Pak. J. Bot., 40(3): 1301-1312, 2008.

ELIMINATION OF *CITRUS TRISTEZA CLOSTEROVIRUS* (CTV) AND PRODUCTION OF CERTIFIED CITRUS PLANTS THROUGH SHOOT-TIP MICROGRAFTING

M. ABBAS[1], M.M. KHAN[1*], B. FATIMA[1], Y. IFTIKHAR[1], S.M. MUGHAL[1], M.J. JASKANI[1], I.A. KHAN[1] AND H. ABBAS[2]

[1]*Institute of Horticultural Sciences, University of Agriculture, Faisalabad-38040, Pakistan.*
[2]*Dr. A.Q. Khan Institute of Biotechnology and Genetic Engineering, University of Karachi, Karachi-75270, Pakistan*

Abstract

Kinnow mandarin (*Citrus reticulata)* and Musambi sweet orange (*C. sinensis*) are the predominant citrus fruits cultivated in Pakistan. Citrus species are highly vulnerable to many types of pathogens. Among the viruses, *Citrus tristeza virus* (CTV) is the most devastating which can cause the death of millions of citrus trees if once established. CTV is a graft-transmissible and can be inadvertently propagated through infected budwood. The production of certified and CTV-free citrus plants could be helpful to restrict the widespread of CTV. The study was designed to optimize the micrografting technique for the propagation of CTV free Kinnow mandarin and Musambi sweet orange plants under aseptic conditions. The MS medium supplemented with 5 mgL^{-1} BA was found better for successful micrografting of both citrus cultivars on rough lemon seedlings. The survival rate of micrografted citrus plantlets was 88% when transferred to soil. The ELISA results showed that more that 90% Kinnow and Musambi plants either grafted on rough lemon or sour orange rootstock were found free from CTV. The foundation block of mandarin and sweet orange free from CTV was successfully established using shoot-tip micrografting technique.

Introduction

Citrus represents one of the most important and a widely grown fruits in the world and Pakistan is one of the major citrus producing countries. Citrus was cultivated on 192274 hectares with annual production of 2458381 tonnes during 2005-06 (Anon., 2006). Since the introduction of Kinnow mandarin (*Citrus reticulata* Blanco.), its production has been increasing steadily to fulfill the growing demands in the country and abroad (Khan, 1992).

Citrus species are highly vulnerable to many types of pathogens. The citrus groves in general are facing a serious problem of decline that is attributed to different causes. The major cause, however, is the prevalence of citrus virus and virus-like diseases which have a significant impact and often become a major constraint in citrus production. In Pakistan, the citrus plantation has had shorter productive life which culminates in low yields and deterioration in fruit quality. Chapot (1970) reported viral diseases in Pakistan for the first time and observed dieback and decline symptoms in large number of citrus trees but he was not sure about the cause of these symptoms. Later on subsequent surveys of citrus groves at number of locations revealed the presence of several virus and virus-like diseases of citrus in Pakistan (Catara *et al*., 1988; Bove, 1995). Anwar & Mirza (1992) confirmed the presence of *Citrus tristeza closterovirus* (CTV) in citrus trees on the basis of survey and the enzyme-linked immunosorbent assay (ELISA).

[*]Corresponding author: mumtaz59pk@hotmail.com

The strategies to control CTV may differ according to the incidence and severity of its strains in each area and with the cultivars and rootstocks used. There is no single control strategy for the CTV that will be applicable in all situations (Garnsey *et al*., 2005).The approach to use CTV tolerant rootstocks can also help to control the CTV induced losses. Tolerance to CTV is seen in the ability of rootstock phloem to withstand the presence of the virus without adverse effects on normal cellular and physiological functions (Bordignon *et al*., 2004). The most effective means of limiting CTV-induced disease is through a certification programme in which bud wood source trees are tested and maintained free of CTV (Garnsey *et al*., 2005).

The shoot-tip micrografting is known technique for the potential elimination of virus and viroid pathogens from citrus germplasm. It is generally utilized in quarantine and certification programs using small trees maintained under glass or screenhouses (Krueger *et al*., 2003). Micrografting technique is important mainly for woody species because in such plants meristem-tip culture is often difficult (Faccioli & Marani, 2005). This technique was used for elimination of different viruses from citrus species (Murashige *et al*., 1972; Navarro *et al*., 1975). The aspects of shoot-tip grafting and its application on citrus virus research were comprehensively reviewed by Navarro (1981). The procedure consists of preparation of rootstock and scion materials, grafting, culture *in vitro* of grafted plantlets and transfer to soil. The study has been taken up to standardize the different factors affecting shoot tip grafting in *C. reticulata* cv. Khasi mandarin (Madhav *et al*., 2001). Higher rate of success (60%) was obtained in shoot tip grafting when a 2-week old etiolated seedling of trifoliate orange was used as a rootstock. Scion size of 0.2-0.3 mm (meristem dome plus three leaf primordial) was found to be optimum for grafting. The successful grafted plantlets were subsequently transferred to soil and 90% plantlets were established in the soil. These plantlets were maintained in aphid proof cage to prevent re-infection of virus through aphids. Preliminary results of indexing indicated recovery of plantlets free from CTV and exocortis. Similarly, the shoot-tip grafting technique was also found 100% efficient in eliminating the CTV, Citrus exocortis viroid (CEVd) and cachexia-xiloporosis viroid from varieties of the citrus germplasm bank at the Centro de Citricultura Sylvio Moreira CCSM-IAC (Carvalho *et al*., 2002). However, 60% efficiency was observed in eliminating the citrus psorosis virus complex. The use of micrografting alone without indexing will not guarantee freedom from viruses in propagative budwood. This can be ascertained only when the techniques of pathogen elimination are combined with a comprehensive indexing programme (Roistacher, 1988). The present study was designed to optimize the shoot-tip micrografting technique for rapid propagation of citrus plantlets *in vitro* to establish a foundation block of certified citrus plants of Kinnow mandarin (*C. reticulata*) and Musmabi sweet orange (*C. sinensis*). The indexing of STG plantlets were conducted through direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

