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Propagation of Grapefruit (Citrus Paradisi Macf) by Shoot Tip Micrografting

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ABSTRACT

This study was conducted to develop an in vitro technique for micrografting of grapefruit on sour orange rootstock during August, 2001 to May, 2002. The best in vitro germination of rootstock seed was obtained when both seed coats (hard and soft) were removed before in vitro culturing on a nutrient medium. The success of micrografting was affected by the age of rootstock seedling, light incubation conditions, source and method of placement of meristem tips and concentration of sucrose in the nutrient medium. High frequency of successful micrografts was obtained when the rootstock seedlings were germinated under darkness till the age of two weeks and then incubated in the same condition for two to three weeks after grafting. Plants grown in the greenhouse were the best source for meristem tips. Placement of scion on an inverted T incision at the point of decapitation was found to be the most suitable method of micrografting. The frequency of successful micro. grafts improved significantly when Murashige and Skoog medium was supplemented with 7.5% sucrose compared with normal sucrose concentration (3%)..

INTRODUCTION

The grapefruit (*Citrus paradisi*) is one of the most important citrus species in Sudan. Sudan ranked second, after south Africa, in production of grapefruit in Africa, with a total production of 65000 metric tons compared to 147000 in South Africa (FAO, 1999). Sudanese grapefruit is well known for its large size, excellent quality and good coloration. A productivity as high as 40 tons per hectare can be obtained (Khalil, 1984).

Commercial citrus species are conventionally propagated by budding on sour orange rootstock. Shield budding is more preferable than chip budding because it is easy to perform (Mohamed, 1999). In Sudan, shield budding (T-budding) is generally used for propagating most citrus species. Buds used for propagation are normally selected from commercial plantings and there are no certified mother trees. This situation resulted in the spread of many virus and virus-like diseases causing poor growth and low yield.

A method to recover citrus plants free of all virus and virus-like diseases and without juvenile characters was thus really needed. The first attempt in this direction was made by in vitro shoot tip culture, a technique widely used to recover healthy herbaceous plants. Murashige *et al*. (1972) were able to obtain a few citrus plants by grafting shoot tips from diseased plants on young rootstock seedlings grown *in vitro*. Some of these plants were free of exocortis and did not have juvenile characters. Since then, several reviews, including different aspects of the shoot tip grafting (STG) technique have been published (Navarro *et al*., 1975; 1976; Navarro *et al*., 1980). This technique is named in vitro shoot tip grafting.

The success of shoot tip grafting for virus elimination is directly correlated to the size of the explant (Nehra and Kartha, 1994). Navarro and Juarez (1977) obtained 100% success when they picked apices with a maximum of two leaf primordia. The frequency of successful grafts increased with a decrease in the size of shoot tip, but the frequency of recovery of healthy plants decreased (Navarro *et al*. (1976). Kitto and Young (1981) successfully obtained proliferation of shoot tip culture of Carrizo citrange, trifoliate orange (*Poncirus Irifoliate* L) and Cleopatra mandarin. They found that BAP was the only cytokinin which induced shoot proliferation, while no beneficial effect was reported when orange juice was added or nitrogen source was altered (Kitto and Young, 1981). In vitro rooting took place on Murashige and Tucker (1969) medium plus adenine sulphate with NAA, the preferred auxin. Approximately, 50% of the shoots which remained unrooted, underwent rhizogenesis during the establishment

stage (George and Sherrington, 1984).

Since citrus industry in Sudan is a growing industry, tissue culture can play an important role in producing virus-free plantlets to be used as mother trees for collection of propagation buds. The objective of this research was, therefore, to investigate the affecting the success of *in vitro* grafting of grapefruit meristem on sour orange rootstock as an important step for production of virus-free plantlets.

MATERIALS AND METHODS

Sour orange seeds were extracted from ripe sour orange fruits at Wad Medani, Gezira State, Sudan, during August, 2001 to May, 2002. The fruits were washed with tap water to reduce the amount of contamination and dust. They were disinfected under a laminar airflow cabinet (Hepaire) by dipping in 70% ethanol for 30 seconds، followed by 10% chlorox (commercial bleach containing 5.25% available chlorine as a surfactant). Three drops of Tween 20 were added to each 100 ml chlorox. The fruits were then rinsed three times with sterilized distilled water. A shallow cut was then made on the rind of the fruit and the fruits were squeezed to discharge the embedded seeds. Seeds were then washed three times with sterilized distilled water, floated and irregular-shaped seeds were discarded.

