The Journal of Animal & Plant Sciences, 27(2): 2017, Page: 567-574
ISSN: 1018-7081

CLONAL PROPAGATION OF JOJOBA BY *IN VITRO* CULTURE AND DETERMINATION OF SEXUALITY OF THE REGENERANTS

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ABSTRACT

The present study was conducted to develop a protocol for in vitro propagation of Jojoba (*Simmondsia chinensis*) and to determine male and female regenerants through molecular markers. Nodal segments were cultured on MS media containing (0.5, 1.0, 2.0 mg/L) BAP, KIN or (0.22, 0.44, 0.88 mg/L) TDZ and 1.5, 2.0, 2.5 mg/L BAP or in combination with auxins (1.25 mg/L IAA, IBA, NAA) for in vitro propagation. As the result of the experiments, the highest number of shoots (9.13 shoots per explant) was obtained on MS medium containing 2.0 mg/L BAP. In vitro propagated shoots were transferred on MS media containing different concentrations of NAA, IAA and IBA for rooting. The best results were obtained on MS media containing 3.0 mg/L NAA or 1.0 mg/L IBA (40%, 30% respectively). The effect of ½ MS, 1.5% sucrose and active carbon addition (0.5%) on rooting was examined. The highest rooting rate (50%) was obtained on ½ MS medium containing 1 mg/L IBA, 1.5% sucrose and 0.5% active carbon. Rooted shoots were successfully acclimatised and showing on 75% survival rate. For sex determination, genomic DNA was isolated from the young leaves of male and female plants and regenerated shoots of unknown sex. PCR applications were performed by using UBC-807 and OPG-5 primers. UBC-807 produced a unique ~1,200 base-pair fragment in the male DNA. The determination of male and female individuals was successfully performed with the gel image obtained from UBC-807 primer.

Key words: dioecious, DNA markers, micropropagation, sex identification, *Simmondsia chinensis.*

INTRODUCTION

Simmondsia chinensis (Link) Schneider, which is commonly known as 'jojoba', is an obligate cross-pollinated and evergreen desert shrub belonging to the *Simmondsiaceae* family native to the State of California and the Sonora desert of northwest Mexico (Gentry, 1958; Mills *et al*., 1997; İnce *et al*., 2010). Jojoba plant has a good economic potential due to its seeds containing a liquid wax used in medicine, plastic industry and cosmetics (Jacoboni and Standarti, 1987). Furthermore, Jojoba oil has certain characteristics similar to those of sperm whale oil (Low and Hackett, 1981). Due to these characteristics, jojoba cultivation has been extensive in the last thirty years for industrial use in producing medicine, non-foaming substances, lubricants resistant to high temperature and pressure, resins and plasticizers (Mills *et al*., 1997). The propagation of jojoba is mainly through seeds. High variation is observed in oil content and seed yield due to pollination by wind and in addition it is not possible to determine female and male plants until flowering occurs. These are serious problems of jojoba cultivation. Molecular marker techniques are among the highly useful ways for determining the sex of dioecious species at an early stage. The determination of male and female jojoba plants at young stage is important for a planned plating (5 male: 1 female) in cultivation. A few molecular marker that can be used for sex determination of jojoba was stated by Agrawal *et al*. (2007) as OPG-5 RAPD primer, by Sharma *et al*. (2008) as UBC-807 ISSR primer and by Ince *et al*., (2010) as JMS900 DNA marker.

The production of clones of known sexuality and of high oil quality and yield is necessary to ensure that commercial plantations will be productive (Kumar *et al*., 2012). Although vegetative propagation is an alternative way for the production of desired clones, propagation through traditional root cutting is not effective due to long procedure and slow growth (Tyagi and Prakash, 2004; Singh *et al*., 2008).

