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IN VITRO STUDIES ON MICROGRAFTING TECHNIQUE IN TWO CULTIVARS OF CITRUS 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 citrus. Some techniques that tend to increase the grafting success were employed. MS media added with 3%, 5% and 7% sugar was used in combination with two grafting methods e.g., inverted-T incision and surface placement in Kinnow mandarin and Succari sweet orange. The grafting was carried out under aseptic conditions by using15 days old etiolated seedlings of rough lemon. Shoot tips (1-2 mm) and 3-leaf primordia (0.3-0.5 mm) were taken from the fresh shoot flushes and grafted *in vitro*. Higher grafting success of 34.7% was recorded with inverted-T incision than surface placement which gave 26.7% successful micrografts. A total of 21% successful micrografts were achieved at 3% sugar level which increased significantly to 33% with increase in sugar level to 5% in both cultivars. Overall, Kinnow mandarin showed relatively better response in combination with inverted-T incision and produced 33.3% successful micrografts. Succari sweet orange responded maximum with surface placement method and yielded 30.7% successful micrografts.

Introduction

Citrus fruits rank on the second position after grapes in the world on the basis of production. In Pakistan it holds first position on the basis of area (183.8 thousand hectares) and production (1943.7 thousand tonnes) (Anon., 2004-05). The province of Punjab contributes 96.3% to the country's total citrus production. Among citrus fruits, Kinnow mandarin (*Citrus reticulata* Blanco), Feutrell's early and sweet oranges (*Citrus sinensis* (L.) Osbeck.) have gained a prime importance due to their exceptionally high yield potential, excellent fruit quality and versatile adaptation to various agro-climatic conditions.

As with many commercial crops, citrus is also subjected to various biotic stresses, where virus and viroids have been recognised as serious problem limiting the vigour, yield and quality. Severe infections have resulted in the exclusion of some cultivars from commercial usage. Santos *et al*., (1984) reported that virus and virus-like diseases are major threats affecting citrus industry. 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 (xyloprosis), citrus greening and stubborn (Arif *et al*., 2005). Average incidence of citrus tristeza closterovirus (CTV) was 27%, citrus variegation virus (CVV) 31%, citrus exocortis viroid (CEVd) 16%, citrus cachexia viroid (CCVd) 4%, citrus greening (*Liberibacter* spp.) 4% and stubborn (*Spiroplasma citri*) 2%. These diseases are graft transmissible through infected bud sticks. Hence,

raising of disease-free foundation plants is imperative to provide certified bud sticks to the growers and to encourage the planting of grafts instead of seedlings (Mukhopadhyay *et al*., 1997). The studies suggested that certain pathogens that are difficult to eliminate by thermotherapy e.g., citrus exocortis and stubborn, can be successfully eliminated by a method of shoot tip grafting *in vitro* (Murashige *et al*., 1972). Shoot tip grafting (STG) *in vitro* was conducted first time by Navarro *et al.,* (1975) to get a citrus nursery free from all kind of viruses and viroids. Presently, STG *in vitro* has been extensively used to recover the disease-free plants from the commercial citrus varieties and became a potential tool for separating virus and virus like agents (Navarro, 1981). This technique is based on the fact that the apical meristem contains little or no virus titre and meristematic cells grows faster than all viruses. Therefore, the production of disease-free foundation plants by micrografting remains the only mean to supply disease-free bud sticks to the growers (DeLange, 1978). In this study the potential use and applicability of micrografting technique in two major cultivars of citrus was investigated.

Materials and Methods

Preparation of rootstock: Rough lemon (*Citrus jambheri* Lash) was used as a rootstock. The seeds were extracted from the fruits, washed in tap water, surface dried at room temperature, dusted with fungicide to avoid fungus attack and placed in polyethylene bags for storage at 5°C until used (Murashige *et al*., 1972). The seeds were then peeled, disinfected by immersing in 7% Sodium hypochlorite solution containing 2-3 drop of 0.1% Tween-20 for 10 minutes. The seeds were then thoroughly rinsed with sterile distilled water and one seed per test tube was cultured in MS medium (Murashige & Skoog, 1962). The pH of the medium was adjusted at 5.7 ± 0.1 prior to autoclaving at 121°C for 15 minutes (Navarro *et al*., 1975). The cultures were kept under etiolated conditions at 27°C and seedlings were grafted after 14 days of development.