An experiment was established to test the pretreatment required to improve the germination of sour orange seeds. Four seed treatments were used: 1. Seeds in which both seed coats were removed. 2. Seeds in which only the hard seed coat was removed. 3. Seeds containing both seed coats. 4. Seeds treated with 5% H_2S0_4. Seeds were cultured individually in culture tubes (25x150 mm) containing solid MS medium. The basal medium used was Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962). The medium was formed from macro and micronutrients, iron source and vitamin mixture. Sucrose (30)g/l) and phytogel (2.5 g/l) were added to the medium. The pH was adjusted to 5.7 prior to autoclaving at 121 ^{o}C and 1.06 bar for 15 minutes. The experimental design was a completely randomized design with 10 replications for each treatment. Seed treatments were carried out under aseptic conditions and incubated at 27±1 ^{o}C under

continuous darkness for two weeks after which the number of germinated seeds were counted and shoot length was measured. Five experiments were initiated to investigate different parameters involved in the grafting technique. The best seed treatment obtained from the previous experiment was utilized in all grafting experiments. An experiment was carried out to study the effect of source of vegetative flushes on success of grafting. Vegetative flushes of 'Miami' grapefruit were obtained from three sources: 1. Seedlings grown under glasshouse conditions at the Tissue Culture Unit, Agricultural Research Corporation. 2. Seedlings grown in nursery conditions at the Department of Horticulture Nursery, University of Gezira and 3. Trees grown under field conditions in an orchard at Wad Medani area. Then shoot tips were excised from newly growing flushes. They were washed with tap water, disinfected in 70% ethanol followed by 10% chlorox (commercial bleach) in which three drops of Tween 20 were added and then rinsed three times with sterilized distilled water.

Two weeks old sour orange seedlings were removed from the culture tubes under aseptic conditions and decapitated leaving about 1.5cm of the epicotyl. Roots of seedlings were cut to a length of 3-5 cm and the cotyledons and their axillary buds were removed. An inverted T-incision was then made on the rootstock seedlings with the aid of sterile dissecting instruments and a microscope. The remaining leaves of the shoot tips except for the youngest three leaf primordia were removed. The apical meristem (0.1-0.2 mm) was placed inside the inverted T-incision of the rootstock. Grafted plants were cultured in liquid MS medium with 75 g/l sucrose and supported with a bridge of filter paper. Cultured plants were incubated at 27±1 ^{o}C and exposed to 16 hours daily light and subcultured every 4 weeks.

An experiment was carried out to determine the effect of light incubation conditions on the success of grafting. Four different incubation conditions were tested: l. Continuous darkness for two weeks, 2. Continuous light for two weeks, 3. Continuous light for the first week and continuous darkness for the second week and 4. Continuous darkness for the first week and continuous light for the

second week. The experimental design used was a completely randomized design with 20 replications for each incubation condition. An experiment was carried out to determine the effect of age of rootstock seedlings on the success of grafting. Four different ages of rootstock seedlings ranging from one to four weeks were tested. The experimental design was a completely randomized design with 10 replications for each age.

An experiment was carried out to study the effects of methods of placement of excised shoot tips on the rootstock seedlings on the success of grafting. Five different methods of placement were used. Propagation of grapefruit by shoot tip micrografting. These were: l. Shoot tip set on cortex surface in inverted-T incision Incision was made 3-4 mm above root system of rootstock seedling 2. Shoot tip set on cortex surface in inverted-T incision. Incision was made at the point of decapitation of epicotyl. 3. Shoot tip placed on cortex exposed at top cut surface of decapitated epicotyl. 4. Shoot tip set on pith exposed at top cut surface of decapitated epicotyl and 5. Shoot tip placed on vascular ring tissue at top of decapitated epicotyl. The experimental design was a completely randomized design with 20 replications for each method of placement.

An experiment was also carried out to determine the effect of sucrose concentration on the success of grafting. Four different concentrations of sucrose in MS medium (2.5, 5.0, 7.5 and 10.0%). were tested. The experimental design was a completely randomized design with 10 replications for each sucrose concentration. The numbers of successful grafts were counted and percentages of successful grafts were calculated. The number of leaves, nodes and length of successful grafts were also determined at weekly intervals for six weeks.

Six-week old micro-grafted plants were transferred to pots containing a soil mix of silt and sand at a ratio of 1:1. Seedlings were grown under glasshouse conditions. Vegetative growth of the seedlings was followed for the next six months. Data in percentage was transformed to square roots before statistical analysis using MSTAT-C computer

programme (MSU, 1993) and mean separation was done according to Duncan's Multiple Range Test(DMRT).

