 and 3.0/mg by placing 10 µl of growth regulator after inserting the shoot tip in inverted 'T' cut.

The micro grafts were cultured in a MS medium fortified with the vitamins of White's medium (White 1943) and sucrose @ 6.0 per cent. The culture tubes were capped to ensure high relative humidity inside the tube. The micrografts were kept at 27 ± 0.5°C in continuous dark for 24 hours and after that exposed daily to 13/11 hr photoperiod. Regeneration of shoot tip from micro graft was closely monitored. After 7-10 days, new growth was observed from the place where inoculated shoot tip was resting. (Fig 1e & f). After 3-4 weeks of inoculation, all responding cultures were sub cultured on freshly prepared medium. During sub culturing, dried leaves and off shoot were removed.

The rooted graft shoots were carefully removed from the culture tubes and their roots were thoroughly washed under running tap water and cleaned with fine brush to remove adhered agar. The grafted shoots were covered with sterilized cotton, wetted with half strength MS medium for 24 hours in culture room. The grafted shoots were treated with Bavistin @ 0.2% for 10 minutes to prevent fungal contamination. These grafted shoots were transferred to pots containing sterilized soil, vermiculite and perlite in equal proportion. These pots were kept in green house at 90 per cent humidity with temperature 26 ± 2^0C. The humidity was continuously lowered within 8-10 weeks up to 60%. During this period the plantlets were irrigated with Hogland solution at three days interval for one month. Thereafter, these plantlets were irrigated with Hogland solution and simple water at an interval of 2-3 days alternately. The observations were recorded on per cent survival of STG, time taken for sprouting, length of sprouted shoots on various rootstocks and on different plant growth regulators treatments. The survived percent of graft after 60 days of transfer in greenhouse on different rootstocks were also recorded. All the treatments were replicated ten times and data analyzed with CRD.

RESULTS AND DISCUSSION

Effect of age and type of rootstock

Perusal of Table 1 indicates that the success of *in vitro* shoot tip grafting was depends on type of rootstock used owing to compatibility difference. Among three rootstocks used, the maximum success percentage of STG (56.60) was observed on 12 days old seedling of Carrizo. Such

Table 1 Effect of age and type of rootstocks seedling on Survival percentage, Days to bud break and Length of shoot after STG in Kinnow Mandarin

Age of seedling (Days)	Rough lemon			Carrizo			Cleopatra		
	Survival (%)	Days to bud break	Length of shoot (cm)	Survival (%)	Days to bud break	Length of shoot (cm)	Survival (%)	Days to bud break	Length of shoot (cm)
11				38.20 (38.17)	18.00	1.03			
12				56.60 (48.45)	19.80	1.43			
13				44.70 (41.96)	20.00	0.97			
14	33.60 (35.43)	18.00	1.03	30.10 (33.27)	19.90	0.95	24.80 (29.33)	20.80	0.80
15	42.80 (40.86)	19.00	1.43	30.00 (33.13)	21.00	0.90	29.90 (33.15)	21.20	0.90
16	52.00 (46.15)	19.00	0.97				40.20 (39.35)	20.00	1.12
17	24.30 (29.33)	20.00	0.95				31.90 (34.39)	22.70	0.81
18	19.90 (26.49)	21.42	0.90				14.60 (22.46)	24.60	0.66
Mean	34.52 (35.97)	19.42	1.06	39.94 (39.17)	19.60	1.06	28.28 (32.01)	21.86	0.86
SEm±	1.69	0.19	0.028	1.43	0.19	0.028	1.21	0.23	0.021
CD (P=0.05)	1.03	0.72	0.059	0.99	0.78	0.059	0.86	0.72	0.058

*Figures given in parentheses are angular transformed values

Table 2 Effect of cytokinins (BAP & kinetin) on different regeneration parameters of shoot tip grafting in Kinnow mandarin, grafted on 12 days old Carrizo seedling

Growth regulators (mg/l)	Per cent survival		Time taken to bud break (Days)		Length of new shoot (cm)	
	BAP	Kinetin	BAP	Kinetin	BAP	Kinetin
0.0	49.90 (44.94)	49.90 (44.94)	19.80	19.80	1.60	1.60
0.5	51.00 (45.57)	50.10 (45.06)	13.80	18.30	1.99	1.62
1.0	56.70 (48.85)	66.50 (54.63)	12.20	13.80	3.10	2.34
2.0	50.20 (45.11)	40.20 (39.35)	16.80	18.20	1.72	1.69
SEm±	0.520	2.026	0.412	0.270	0.100	0.062
CD (P = 0.05)	1.618	1.158	0.715	0.691	0.187	0.091