The propagation of jojoba through tissue culture techniques can be another alternative. Today, agricultural production through micropropagation is effectively used especially in ornamental plants. Clonal propagation of jojoba plants of elite individuals of known sexuality can be achieved through tissue culture methods (Mills *et al*., 1997; Tyagi and Prakash, 2004; Kumar *et al*., 2012). Indeed, micropropagation ensure the production of multiple planlets from the male or female plant of jojoba in a short time for commercial plantations. A single explant source could conceivably provide thousands of new true-to-type plantlets per year i.e., those plants that are genetically similar to the parental stock and that could

potentially maintain the genetic line (Bekheet, 2015). Propagation studies have been conducted on different genotypes of jojoba and it was reported that genotype was effective on the success of the propagation of jojoba (Tyagi and Prakash, 2004; Bashir *et al*., 2008; Mohammed *et al.,* 2013). Generally nodal segments were used as explants sources in the previous studies on the propagation of jojoba (Tyagi and Prakash, 2004; Singh *et al*., 2008; Bekheet, 2015).

The present study aim to determine the clonal propagation ability of jojoba through tissue culture methods by using nodal explant of jojoba cultivated in Turkey. Furthermore, the sexuality of the regenerants was determined through OPG-5 and UBC-807 primers.

MATERIALS AND METHODS

Seeds belonging to the *Simmondsia chinensis* (Link) Schneider species supplied by Prof. Dr. Mehmet Babaoğlu (Selçuk University, Faculty of Agriculture, Department of Field Crops, Konya, Turkey) and shoots of jojoba plants of known sex cultivated at Alata Horticultural Research Institute were used as material in this study.

At the beginning, the seeds were agitated in 50% (v/v) ethanol for 30 seconds prior to washing with sterile distilled water. The seeds were continuously rinsed in 30% (v/v) commercial bleach (HES) with 1-2 drops of Tween-20 for 15 minutes followed by 3 times rinses with sterile distilled water. The sterilized seeds were incised with a sterile scalpel and transferred into magenta culture vessels containing 50 ml MS (Murashige and Skoog, 1962) medium for germination. The aseptic seedlings were used as explant source.

Shoots of male and female plants were separated from their leaves and then, they were cut at certain intervals in a way that would not harm the nodal segments. Surface sterilization of the pieces of shoots were carried out with different concentrations of solutions containing ethanol, commercial bleach, mercury chloride ($HgCl_2$) and Tween-20, before rinsing with sterilized distilled water. The treatments performed are given in Table 1.

Table 1. Treatments for surface sterilization of shoots.

Tr. No	Presterilization	Sterilization
1	A	Rinsing in 10% commercial bleach + 1-2 drop tween-20 for 15 min. followed by 3 rinses with sterile distilled water.
2	A	Rinsing in 30% commercial bleach + 2 drops tween-20 for 15 min. followed by 3 rinses with sterile distilled water.
3	A	Rinsing in 60% commercial bleach + 2 drops tween-20 for 15 min. followed by 3 rinses with sterile distilled water.
4	A and continuously agitated in 50% ethanol for 30 second prior to washing with sterile distilled water.	Rinsing in 20% commercial bleach + 2 drops tween-20 for 10 min. followed by 3 rinses with sterile distilled water.
5	A and continuously agitated in 50% ethanol for 30 second prior to washing with sterile distilled water.	Rinsing in 30% commercial bleach + 2 drops tween-20 for 15 min. followed by 3 rinses with sterile distilled water.
6	A	Rinsing in 30% commercial bleach + 2 drops tween-20 for 30 min. and then agigated 96% ethanol for 2-4 second followed by 3 rinses with sterile distilled water.
7	A	Rinsing in 40% commercial bleach + 2 drops tween-20 for 20 min. and then agigated 96% ethanol for 2-4 second followed by 3 rinses with sterile distilled water.
8	A	Rinsing in 50% commercial bleach + 2 drops tween-20 for 20 min. and then agigated 96% ethanol for 2-4 second followed by 3 rinses with sterile distilled water.
9	A	Rinsing in 0,05% $HgCl_2$ for 20 min. followed by 4 rinses with sterile distilled water.
10	A	Rinsing in 0,1% $HgCl_2$ for 15 min. followed by 4 rinses with sterile distilled water.
11	A	Rinsing in 0,2% $HgCl_2$ for 10 min. followed by 4 rinses with sterile distilled water.
12	B and continuously agitated 70% ethanol for 30 seconds before rinsing with sterile	Rinsing in 30% commercial bleach + 2 drops tween-20 for 15 min. followed by 4 rinses with sterile distilled water.

	distilled water.	
13	B and continuously agitated 96% ethanol for 30 seconds before rinsing with sterile distilled water.	Rinsing in 30% commercial bleach + 2 drops tween-20 for 15 min. followed by 4 rinses with sterile distilled water.
14	B	Rinsing in 0,2% $HgCl_2$ for 10 min. followed by 4 rinses with sterile distilled water.