RESULTS AND DISCUSSION

The effect of sour orange seed treatments on percentage of germi. nation and shoot length after two weeks is shown in Table l. Results indicated significant differences between treatments ($P<0.05$). High percentage of germination and the longest shoot length of sour orange seedlings were obtained when both seed coats and only the hard seed coats were removed from sour orange seeds. Although these two treatments gave comparable results, but technically removal of both seed coats was easier. Seeds treated with 5% H_2S0_4 resulted in the lowest germination percentage and the lowest shoot length.

Table l. Effect of sour orange seed treatment on percentage of germination and seedling shoot length after two weeks.

Seed treatment	Germination (%)	Shoot length(mm)
Removal of both seed coats	100.0a	28.7a
Removal of hard seed coat only	90.0a	25.4a
Seeds containing both seed coats	60.0b	2.8 b
Seeds treated with 5% H_2S0_4	30.0c	1.5 c
CV (%)	13.8	25.0
SE+	0.5	1.1

Means having the same letter(s) within each column are not significantly different at 5% probability level according to Duncan Multiple Range Test (DMRT).

The use of citrus rootstock seeds in which both seed coats were removed in shoot tip grafting in vitro has been practiced by many researchers. Murashige *et al*. (1972) used Troyer citrange and trifoliate orange in which both seed coats were removed and obtained the highest germination *in vitro*. Navarro et al. (1975) used the same rootstock in addition to rough lemon and obtained similar results. The resulted sour orange rootstock seedlings are assumed to be disease-free seedlings because it is well known that citrus seedlings arising *in vivo* or in *vitro* from polyembryonic species like sour orange are free from most pathogenic viruses (Rangan *et al.,* 1968)..

The effect of source of grapefruit shoot tips on percentage of successful grafts after four weeks from grafting-is shown in Table 2. High percentage of successful grafts was obtained when shoot tips of "Miami" grapefruit -were obtained from seedlings grown under glasshouse and nursery conditions. The lowest percentage of successful grafts was obtained when shoot tips were obtained from flushes in trees grown under field conditions. Several authors (Navarro et al., 1975; Navam *et al.*, 1980) recommended that flushes from potted plants grown in glasshouse conditions were the best source of citrus shoot tips. The occurrence of flushes on trees grown under field conditions is season dependent whereas potted plants grown in glasshouse conditions have the advantage that they can be induced when necessary, avoiding the seasonal dependency on orchard trees.

Table 2. Effect of source of grapefruit shoot meristem on percentage of successful grafts four weeks after grafting.

Source of shoot tips	Successful grafts(%)
Potted plants grown in glasshouse	60.0 a
Potted plants grown in the nursery	40.0 ab
Trees grown in the field	20.0b
CV (%)	16.9
SE+	0.5

Means having the same letter(s) within each column are not significantly different at 5% probability level according to DMRT.

The effects of light incubation conditions for two week old sour orange seedlings on percentage of successful sour orange/grapefruit grafts after four weeks is shown in Table 3. There were variations in percentage of successful grafts according to exposure of seedlings to light. The frequency of successful grafts was greater when rootstock seedlings were obtained from seeds germinated under continuous darkness for two weeks, followed by rootstock seedlings exposed to light for the first week and darkness for the second week. However, there was no significant difference between the two treatments. The lowest percentage of successful grafts was obtained when sour orange

rootstock seedlings were obtained from seeds germinated under continuous light for two weeks. Results obtained agree with Herman and Hess (1963) who observed that the regeneration of roots in bean epicotyl was better if cuttings were taken from etiolated rather than green seedlings. Navarro *et al*. (1975) reported a very low frequency of successful grafts using Troyer citrange seedlings grown under continuous light compared to seedlings grown in continuous darkness‹. The effect of age of sour orange seedlings at the time of grafting on percentage of successful shoot tip grafts in vitro after 4 weeks from grafting is shown in Table 4. The age of sour orange seedlings significantly affected the percentage of successful sour orange/ grapefruit grafts. The highest percentage of successful grafts was obtained when 2 to 3 week old sour orange seedlings were used. Four week old seedlings resulted in a significantly high percentage of unsuccessful grafts. The unsuccessful grafts on older seedlings (4 week-old) shows shoot tips which dried, turned brown and died. One week old seedlings resulted in a significantly high percentage of unsuccessful grafts with callus. Shoot meristem grafted on younger

Table 3. Effect of light incubation conditions for two week old sour orange seedling on percentage of successful sour orange/grapefruit grafts four weeks after grafting.