*Figures given in parentheses are angular transformed values

compatibility differences have been observed by many other workers (Jonard *et al.* 1983, Jonard 1986, Vijaykumari *et al.* 1994, Mukhopadhyay *et al.* 1997 and Parthasarathy *et al.* 1997). Dass *et al.* (1997) reported that the overall success of *in-vitro* grafts were the maximum in troyer citrange (38.25 %) followed by Carrizo citrange (29.60 %) and rough lemon (25.78 %).

The success of STG depended on age of seedling which was different for all the three root stocks because of difference in germination time. Three rootstocks took different time for growing to grafting stage. The optimum age to carry out STG of three rootstock was apparently different. Age of rootstock has not shown any definite trend on success of grafting. In Carrizo success was better at the age of 12-13 days, whereas it was 15-16 days old in rough lemon and 16-17 days in Cleopatra mandarin (Table 1). In general, age is only the indicative of seedling growth which may be dependent on temperature, seed batch and concentration of disinfecting solution used for shoot sterilization (Navarro *et al.* 1975).

In case of Carrizo, the maximum per cent of successful STG was obtained when 12 days old seedling was used as rootstock, whereas in rough lemon and Cleopatra mandarin, the maximum survival of STG was observed when rootstock seedling age was 16 days old. Both younger seedlings as well as older one (more than 15-18 days) were found to be inferior. The unsuccessful grafts on older seedlings showed greater proportion of shrivelled scion shoot tip which later on turned in brown colour and dried, whereas those of younger age seedlings, the scion shoot tip became quiescent and buried in the callus (precocious callus formation) produced by the rootstock tissue and exhibited higher incidence of browning and drying of shoot tips indicates the grafts failure and failure of older seedlings were probably related to moisture inadequacy.

Results of the study revealed that the length of new shoot on different rootstock varied after 45 days of grafting.The maximum shoot length was observed on Carrizo (1.43 cm) at 12 days old seedling followed by rough lemon (1.43 cm) on 15th days old seedling and Cleopatra (1.12 cm) on 16 days old seedling. This difference in shoot length on different root stock may be attributed due to the growth habit of rootstock, graft compatibility and time taken in union between both the rootstock and scion.

Effect of PGRs on various parameters of shoot tip grafting

On the basis of observations, Carrizo citrange was selected as root stock for further study to see the effect of plant growth regulators on success of STG. Data related to success of shoot tip grafting in scions pretreated with cytokinins (BAP and Kinetin) are presented in Table 2 & 3. The levels and kinds of plant growth regulators significantly affected the survival per cent, time taken in sprouting and length of shoot of micro grafting. In case of cytokinins BAP and Kinetin, the maximum survival was recorded in kinetin (66.50%) followed by BAP (56.70%) at 1.0 mg/l. Kinetin was found superior than BAP because it gave significantly higher survival (66.50%,). These two pre-treatments probably helped to prevent desiccation of shoot tip. These findings are in close agreement with the findings of Vijayakumari and Singh (2000) and reported that the dipping shoot tips and decapitated rootstocks in kinetin and BAP @ 1.0 mg/l improved the success of grafting in Nagpur mandarin as compared to control. Similar observations for response to pre treatment were also reported by Driss and Burger (1984) in citrus.

Pretreatment of shoot tips with auxins also enhanced survival of STG. A peep of Table 3 revealed that the IAA and NAA produced almost similar response for survival, time taken in bud break and shoot length. The response for the per cent survival of STG was the maximum at 2.0 mg/l level of 2, 4-D, whereas it was 0.5 mg/l for IAA and 0.1mg/l for NAA for survival, whereas shoot length was better when IAA and NAA were applied % 0.5 mg/l. Interestingly, 2, 4-D elicited a bette response than IAA/NAA.The increasing concentration of 2, 4-D significantly increased success rate of STG probably it may be due to prevention of meristem desiccation and induction of early

Table 3 Effect of Auxins (NAA, IAA & 2, 4-D) on different regeneration parameters of shoot tip grafting in Kinnow mandarin, grafted on 12 days old Carrizo seedling