A: Washing under running tap water for 30 min.
B: Rinsing in tap water with 1 drop tween-20 for 15 min. transferred to sterile distilled water

Although the seeds were sterilized at a success rate of 100%, the sterilization studies conducted on vegetative shoots did not yield the expected results. Therefore, aseptic seedlings obtained through the germination of seeds were used as explant source in clonal propagation studies.

Nodal segments of 12-week of age aseptic seedlings which were cut into pieces of approximately 1.5 cm in length in a way to contain a node were used as explant in the study. The explants were cultured on MS media containing 0.5, 1.0, 2.0 mg/L BAP or KIN or 0.22, 0.44, 0.88 mg/L TDZ to determine the best cytokinin source for shoot propagation. Afterwards, a new experiment was set up to determine the effect of cytokinin x auxin interaction on shoot propagation and the explants were cultured on MS medium containing BAP (1.5, 2, 2.5 mg/L), which was determined as the best cytokinin for shoot propagation at preliminary experiment, and different auxin (1.25 mg/L NAA, IAA, IBA) combinations.

Regenerated shoots were transferred on MS media containing different concentrations of NAA (1.0, 3.0, 5.0, 7.0 and 9.0 mg/L), IAA (1.0 and 3.0 mg/L) or IBA (1.0, 3.0, 5.0, 10.0 and 15.0 mg/L) for rooting. This preliminary experiment showed that NAA (3mg/l) and IBA (1 mg/l) resulted in better rooting response compared to IAA and other concentrations of NAA and IBA (data not shown). In order to increase the rooting rate, the experiment provided the best rooting responses was also tested with ½ MS and 1.5 or 3.0 % sucrose and with or without active carbon (AC). Rooted shoots were washed with tap water to remove residual rooting media, and transferred to small pots containing sterilized turf in a growth chamber.

MS mineral salt and vitamins solidified with 0.8% (w/v) agar were used as a basal medium in the experiments. The pH of media was adjusted to 5.8 before autoclaving at 121°C for 20 min. All cultures were maintained at 24 ± 2 °C in growth chamber (SANYO, MLR-351H, Japan) with fluorescent light (112 µmol $m^{-2}s^{-1}$) and 16-h light and 8-h dark photoperiod.

In shoot propagation studies, each experiment contained 5 explants with 3 replicates. Rooting studies were set up by putting one shoot in each culture tube and ten replicates were used per experiment. The obtained data was analyzed on the computer using MSTAT-C statistical software and the results that were found to be significant were subjected to LSD multiple comparison test.

Genomic DNA was extracted from leaves of jojoba plants of known sex (male or female) and regenerants of unknown sex by using a modified method from Doyle and Doyle (1987) and Özkaya *et al*. (2009). The concentration and purity of the extracted DNA were determined using a spectrophotometer (Nanodrop ND-100). The DNA samples were run in 1% agarose gel and the presence of the DNA was detected in the imaging device (Vilber Laurmat, France).

Two primers OPG-5 (Agrawal *et al*., 2007) and UBC-807 (Sharma *et al*., 2008), which were reported to yield successful results for sex determination in jojoba, were used to determine the sexes of the regenerants. Each reaction mix contained 1 µl of 20 ng DNA template, 2.5 µl x10 PCR buffer (Fermentas), 2.5 µl of 25 mM Mg^{+2} (Fermentas), 0.4 µl of 25 mM each of dNTP (Fermentas), 0.3 µl of 500 U Taq DNA polymerase (Fermentas), and 0.5 µl of 50 pmol. μl^{-1} primer, in a final reaction volume of 25 µl. The reactions were carried out in Eppendorf mastercycler gradient device according to the PCR conditions given in Table 2.

Table 2. The conditions for PCR reaction of each primer.