Preparation of scion: Two cultivars, Kinnow mandarin and Succari sweet orange were used as source of scion materials. Shoots were collected from the fresh flushes of field grown plants. Shoot apices (1-2 mm) and 3-leaf primordia (0.3-0.5 mm) were cut with a sterile razor blade and sterilized with 70% ethanol for 1-2 minutes followed by 3 washing with autoclaved distilled water.

Micrografing of scion: Two weeks old seedlings were decapitated leaving about 2 cm of epicotyl under the laminar flow system. Cotyledons were detached and roots were cut to a length of 2-3 cm. An inverted T-incision was made on each epicotyl through the cortex with a sterile razor blade and the excised shoot tips were carefully placed inside the incision which is termed here as inverted-T incision. In the second method, the leaf primordia were placed on the vascular ring tissue at the top of the decapitated epicotyl, called as surface placement. Then the micrografts were aseptically cultured in MS medium without agar but supported with perforated paper bridges. Activated charcoal (5 g/l) and different concentrations of sugar (3, 5 and 7%) were also added in the media. The grafted cultures were kept at constant temperature of 27°C and exposed to 1000 lux illumination for 16h per day.

The data on successful micrografts influenced by the sugar levels, methods of grafting and cultivars was recorded and subjected to the analysis of variance in a factorial arrangement (Steel & Torrie, 1984).

Table 1. Germination of rough lemon rootstock seeds and frequency of mono- and polyembryonic seedling emergence *in vitro*.

Parameters	Observations
No. of seeds cultured	100
Seed germination percentage	94
Monoembryonic seedlings percentage	67.6
Polyembryonic seedlings percentage	32.4

Table 2. The analysis of variance for micrograft success influenced by cultivars, methods of grafting and sugar levels with their relative interaction.

S. O. V.	D.F	SS	MS	F-value
Cultivars	1	0.001	0.001	0.00^{NS}
Method of grafting	1	960.00	960.00	4.96^{*}
Sugar levels	2	3053.33	1526.67	7.89^{**}
Cultivars X method of grafting	1	106.67	106.67	0.55^{NS}
Cultivars X sugar levels	2	280.00	140.00	0.72^{NS}
Method of grafting X sugar levels	2	280.00	140.00	0.72^{NS}
Cultivars X method of grafting X sugar levels	2	13.33	6.67	0.04^{NS}
Error	48	9280.00	193.33	
Total	**59**	**13973.00**		

^{NS} = Non-significant; * = Significant (P<0.05); ** = Highly significant (P<0.01)

Results and Discussion

Response of rough lemon as rootstock: A total of 100 cultures were studied for this purpose out of which 94% were successful. Similar results were observed by Mukhopadhyay *et al*., (1997) while raising seedling of the rootstock rough lemon, sour orange and Ranpur lime in MS medium under standard conditions of temperature, light and pH. The frequency of monoembryonic and polyembryonic seedling was also calculated where 67% seedlings showed monoembryony and 32.4% polyembryony (Table 1). The results are almost in line with the findings of Andrade-Rodriguez *et al*., (2004) where they found 31%, 44% and 54% in three years, respectively, of the polyembryonic seedling while working on the identification of polyembryony in Volkamerian lemon by using RAPD markers.

Analysis of variance for successful micrografts: In Table 2 the results of variance analysis are shown in response to grafting success influenced by grafting methods, sugar levels and cultivars together with their respective interactions. The F-value (4.9) at $P < 0.05$ indicated that there was significant difference between the performance of inverted-T-incision and surface placement methods of grafting in response to successful grafts. Likewise, highly significant differences were calculated between 3% and 5% sugar levels with an F-value of 7.9 at $p<0.01$.

Comparison of methods of grafting in both cultivars: By inverted-T incision there were 36% and 33.3% successful micrografts in Kinnow mandarin and Succari sweet orange, respectively (Fig. 1). Significantly lower success of 25.3% in Kinnow mandarin and 28% in Sweet orange was observed when scion was grafted by surface placement with a mean value of 26.7%. The mean values showed that both methods of grafting influenced the grafting success significantly, while the cultivars responded almost similarly (Fig. 1). Navarro *et al*., (1975) found no difference but higher success (45-50%) in cultivars Cadenera de Carcagente, Para and Tample when the shoot tip was placed by surface placement or inverted-T incision. However, they adopted inverted-T incision method as standard because adventitious shoot from the rootstock arise readily in surface placement method.