Light incubation conditions	Successful grafts (%)
Continuous darkness for two weeks	50.0 a
Continuous light for two weeks	5.0 b
Continuous light for the first week and continuous darkness for the second week	35.0 a
Continuous darkness for the first week and Continuous light for the second week	5.0 b
CV (%)	30.4
SE+	0.6

Means having the same letter(s) within each column are not significantly different at 5% probability level according to DMRT.

seedlings (one week old) shows shoot tips that had become quiescent and buried in callus. These results are consistent with earlier work of Navarro *et al* (1975) who reported that the highest rate of successful

grafts using Troyer citrange as rootstock was obtained with 2 weeks old seedlings.

Table 4. Effect of age of sour orange seedlings at the time of grafting on percentage of successful shoot tip grafts *in vitro* four weeks after grafting.

Age of seedlings	Successful grafts(%)	Unsuccessful grafts with dead scion(%)	Unsuccessful grafts with callus(%)
One week	10.0c	10.0c	80.0a
Two weeks	60.0a	20.0bc	20.0bc
Three weeks	40.0ab	30.0b	30.0b
Four weeks	20.0b	70.0a	10.0c
CV (%)	30.1	24.1	31.8
SE+	0.8	0.7	0.9

Means having the same letter (s) within e h column are not significantly different at 5% probability level according to DMRT.

Effect of method of placement of excised shoot tips of grapefruit on sour orange rootstock seedlings on percentage of successful grafts after 4 weeks from grafting is shown in Table 5. The highest success of grafts was obtained when the shoot tip of grapefruit was placed either in the inverted-T incision made at the top of the decapitated epicotyl or over the vascular ring at the top cut surface of the decapitated epicotyl. These two methods of placement of shoot tip were comparable and significantly higher than the other treatments Successful grafts decreased significantly when shoot tip was placed on pith exposed at top cut surface of decapitated epicotyl. Low success of grafting was also obtained when shoot tip was placed either on cortex exposed at top cut surface of decapitated epicotyl of seedling or on cortex surface in inverted-T incision made 3-4 mm above root system of rootstock seedling. These results agreed with Platt and Opitz (1973) who reported that placement of the shoot tip in the inverted-T incision at the top of decapitated epicotyl gave high success of grafts in contrast to the placement of shoot tip in inverted-T at 3-4 mm above root system of seedling. Navarro et ale (1975) also reported several techniques of shoot tip placement that can be used but the inverted-T incision method was the best.

Table 5. Effect of method of placement of shoot meristem of 'Miami' grapefruit on sour orange seedling on percentage of successful grafts after four weeks.

Method of placement of shoot tip	Successful grafts(%)
Shoot tip set on cortex surface in inverted-T incision made 3-4 mm above root system of seedling.	5.0c
Shoot tip placed on cortex exposed at top cut surface of decapitated epicotyl.	10.0c
Shoot tip set on pith exposed at top cut surface of decapitated epicotyl.	25.0b
Shoot tip placed on vascular ring tissue at top of decapitated epicotyl	40.0a
Shoot tip set on cortex surface in inverted-T incision made at point of decapitated epicoty1	50.0a
CV (%)	25.0
SE+	0.6

Means having the same letter(s) within each column are not significantly different at 5% probability level according to DMRT.

Effect of sucrose concentration on MS liquid nutrient medium on percentage of successful sour orange/grapefruit grafts and vegetative growth of seedling after 4 weeks from grafting is shown in Table 6. Sucrose concentration of nutrient medium significantly affected percentage of successful sour orange/grapefruit grafts. The highest percentage of successful grafts was obtained when MS medium was supplemented with 7.5% sucrose. Vegetative growth of sour orange/ grapefruit grafts was also influenced by sucrose concentration. The highest number of new leaves, length of scion and number of nodes were also obtained on a medium containing 7.5% sucrose. Increasing sucrose concentration to 10% showed no additional benefit. These results agree with Navarro et al. (1975) who reported that sucrose concentration of nutrient medium of grafted plants played a significant role and that the highest rate of successful grafts was obtained with 75 g/l of sucrose. They also reported that 7.5% sucrose concentration had been selected as optimum in the nutrient medium for *in vitro* grafting of citrus species.

Table 6. Effect of sucrose concentration of MS liquid nutrient medium on percentage of successful sour orange/grapefruit grafts and vegetative growth of seedlings four weeks after grafting.

Sucrose Concentration(%)	Successful Grafts (%)	Number of leaves	Length of Scion (mm)	Number of nodes
2.5	30.0 c	3.0b	8.7a	1.0b
5.0	40.0 bc	4.0ab	10.7a	2.0ab
7.5	60.0 a	6.0a	13.8a	3.0a
10.0	50.0ab	5.0ab	12.0a	2.0ab
CV(%)	12.3	19.7	21.1	19.6
SE±	0.4	0.2	0.1	0.1

Means having the same letter(s) within each column are not significantly different at %5probability level according to DNRT.

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