OPG-5 (5’ CTGAGACGGA 3’)		**UBC-807** (5’AGAGAGAGAGAGAGAGT 3’)	
94 °C 3 min		94 °C 3 min	
94 °C 1 min		94 °C 20 sec	
30 °C 1 min	x38	50 °C 1 min	x35
72 °C 2 min		72 °C 1.5 min	
72 °C 10 min		72 °C 7 min	

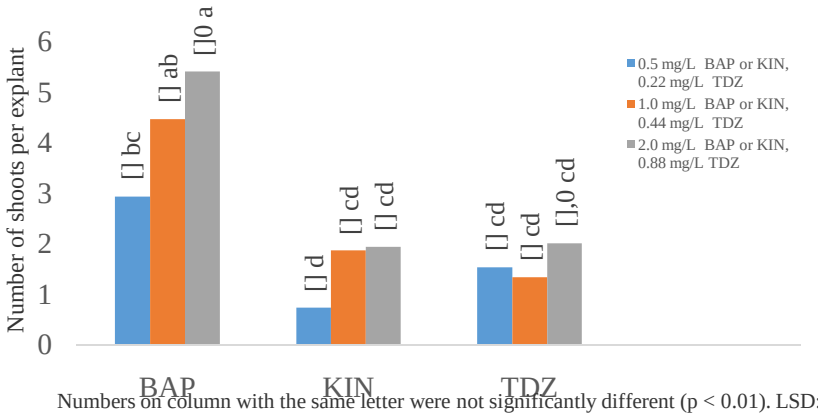
Amplified products were electrophoresed onto a 2% (w/v) agarose (Serva) gel in the presence of a 200bp DNA ladder (Fermentas, SMO633) and followed by visualization in a Vilber Lourmat (France) imaging system. PCR applications were repeated twice for both primers. The results were assessed for both primers based on the existence of band, which is reported to be used in sex determination, on the gel.

RESULTS AND DISCUSSION

Firstly, the effect of different cytokinins on shoot propagation of jojoba was tested. It was observed that new buds started to form on the sides of the explants after 10 days of culture but shoot development was slow. The number of shoots per explant was determined after 8 weeks. The results obtained from the media containing BAP were found to be more successful compared to those obtained from the media containing KIN or TDZ. The highest number of shoots per explant (5.40) was obtained on MS medium containing 2.0 mg/L BAP, followed by 1.0 mg/L BAP (4.46). The lowest number of shoots per explant (0.73) was obtained on MS medium containing 0.5 mg/L KIN (Figure 1).

Secondly, the explants were cultured on MS media including BAP and different type of auxin (NAA, IAA or IBA) to determine the effect of cytokinin x auxin interaction on shoot propagation. After 6 weeks, the number of shoots per explant was counted and subcultured on the same medium. After 6 weeks of subculture, the number of shoots was recounted. It was found that cytokinin x auxin interaction was effective on shoot propagation and subculturing caused an increase in the number of shoots (Table 3). Similarly, Singh *et al*. (2008) also reported that the number of shoots showed an increase throughout the subculture period. After 6 weeks of culture, the best results were obtained on media containing 2.0 mg/L BAP and 1.5 mg/L BAP x 1.25 mg/L IAA (3.53 and 3.46 shoots per explant respectively) whereas after subculture, MS medium containing 2.0 mg/L BAP yielded the highest result with 9.13 shoots per explant (Table 3; Figure 2a-b).

It was reported in the literature that in the studies in which nodal segments were used as explant sources and media containing different concentrations of BAP were used, the highest results were obtained as 10-15 shoots per explant on media containing 6 mg/L BAP over a 12 week period (Roussos *et al*., 1999) and 1 mg/L BAP plus 13 mg/L adenine after repeated subcultures (Singh *et al*., 2008). Male and female nodal explants of jojoba produced maximum 9.3-10 shoots per explant, respectively on MS including 2.25 mg/L BAP in 90-d-old cultures (Tyagi and Prakash, 2004).



Numbers on column with the same letter were not significantly different ($p < 0.01$). LSD: 2.082

Figure 1. Effects of different concentrations of BAP, KIN or TDZ on in vitro shoot propagation of *S. chinensis*.