Influence of sugar levels on micrograft success in both cultivars: Micrograft success improved with increase in sugar levels in both cultivars. The mean values were 21% at 3% sugar level which rose up to 33% at 5% sugar level. The grafting success increased to 38% in Kinnow at 7% sugar level but values remained almost similar in Succari. The mean values indicated that sugar levels influenced the graft success whereas both cultivars responded almost similarly (Fig. 2). Generally the *in vitro* growth and development increases with increased sugar concentration (Pierik, 1987). On the basis of these data, 7% sucrose concentration was found optimum in the nutrient medium of citrus plants grafted *in vitro*. Navarro *et al*., (1975) also reported most successful grafts and progressive increase in the number of new leaves arising in shoot tips of successful grafts at 7.5% sucrose.

Interactions of cultivars, methods of grafting and sugar levels: The interaction effects of all factors over the grafting success are shown in Fig. 3. Grafting success increased significantly with increase in sugar levels from 3% to 5% in combination with both grafting methods and cultivars. A further increase in sugar level to 7% increased the number of successful micrografts in Kinnow mandarin. There was variable response between cultivars and method of grafting in their respective combinations at 3%, 5% and 7% sugar levels. Inverted-T incision in Kinnow mandarin gave slightly better success than surface placement in Kinnow mandarin. The interaction of grafting method and cultivars was statistically at par (Fig. 3).

Citrus industry is under serious virus threat in many parts of the world which has resulted in reduced productivity of existing orchards. Shoot tip micrografting is being used successfully in many budwood certification programs of citrus producing countries. Hence we have tried to standardize this technique to produce disease free citrus nursery plants of commercial cultivars under indigenous environmental conditions. The initial tests of successful micrografts proved free from citrus tristeza virus which is a major threat in citrus cultivation globally.

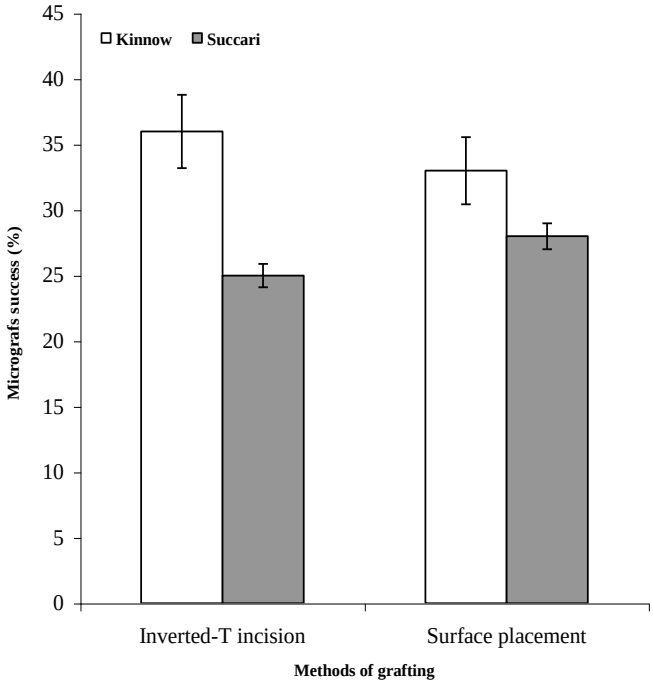

Fig. 1. Effect of different scion placement methods on micrografting success in Kinnow mandarin and Succari sweet orange. The bars are showing the standard deviations of each method in both cultivars.