PGRs	Per cent survival of STG			Time taken to bud break (Days)			Length of new shoot (cm) after 45 days STG		
	NAA	IAA	2,4-D	NAA	IAA	2,4-D	NAA	IAA	2,4-D
0.0	49.90 (44.94)	49.90 (44.94)	49.90 (44.94)	19.80	19.80	19.80	1.59	1.59	1.59
0.1	52.50 (42.99)	50.60 (40.16)		19.00	20.30		1.42	1.51	
0.5	44.10 (42.76)	53.60 (47.06)		21.30	18.10		1.66	1.70	
2.0	39.80 (40.86)	36.60 (42.48)		22.20	22.20		0.90	1.32	
1.0			54.30 (47.47)			19.20			1.60
2.0			54.50 (47.58)			18.10			1.72
3.0			66.90 (54.88)			17.20			1.79
SEm±	0.247	0.731	1.106	0.268	0.305	0.265	0.048	0.031	0.016
CD (P = 0.05)	0.585	0.626	0.856	0.850	0.830	0.663	0.049	0.059	0.045

*Figures given in parentheses are angular transformed values

callusing. These findings are similar to those reported by Vijayakumari and Singh (2000) and Starrantino *et al.* (1986) in Nagpur mandarin who had demonstrated an increase in success per cent grafts by application of one drop of 2, 4-D (10 mg/l) at the time of graft union.

Data presented in Table 2 revaled that the time required for sprouting of graft was minimum (12.20 and 13.80 days) with cytokinins; BAP and Kinetin @1.0 mg/l, respectively whereas it was delayed in all concentrations of Auxin (IAA/ NAA). However, this delay was reduced on various levels of 2, 4-D. Similar findings were also reported by Vijayakumari and Singh (2000) and Starrantino *et al.* (1986) in Nagpur mandarin.

The length of new shoot was also significantly affected by the type and levels of plant growth regulators. Among cytokinins (BAP and kinetin), the maximum length of new shoot (3.10 cm) was recorded with BAP @ 1.0 mg/l followed by kinetin (2.34cm). This treatment was significantly superior than other treatments including control (1.60 cm). In case of various levels of kinetin, the maximum length (2.34 cm) was observed with 1.0 mg/l which was found to be better than lower and higher levels of kinetin. These findings are in close conformity to the findings of Navarro *et al.*, (1975) who has reported the progressive increase in growth of shoots up to a concentration of BAP @ 1.0 mg/l, while higher concentration was found inhibitory effect in citrus. Vijayakumari and Singh (2000) also reported the similar effect of BAP and kinetin on length of new shoot.

It is evident from the Table 3 that the NAA and IAA were not effective in increasing the growth of graft shoot considerably except at 0.5 mg/l level, where significant growth was observed 1.66 cm, 1.70 cm in IAA and NAA respectively. However, higher concentration of 2, 4-D (1.0 - 3.0 mg/l) was significantly more effective than lower level.

Table 4 Percent Survival of grafted plant on different root stock in polyhouse

Percentage of plantlets survival after (in days)	Name of citrus species		
	Carrizo	Rough lemon	Cleopatra
15	92.50 (74.11)	87.50 (69.30)	77.50 (77.75)
30	90.00 (71.57)	87.50 (69.30)	72.50 (58.37)
60	90.00 (71.57)	87.50 (69.30)	72.50 (58.37)

*Figures given in parentheses are angular transformed values

Acclimatization of micro grafts

The data related to percentage success of micro grafts on different root stock are presented in Table 4. Results of study revealed that the maximum success of grafts was recorded in kinnow/ Carrizo (92.5%) followed by kinnow/ Rough lemon (87.50 %) at 15, 30 and 60 days after transfer of plants for acclimatization in green house (Fig 1 g & h). The minimum survivality (72.50 per cent) was recorded in Kinnow/Cleopatra (Table 4), whereas the maximum survival damage of grafts in green house was recorded in Kinnow/ Carrizo. The similar findings are also reported by Kumar *et al.* (2010) who had reported 72.50 to 90 per cent survival in *in-vitro* generated plants of citrus. The grafted plants should be established in screen proof net house, so it can be further used as mother stock for multiplication of Kinnow

commercially. Based on the above observations, it may be inferred that the protocol is effective and reproducible which can effectively be used for production of virus free plants of Kinnow mandarin commercially.

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