Table 3. Effects of BAP and different auxins combinations on in vitro shoot propagation of *S. chinensis*.

	No. of shoots per explants							
BAP	**-**		**IAA (1.25 mg/L)**		**IBA (1.25 mg/L)**		**NAA (1.25 mg/L)**	
(mg/ L)	**6. week**	**12.week**	**6. week**	**12. week**	**6. week**	**12.week**	**6. week**	**12. week**
1.5	2.26 ^{BC}	3.13 ^{cde**}	3.46 ^{A}	6.26 ^{b}	2.26 ^{BC}	6.33 ^{b}	1.26 ^{D}	2.33^{de}
2	3.53 ^{A*}	9.13 ^{a}	2.60 ^{B}	6.06 ^{b}	1.00 ^{D}	4.40 ^{bcd}	1.13 ^{D}	1.80 ^{e}
2.5	2.73 ^{B}	5.80 ^{b}	1.73 ^{CD}	4.93 ^{bc}	1.66 ^{CD}	5.13 ^{bc}	2.53 ^{B}	3.20 ^{cde}
Mean	2.84	6.02	2.60	5.76	1.64	5.29	1.64	2.44

Numbers in column with upper and lower case were significantly different. ($p<0.01$) * LSD:0.77 **LSD:2.54

According to the results obtained from shoot propagation studies, the highest number of shoots per explant (9.13 shoots) was obtained on the medium containing 2 mg/ l BAP. So the small number of nodal segments that were obtained at vegetative shoots sterilizations of plants of known sex were cultured on MS containing 2 mg/L BAP and a mean number of 4.5 shoots per explant were obtained at the end of 4 weeks (Figure 2c). Furthermore, it was observed that the development of the regenerant shoots was faster and more healthy shoots were obtained than shoots from the explants from aseptic seedlings

Regenerated shoots transferred into rooting media and root formation occurred in 4-6 weeks. At the end of 8 weeks, the number of rooting shoots was determined. In the first experiment, rooting was obtained on MS media containing IBA and NAA, whereas no root formation was observed on media containing IAA. The best results (40% and 30%) were obtained on MS medium containing 3 mg/L NAA and 1 mg/L IBA, respectively (data not shown). In the second experiment, which was set up in light of the data obtained from the preliminary experiment, the highest rooting rate (50%) was obtained on ½ MS medium containing 1 mg/L IBA, 1.5% sucrose and 0.5% AC. The leaves of the shoots in this medium turned slightly yellow. However, this change of color stopped after the plantlet was transferred to the outdoor conditions and the plant continued its normal development (Table 4; Figure 2d).

Table 4. Effect of auxins, sucrose concentrations and AC in ½ MS on rooting of *S. chinensis*.

½ MS	Plant growth regulator	Percentage of rooting (%)
3% sucrose	-	0 d
	1 mg/ l IBA	20 bcd
	3 mg/ l NAA	30 abc
1.5 %sucrose	-	0 d
	1 mg/ l IBA	30 abc
	3 mg/ l NAA	40 ab
1.5 %sucrose + 0.5 % AC	1 mg/ l IBA	50 a
	3 mg/ l NAA	10 cd

Numbers in column with the same letter were not significantly different ($p<0.05$).LSD:26.16

Different doses of IBA, IAA and NAA were used in the studies conducted for rooting of regenerated shoots in jojoba. They reported that higher results were obtained from the media containing IBA (Tyagi and Prakash, 2004; Bashir *et al*., 2008). Furthermore, the addition of AC (Tyagi and Prakash, 2004) and the transfer of shoots to the ½ MS medium (Agrawal *et al*., 2002; Singh *et al*., 2008) increased the success of rooting in jojoba. *Astragalus maximus* (Turgut-Kara and Arı, 2006) and *Viola patrinii* (Chalageri and Babu, 2012) species were also reported to have more successful rooting on ½ MS media with degreased dose of sucrose. In this study, the best result (50%) was obtained on ½ MS medium containing 1 mg/L IBA, 1.5% sucrose and 0.5% AC.