Materials and Methods

Shoot-tip micrografting: Micrografting was employed for the production of CTV-free citrus plants to develop a clean foundation block for future propagation. Shoot-tip grafting was used as described by Navarro *et al*., (1975) and Navarro (1981). Rough lemon (*C. jambhiri*) and sour orange (*C. aurantium*) were used as a rootstock, and scions were taken from CTV-infected tissues of mandarins and sweet oranges. The sterilized seeds of rough lemon and sour orange were germinated in test tubes containing MS medium (Murashige & Skoog, 1962) solidified with 1% bactoagar. One seed was sown

per tube and maintained at 25±2°C for two weeks under continuous darkness. The shoot tips were obtained from axillary buds of CTV infected Kinnow mandarin (*C. reticulata*) and Musambi sweet orange (*C. sinensis*). The leaves were removed from twigs which were cut into sections containing 2-3 axillary buds. The sections were rinsed with 95% ethanol followed by washing under running tap water to remove dust. Then dipped for 10 minutes in 0.5% Sodium hypochlorite (NaOCl) followed by 3 times washing with sterilized distilled water. The scions were excised from these sterilized sections. Two weeks old rough lemon and sour orange rootstock seedlings were removed from test tubes and decapitated to remove all leaves. The cotyledons and their axillary buds were also detached. The upright cut was made on the central part of the rootstock and the scion with a V shape base excised from the sterilized twig sections was fit into the cut. The grafted plantlets were cultured in MS medium supplemented with different levels (0, 1, 3 and 5 mg) of Benzyl Adenine (BA). The grafted plants were exposed to 16 hour daily 1000-lux illumination and maintained at the temperature of 25±2°C.

The grafted plants having at least two expanded leaves in the scion were transferred to plastic pots containing a medium of garden soil, sand and compost (3:1:1). The pots were covered with polythene bags to maintain humidity and to avoid any damage from desiccation. One week later the bags were opened and after second week bags were removed. The grafted plants were allowed to continue growth under glasshouse conditions (25±2°C). The successfully established plants were transferred to plastic containers, filled with garden soil only and after hardening the plants were transferred to screenhouse.

CTV indexing: The grafted plants in screenhouse were assayed through double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for CTV infection. The leaves of survived citrus plants were harvested at the age of 4-6 months for virus indexing. The ELISA procedure using CTV specific polyclonal antibodies (Catalogue No. 1102, Adgen, UK) was performed as described by Clark & Adams (1977). The 96-well microtiter plates were coated with 200 µl of antibodies (diluted at 1:200). The plates were then covered and incubated at 4°C in refrigerator for over night. The plates were washed with PBS-Tween buffer three times at five minute intervals using ELISA plate washer (Model: EL_X 50, BIO-TEK® USA). The tissues were extracted in extraction buffer and 200 µl of plant extract was added to duplicate the wells. The plates were incubated over night at 4°C and washed accordingly. After washing wells were charged with 200µl of alkaline phosphatase enzyme conjugate and the plates were incubated at 4°C for over night. After washing the plates 200 µl aliquots of freshly prepared substrate buffer were added to each well and the plates were incubated at room temperature (25±2°C) for 30-60 minutes. The reaction was stopped by adding 50 µl of 3M NaOH in each well and the absorbance was measured at 405 nm in Universal Microplate Reader (Model: EL_X 800, BIO-TEK® USA). The absorbance value recorded three times higher compared to healthy tissues was considered as CTV positive (Lbida *et al*., 2005).

Data collection and analysis: The data was collected for successful grafts, days to establish union, days to sprout 1st leaf and percent of CTV elimination (ELISA). The experiment for shoot-tip grafting was laid out under completely randomized design and three replications (100seeds/repeat) with four treatments were used for each rootstock. The data collected was analyzed statistically and means was compared using Duncan's Multiple Range (DMR) test (Steel *et al*., 1997).