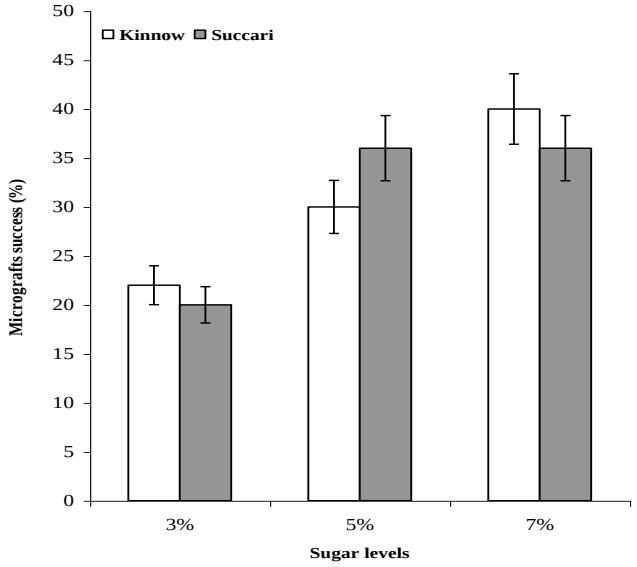

Fig. 2. Effect of different sugar levels added in media on micrografting success in Kinnow mandarin and Succari sweet orange. The bars are showing the standard deviations of each method in both cultivars.

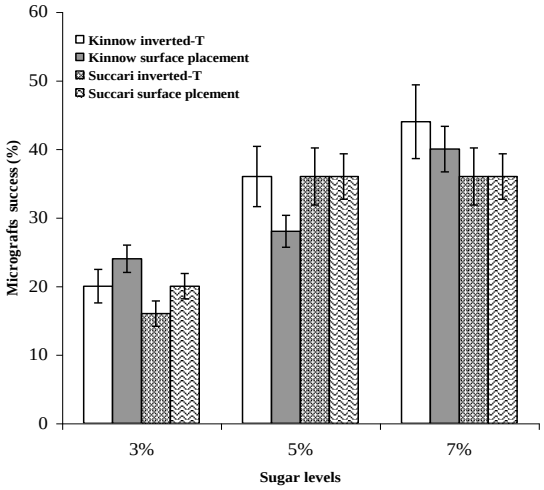

Fig. 3. The cumulative effect of scion placement method and sugar levels on grafting success in Kinnow mandarin and Succari sweet orange. The bars are showing the standard deviations of each method in both cultivars.

References

Andrade-Rodriguez, M., A.Villegas-Monter and G. Carrillo-Castaneda. 2004. Polyembryony and identification of Volkamerian lemon zygotic and nucellar seedlings using RAPD. *Pesquisa Agropecuaria Brasileira*, 39: 551-559.

Anonymous. 2004-05. *Agriculture statistics of Pakistan*. Ministry of Food Agriculture and co-operatives, (Planning Unit), Islamabad, Pakistan.

Arif, M., A. Ahmad, M. Ibrahim and S. Hassan. 2005. Occurrence and distribution of virus and virus-like diseases of citrus in North-West Frontier Province of Pakistan. *Pak. J. Botany*, 37: 407-421.

DeLange, J.H. 1978. Shoot-tip grafting, A modified procedure. *Citrus and Subtropical Fruits Journal*, 539: 13-15.

Mukhopadhyay, S., J. Raj, B.C. Sharma, A. Gurung, R.K. Sengupta and P.S. Nath. 1997. Micropropagation of Darjeeling orange (*Citrus reticulata* Blanco) by shoot-tip grafting. *J. Hortic. Sci.*, 72 (3): 493-499.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.

Murashige, T., W.P. Bitters, E.M. Rangan, E.M. Naue, C.N. Roistacher and P.B. Holliday. 1972. A technique of shoot apex grafting and its utilization towards recovering virus-free citrus clones. *HortScience*, 7: 118-119.

Navarro, L. 1981. Shoot-tip grafting *in vitro* (STG) and its application: A review. *Proc. Int. Soc. Citriculture*, 1: 452-456.

Navarro, L., C.N. Roistacher and T. Murashige. 1975. Improvement of shoot-tip grafting *in vitro* for virus-free citrus. *J. Amer. Soc. Hort. Sci.*, 100: 471-479.

Pierik, R.L.M. 1987. *In vitro culture of higher plants*. Martinus Nijhoff Publishers, Dordrecht, Netherlands, pp. 60-63.

Santos Filho, H.P., O.R. Paguio, Y.S. Coelho and V.M. Medina Urrutia. 1984. The citrus variety improvement program in Brazil. *Fifth Int. Citrus Cong.*, 2: 325-327.

Steel, R.G.D. and J.H. Torrie. 1984. *Principles and procedures of statistics*. 2nd Ed. McGraw Hill Book Co. Inc., UK.

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