In the process of purifying the rooted shoots from the medium, the roots of the plantlets collected from the medium containing NAA were ruptured from the shoot. It was seen that these ruptures occurred because micro callus formation occurred before root formation. However, callus formation was not observed on media containing IBA. The adaptation of the plantlets to the outer medium was maintained and it was observed that 75% of the plantlets collected from the medium containing IBA survived (Figure 2 e-f-g).

Two primers were used to determine the sexes of the regenerants of unknown sex. As the result of the PCR conducted using OPG-5 RAPD primer, the agarose gel images did not reveal the bands at the desired length that could be used for sex identification which were reported by Agrawal *et al*., (2007). This might have been the result of the low repeatability rate of the RAPD markers.

ISSR markers are more reproducible compared to RAPD markers. When the agarose gel images of the PCR products obtained via the UBC-807 ISSR primer used in this study were observed, it was seen that the band viewed in the individual in which it was visible that the marker was cross male with a length of 1200 bp was also observed in the R5, R6 and R7 individuals of unknown sex. The equivalent of this band accompanying the marker corresponds to the area stated by Sharma *et al*. (2008). This band did not occur in the individual which was known to be female and in other regenerants of unknown sex (Figure 3).

In conclusion, the nodal segments of aseptic seedlings obtained from the seeds of jojoba plants cultivated in Turkey were used as an explant, and a protocol was developed for the micropropagation of jojoba. Although jojoba is a plant with high economic value, the plants that gain economic importance are females. The sex of the jojoba plant becomes visible after a long period of 1 to 4 years. Sex determination at earlier stages is important in plantation for commercial

production of jojoba. The identification of male and female individuals was successfully realized using the gel image obtained in UBC-807.