Results

Micrografting success: Highly significant ($p<0.05$) difference were observed among the treatments for micrografting success of mandarin and sweet orange scions on rough lemon rootstock (Fig. 1). The results revealed that MS medium supplemented with 5 mgL^{-1} BA varied significantly ($p<0.05$) from the remaining treatments for successful micrografting of both citrus cultivars on rough lemon seedlings. Among other treatments, MS medium supplemented with 3 mgL^{-1} BA was found statistically significant ($p<0.05$) for micro-grafting success compared to other treatments (Fig. 1). The MS media supplemented with 1, 3 and 5 mgL^{-1} BA were found statistically similar for sweet orange scion grafted on sour orange but showed significant ($p<0.05$) difference with control (Fig. 2). The results were highly significant ($p<0.05$) for micrografting success of mandarin scions grafted on sour orange rootstock seedlings (Fig. 2). The grafting success was significantly ($p<0.05$) high in MS medium containing 5 mgL^{-1} BA for mandarin scions grafted on sour orange. The 3 mgL^{-1} of BA in MS medium was found second best treatment for successful grafting of mandarin scion on sour orange rootstock seedlings (Fig. 2).

Days to establish union of scions on rootstock seedlings: The results were statistically significant ($p<0.05$) for the number of days taken by sweet orange scions for a successful union with rough lemon rootstock seedlings (Fig. 3). The MS medium without BA (control) significantly ($p<0.05$) took higher number of days for successful graft union of sweet orange scions with rough lemon. The scions taken from sweet oranges also took more days (statistically similar with control) for successful union with rough lemon rootstock, cultured in MS medium containing 5 mgL^{-1} of BA. The sweet orange grafted plantlets took less number of days for successful scion-stock union when cultured in MS medium supplemented with 1 and 3 mgL^{-1} of BA (Fig. 3). The results were non-significant for number of days taken by scions of both cultivars when grafted on sour orange rootstock seedlings (Fig. 4).

Days to sprout first leaf of scions grafted on *In vitro* grown rootstock: The results showed highly significant ($p<0.05$) differences among the treatments for days taken by scions of both cultivars micrografted on rough lemon rootstock to sprout first leaf after successful union. The grafted mandarins cultured in MS medium (control) and MS medium supplemented with 3 mgL^{-1} of BA took maximum days to sprout first leaf (Fig. 5). The grafted mandarins took minimum number of days to sprout first leaf cultured in MS medium containing 5 mgL^{-1} BA. The sweet orange scions grafted on rough lemon rootstock seedlings took minimum days to sprout first leaf when cultured in MS medium supplemented with different concentrations of BA. The culture media supplemented with 1, 3 and 5 mgL^{-1} BA showed significant ($p<0.05$) difference from control (Fig. 5).

The results were highly significant ($p<0.05$) for the number of days taken by scions of both cultivars grafted on sour orange rootstock to sprout first leaf. The mandarin scions took maximum days to sprout first leaf when cultured in MS + BA (3 mgL^{-1}) and MS + BA (5 mgL^{-1}) media (Fig. 6). The mandarin plantlets cultured in MS medium (control) and MS medium containing 1 mgL^{-1} BA took minimum days to sprout first leaf. The differences were highly significant ($p<0.05$) for the sweet orange scions grafted on sour orange rootstock seedlings to produce their first leaf (Fig. 6). The *in vitro* grown sweet orange plants took higher number of days to sprout first leaf after successful graft union when cultured in MS medium (control) and MS medium containing 3 mgL^{-1} BA. The sweet orange plants cultured in MS medium supplemented with 1 mgL^{-1} BA took minimum days to sprout the first leaf (Fig. 6).

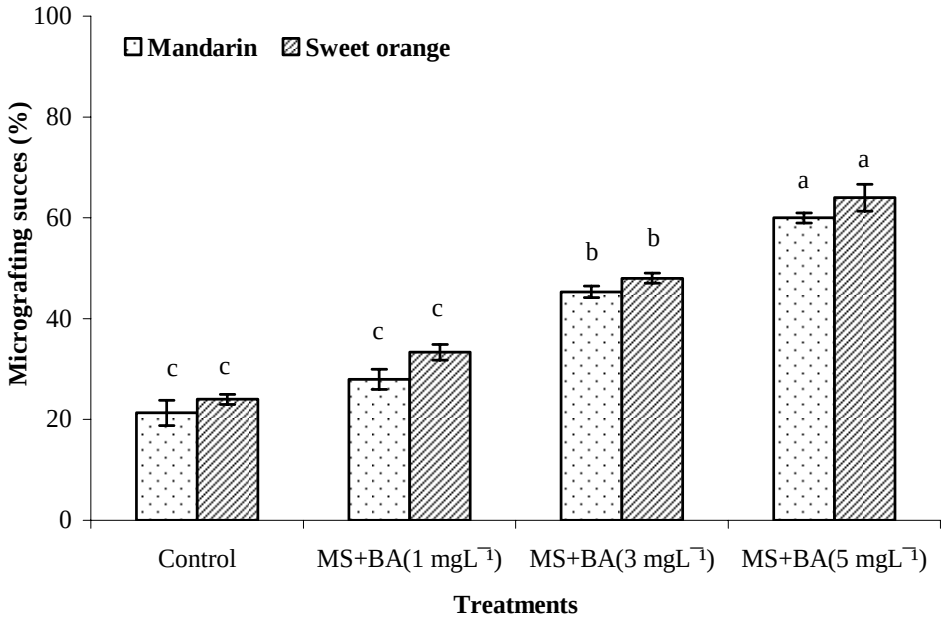

Fig. 1. Successful grafting of both cultivars on Rough lemon rootstock.
Bars with different letters are significantly different by DMRt (5% level)