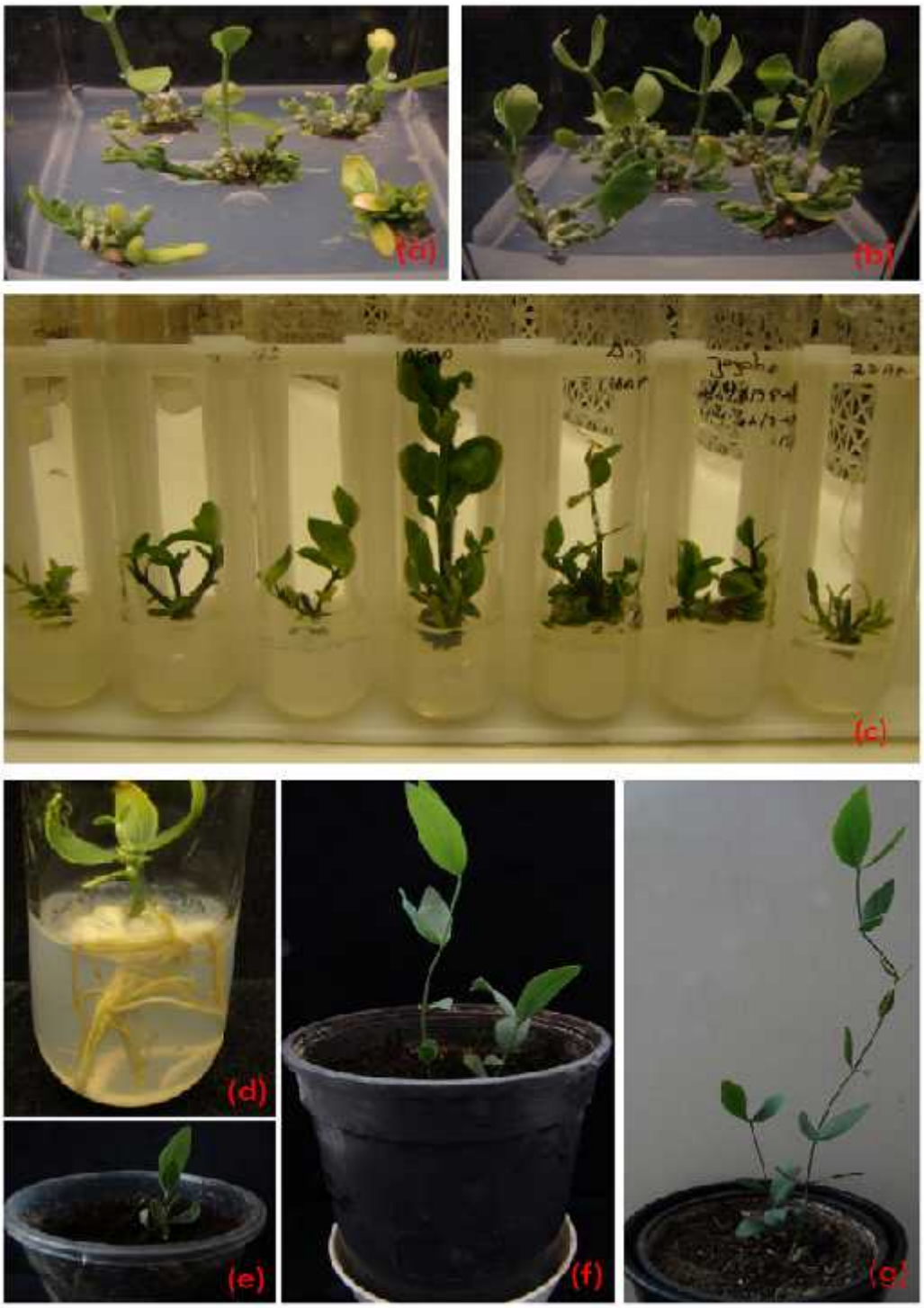


Figure 2. In vitro propagation from nodal segment of *S. chinensis* (α-b): Axillary shoots on explants collected from aseptic seedling on MS medium containing 2 mg/L BAP (6.-12.week), c: Axillary shoots on explants collected from male and female plants on medium containing 2 mg/L BAP (d): Rooted shoot on ½ MS medium containing 1.5% sucrose + 0.5%AC+1 mg/L IBA, (e-f-g): Acclimatized plantlets after transfer to outdoor conditions e:3 weeks, f:2 months, g:3 months.

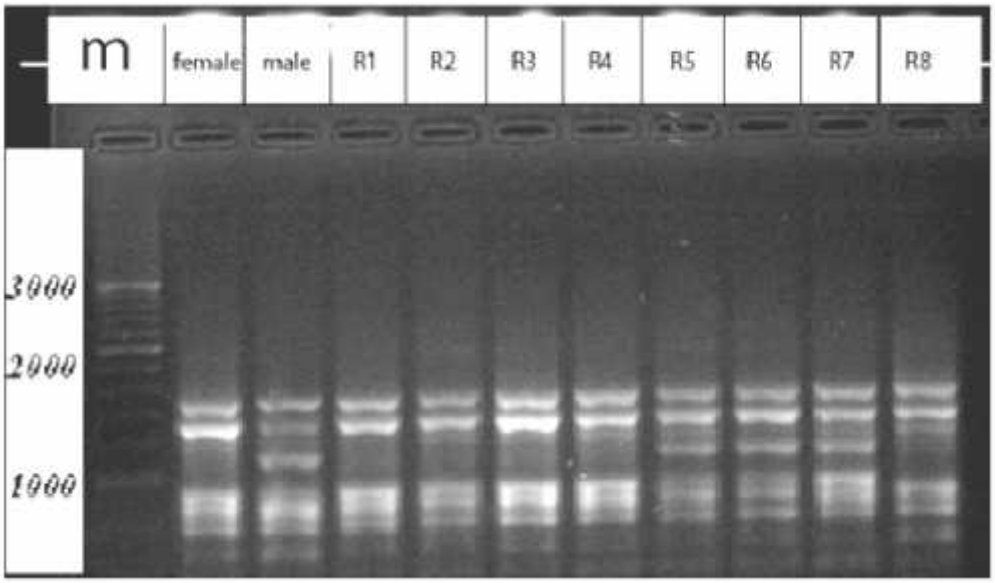


Figure 3. ISSR profile using primer UBC-807 of female, male and regenerated plants of *S. chinensis*. (m: marker; R1–8: regenerated plants).

Acknowledgements: The authors thank Dr. M. Yorgancılar and Dr. E. Atalay (Selçuk University, Agricultural Faculty, Plant Biotechnology Lab. Konya/Turkey) for assistance with molecular analysis.

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