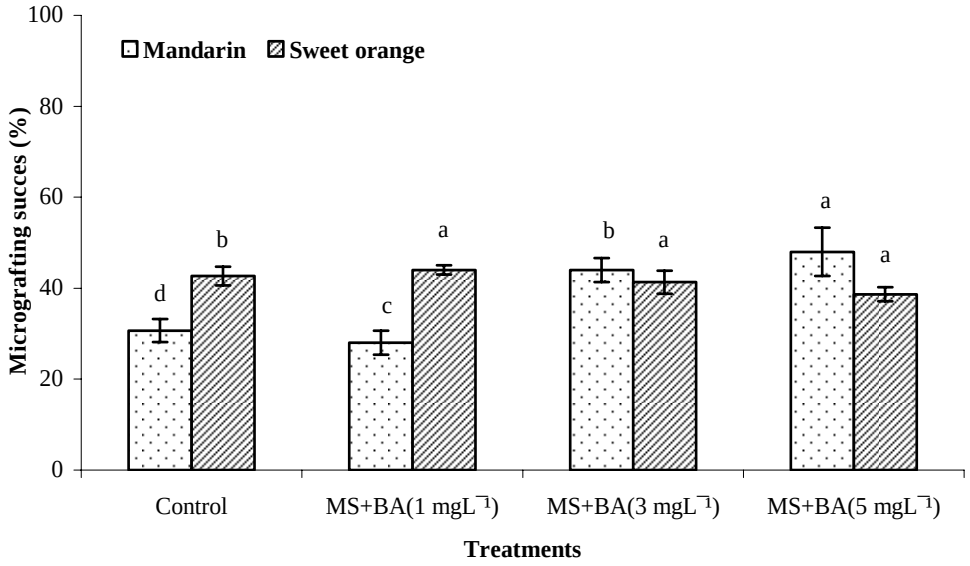

Fig. 2. Successful grafting of both cultivars on Sour orange rootstock.
Bars with different letters are significantly different by DMRt (5% level)

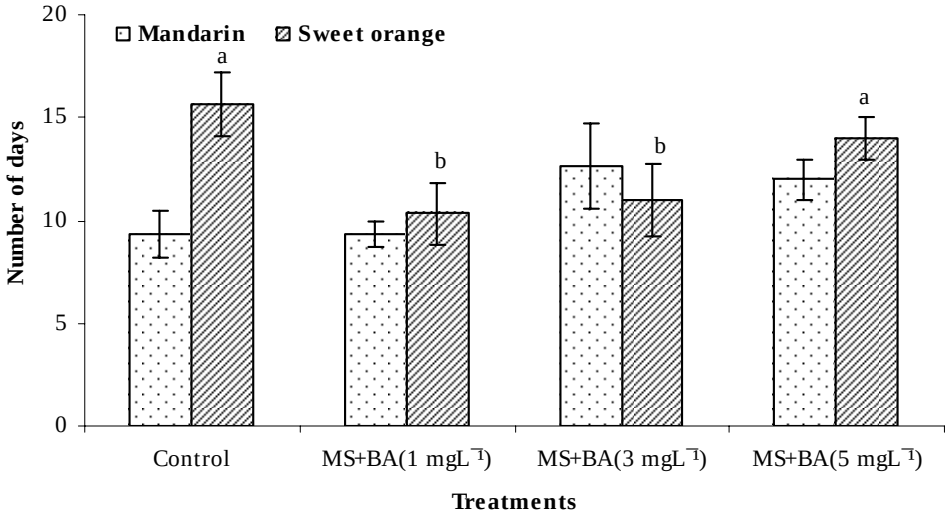

Fig. 3. Days to establish union of both cultivars micrografted on Sour orange.

Bars with different letters are significantly different by DMRt (5% level)

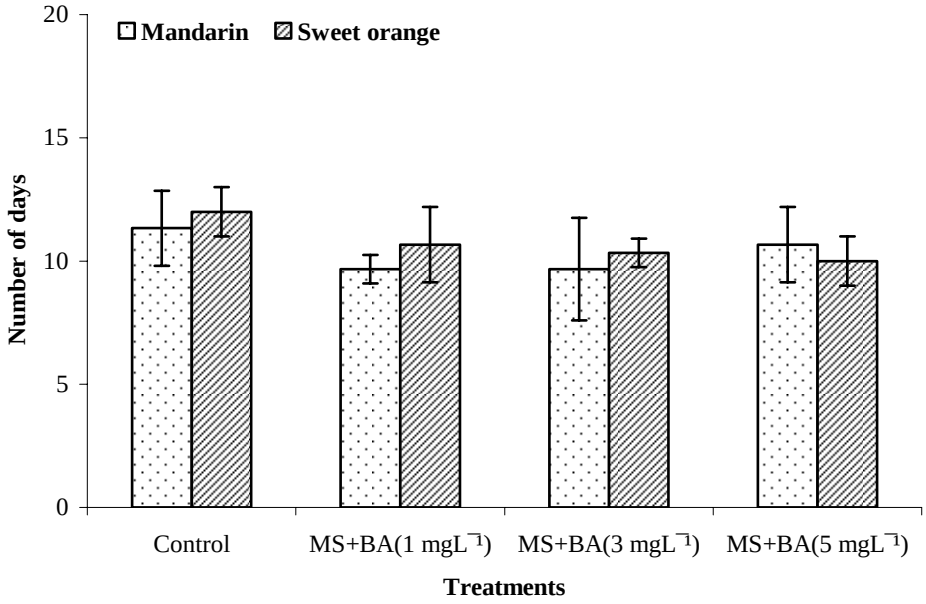

Fig. 4. Days to establish union of both cultivars micrografted on Rough lemon.

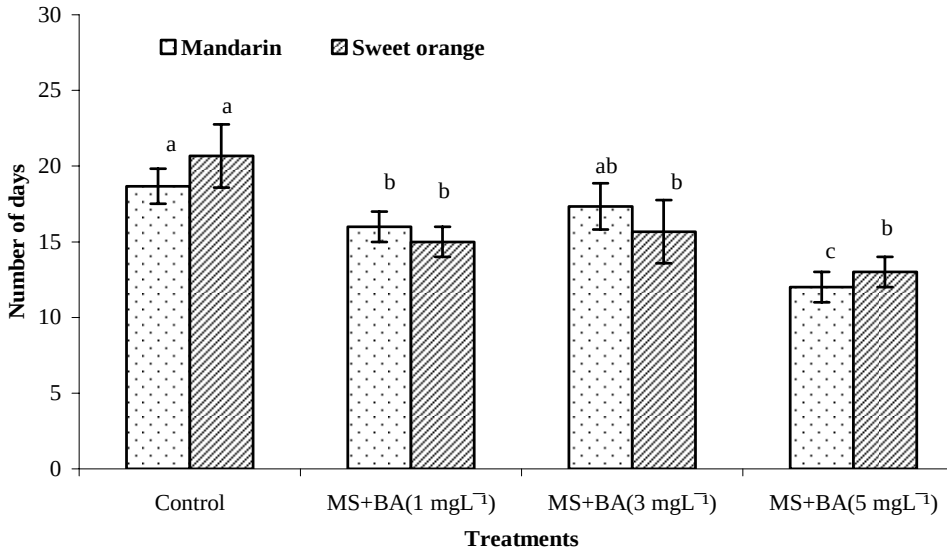

Fig. 5. Days to sprout first leaf of both scions grafted on Rough lemon rootstock.

Bars with different letters are significantly different by DMRt (5% level)

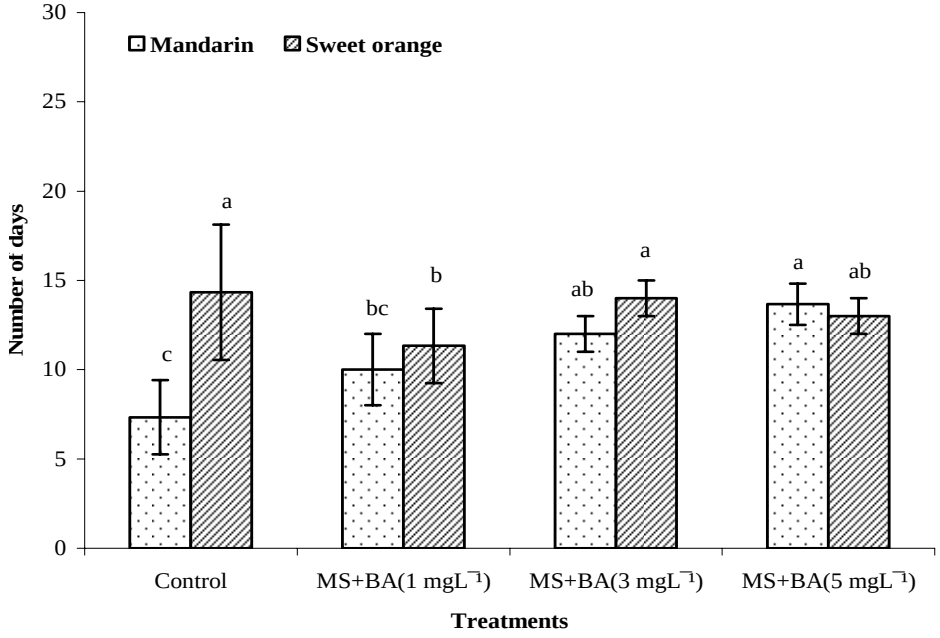

Fig. 6. Days to sprout first leaf of both scions grafted on Sour orange rootstock.

Bars with different letters are significantly different by DMRt (5% level)

Table 1. Status of CTV in shoot-tip micrografted citrus plants under screenhouse.

Scion	Rootstock	No. of micro-grafted plants	No. of survived plants	Infected/ Examined	Percentage (%) of CTV free Plants	Healthy (micrografted plants)	Infected (scion tree)
Mandarin (Kinnow)	RL	200	185	11/185	94	0.173-0.191	0.419 - 1.542
	SO	100	89	8/89	91		
Sweet orange (Musambi)	RL	100	80	4/80	95	0.187-0.201	0.453-1.732
	SO	50	42	3/50	94		
Total		**450**	**396 (88%)**	**26/396**	**93.43**		

* Absorbance value recorded on ELISA microplate Reader
RL: Rough lemon
SO: Sour orange

Indexing of micrografted citrus plants through ELISA: The micrografted citrus plants with seven to nine fully expanded leaves were transferred in insect proof netted screenhouse. A total of n=300 *in vitro* grown citrus plants grafted on rough lemon rootstock comprising of mandarin (n=200) and sweet orange (n=100) were transferred to soil under screenhouse. The *in vitro* grown citrus plants (100 mandarin and 50 sweet orange) grafted on sour orange rootstock were also transferred to soil under screenhouse. Out of 450 transferred citrus plants 396 were survived which showed survival rate of 88% (Table 1). The survived citrus plants were indexed for CTV through ELISA. The results revealed that more than 90% mandarin and sweet orange plants either grafted on rough lemon or sour orange rootstock were found free from CTV. The overall percentage of CTV-free citrus plants was 93.43% (Table 1). The foundation block of mandarin and sweet orange free from CTV was successfully established using shoot-tip micrografting technique.

Discussion

The present study was designed to optimize the micrografting technique for the propagation of CTV-free citrus plants under aseptic conditions. The objectives of optimizing this technique were to produce virus free citrus plants, establishment of foundation block of healthy plants and to provide healthy budwood to citrus growers, as reported by Navarro *et al*., (1975) and Navarro (1976).

The shoot-tip micrografting protocol was standardized keeping in view the limited available resources (chemicals and tissue culture facilities). Two rootstocks (rough lemon and sour orange) and two scions (kinnow mandarins and musambi sweet orange) were evaluated for micrografting technique. The results suggested that increasing concentration of BA in MS medium significantly effect the micrografting percentage of mandarin and sweet orange scions grafted on rough lemon rootstock. The grafting success was significantly increased with the increase of BA concentration in MS medium for the mandarin scions grafted on sour orange rootstock. However, micrografting success was not considerably affected by different concentrations of BA in MS medium in case of sweet orange grafted on sour orange rootstock. The results reported in this study for micrografting success in mandarin grafted on rough lemon and sour orange and sweet orange grafted on rough lemon are contrary to Navarro *et al*., (1975). However, in the present study, BA did not influence the grafting success in sweet orange grafted on

sour orange which is in accordance with Navarro *et al*., (1975). The micrografting success percentage reported here was high in sweet orange compared to mandarin grafted on rough lemon rootstock and this is in accordance with Vijayakumari *et al*. (2006) who reported the maximum grafting success in sweet orange followed by mandarin.

The present findings suggested that addition of BA in MS medium did not significantly affect the number of days taken by both cultivars either grafted on rough lemon or sour orange. However, minimum days were taken by mandarin and sweet orange grafted on rough lemon to sprout first leaf with the increase in level of BA in MS medium but when mandarin scions were grafted on sour orange number of days taken to sprout first leaf were increased with the increase in BA concentration. Many factors affect the grafting success, days to establish graft union and sprouting of first leaf in micrografetd citrus plants. The frequency of grafting success and other factors can be affected by type of scion and rootstock used. The grafting success also depends on rootstock age and on extent of tissue differentiation. All these conditions can be varied with different rootstocks used in micrografting of citrus plants (Navarro, 1981). In addition genotype can also significantly affect the successful micrografting of citrus plants which ranged from 5-40% (Murashige *et al*., 1972). In this study, the concentration of BA significantly effect the days taken by scions to produce their first leaf and it has been well reported that cytokinins like BA are often used to stimulate growth and development of *in vitro* grown plantlets (Chawla, 2000).

The micrografting technique was successfully applied for the elimination of CTV in mandarins and sweet oranges. This technique was successfully and efficiently used for elimination of different viruses from citrus species (Murashige *et al*., 1972; Navarro *et al*., 1975; Naz *et al*., 2007). Similarly, Vijayakumari *et al*., (2006) used micrografting in quarantine greenhouse to produce the clean stock of diverse citrus germplasm to strengthen the citrus gene bank. The shoot-tip grafted citrus plants were found 76 and 86.95% negative for CTV and greening disease, respectively. However, percentage of CTV free citrus plants reported in this study was 93.43%. The present study suggested that 88% micrografted citrus plants were successfully established under screenhouse conditions, theses results are contrary to Madhav *et al*., (2001) who reported 90% micrografted mandarin plantlets were established in the soil. Recently, Reza *et al*., (2007) reported that 100% CTV elimination in sweet oranges and 90% survival rate when transferred to greenhouse. In present studies less success rate for survival of transferred plantlets was observed, which suggested that the utmost care should be taken at the stage of acclimatization and adoption under screenhouse conditions. Similarly, our results are similar to Carvalho *et al*., (2002) that the micrografting technique was found efficient to successfully eliminate CTV. They reported that 100% micrografted plants were CTV free, however, in our case this percentage was 93.43%. This is because the shoot-tips used in this study were of large size, which can affect the percentage of virus elimination in micrografted plants (Suarez *et al*., 2005). The reason for use of large size shoot-tips is that it enhanced the bud uptake with better micrografting success rate for the people who are less experienced in this technique (Sharma *et al*., 2007).

The CTV was successfully eliminated through this technique without the combination of thermotherapy with micrografting. However, it is well reported that the process of elimination of some viruses and viroids can be improved by using thermotherapy coupled with shoot-tip grafting. The combination of thermotherapy and shoot-tip grafting (STG) is recommended for the elimination of all known major citrus

pathogens (Roistacher, 1988). The re-indexing of 427 plants recovered by shoot tip grafting associated with thermotherapy showed 100% success in eliminating *Citrus tristeza virus*, exocortis, psorosis and cachexia/xyloporosis (Carvalho *et al*., 2001). However it is important that budwood be thoroughly indexed before and after therapy. The results are also similar to Sertkaya (2004) that is a good number of citrus plants reached to the size of indexing for virus diseases in a short time by micrografting method. Many techniques besides micrografting are now available which can efficiently be used for the propagation of virus free citrus plants. Microbudding technique was also found to be efficient for production of CTV-free sweet orange (Abbas *et al*., 2006). However, percentage of virus free citrus plantlets is low because the scion wood used in microbudding was of large size. Ghazvini *et al*., (2001) produced CTV-free citrus plants using unfertilized ovules of suspected CTV-infected plants by under aseptic conditions. The *In vitro* citrus plants when budded on lime (indicator host) did not show any CTV positive reaction. Similarly, somatic embryogenesis was found to be a very promising technique for the production of virus-free citrus plants (D'Onghia *et al*., 2001). Citrus psorosis virus CPsV was not detected in any of the mandarin and sweet orange plant, obtained using somatic embryogenesis even after 24 months regeneration.

The use of thermotherapy or STG alone without indexing will not guarantee freedom from viruses in propagative budwood. This can be ascertained only when the techniques of pathogen elimination are combined with a comprehensive indexing programme (Roistacher, 1988). The shoot-tip micrografting (STG) is a well established technique for the potential elimination of virus and viroid pathogens from citrus germplasm. It is generally utilized in quarantine and certification programs using small trees maintained under glass or screen (Krueger *et al*., 2003). Shoot-tip micrografting technique was successfully optimized and employed for the rapid propagation virus-free citrus nursery plants. This technique successfully produced the CTV-free citrus plants for the establishment of foundation block of healthy mandarin and sweet orange plants to provide healthy budwood to citrus growers.

Acknowledgement

The authors highly acknowledge the financial support of Ministry of Science and Technology, Government of Pakistan for the completion of this study.

References

Abbas, M., M.M. Khan, S.M. Mughal, M.J. Jaskani and H. Abbas. 2006. Propagation of CTV-free sweet orange [*Citrus sinensis* (L.) Osbeck] plants through microbudding technique. *Pak. J. Bot.*, 38(3): 583-587.

Anonymous. 2006. Fruit, vegetables and condiments statistics of Pakistan 2005–2006. Government of Pakistan, Ministry of Food, Agriculture & Livestock (Economic Wing), Islamabad. pp. 1-10.

Anwar, S.M. and S.M. Mirza. 1992. Survey of citrus tristeza virus in Punjab. In: *Proceedings 1st Int. Seminar on Citriculture in Pakistan.* University of Agriculture Faisalabad, Pakistan, 413-416.

Bordignon, R., H.P. Medina-Filho, W.J. Siqueira and J.T. Sobrinho. 2004. The genetics of tolerance to tristeza disease in citrus rootstocks. *Gen. Mol. Biol.*, 27(2): 199-206.

Bove, J.M. 1995. Virus and virus-like disease of citrus in the Near East region. FAO, Rome. 239-266.

Carvalho, S.A., F.A. Santos and M.A. Machado. 2002. Psorosis virus complex elimination from citrus by shoot-tip grafting associated to thermotherapy. *Fitopatol. Brasileira*., 27(3): 306-308.

Carvalho, S.A., M.A. Machado, G.W. Müller and H.D. Coletta Filho. 2001. Basic budwood for production of healthy citrus nursery-trees in São Paulo State. *Laranja*., 22(1): 185-201.

Catara, A., A. Azzaro, S.M. Mughal and D.A. Khan. 1988. Virus, viroids and prokaryotic diseases of citrus in Pakistan. In: *Proceedings 6th Int. Congress of IOCV*., pp. 957-963.

Chapot, H. 1970. Les problèmes de la production des agrumes en Proche-Orient at en Afrique du Nord. Prog. Des Nations Unies le Develop. FAO, Rome, 2870.

Chawla, H.S. 2000. *Nutrition medium. Introduction to Plant Biotechnology*. Oxford & IBH Publishing Co. Pvt. Ltd. New Delhi, India. pp. 13-23.

Clark, M.F. and A.N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol*., 34: 475-483.

D'Onghia, A.M., F. Carimi, F. dePasquale, K. Djelouah and G.P. Martelli. 2001. Elimination of *Citrus psorosis virus* by somatic embryogenesis from stigma and style cultures. *Plant Pathol*., 50(2): 266-269.

Faccioli, G. and F. Marani. 2005. Virus elimination by meristem tip culture and tip micrografting. In: *Plant Virus Disease Control*. (Eds.): A. Hadidi, R.K. Khetarpal, H. Koganezawa. Second Printing, APS Press, St. Paul. Minn. pp. 346-380.

Garnsey, S.M., T.R. Gottwald and R.K. Yokomi. 2005. Control strategies for citrus tristeza virus. In: *Plant Virus Disease Control*. (Eds.): A. Hadidi, R.K. Khetarpal, H. Koganezawa. Second Printing, APS Press, St. Paul. Minn. pp. 639-658.

Ghazvini, R.F., Y. Hammidoghli and Y. Ebrahimi. 2001. Production of virus-free clones from unfertilized ovules of Satsuma mandarin cultivars. *J. Sci. Tech. Agri. Natural Resources*, 5(2): 27-37.

Khan, I.A. 1992. Virus and virus-like diseases of citrus. In: *Proceedings 1st Int. Seminar on Citriculture in Pakistan*. University of Agriculture Faisalabad, Pakistan, 343-352.

Krueger, R.R., T.L. Kahn, D.J. Gumpf, P.M. Ballance, O.J. Bier and J.A. Bash. 2003. Shoot-tip micro grafting for rescue or therapy of field-grown citrus germplasm accessions. In: *Proceedings 9th ISC Congress* (Abstract).

Lbida, B., A. Bennani, M.N. Serrhini and M. Zemzami. 2005. Biological, serological and molecular characterization of three isolates of *Citrus tristeza closterovirus* introduced into Morocco. *EPPO Bulletin*, 35(3): 511-517.

Madhav, M.S., B.N Rao, S. Singh and P.C. Deka. 2001. *In vitro* shoot tip grafting in *Citrus reticulata* Blanco cv. Khasimandarin. *Res. Crops*, 2(3): 338-345.

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

Murashige, T., W.P. Bitters, E.M. Naver, C.N. Roistacher and P.B. Holiday. 1972. A technique of shoot tip grafting and its utilization towards recovering virus-free citrus clones. *Hort. Sci*., 7: 118-119.

Navarro, L. 1976. The citrus variety improvement program in Spain. In: *Proceedings 7th Int. Congress of IOCV*., pp. 198-203.

Navarro, L. 1981. Citrus shoot-tip grafting *In vitro* (STG) and its applications: a review. *Proc. Int. Soc. Citriculture*., pp. 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.

Naz, A.A., M.J. Jaskani and H. Abbas. 2007. *In vitro* studies on micrografting technique in two cultivars of citrus to produce virus free plants. *Pak. J. Bot*., 39(5): 1773-1778.

Reza, F., B. Golein, H. Taheri and Y. Tadjvar. 2007. Elimination of Citrus tristeza virus of Washington Navel orange (*Citrus sinensis* [L.] Osbeck) through shoot-tip grafting. *Int. J. Agri. Biol*., 9(1): 27-30.

Roistacher, C.N. 1988. Concepts in detection and control of citrus virus and virus-like diseases. In: *Proceedings 6th Int. Citrus Congress Middle-East*. *Int. Soc. Cit*., pp. 853-861.

Sertkaya, G. 2004. Effects of different rootstocks in micrografting on growing of washington navel orange plants obtained by shoot tip grafting. *Biotechnology & Biotechnological Equip*., 18(2): www.diagnosisp.com

Sharma, S., B. Singh, G. Rani, A.A. Zaidi, V. Hallan, A. Nagpal and G.S. Virk. 2007. Production of Indian citrus ringspot virus-free citrus plants of Kinnow mandarin (*Citrus nobilis* Lour x *C. deliciosa* Tenora) employing phytotherapy coupled with shoot tip grafting. *In Vitro Cell Dev. Biol*., 43: 254-259.

Steel, R. G.D., J.H. Torrie and D. Dickey. 1997. *Principles and procedures of statistics: a biometrical approach*. 3rd Ed. McGraw Hill Book Co. Inc., New York.

Suarez, I.E., R.A. Schnell, D.N. Kuhn and R.L. Litz. 2005. Micrografting of ASBVd-infected Avocado (*Persea americana*) plants. *Plant Cell, Tissue Organ Cult*., 80: 179-185.

Vijayakumari, N., D.K. Ghosh, A.K. Das, A. Singh and S. Singh. 2006. Elimination of citrus tristeza virus and greening pathogens from exotic germplasm through in vitro shoot tip grafting in citrus. *Ind. J. Agric. Sci*., 76 (3): 209-210.

(Received for publication 31 March 2008)