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Determination and assesment of the sex chromosomes of male trees of pistachio (*Pistacia vera* L.) using *in vitro* culture

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Abstract

The chromosome number and karyotype analysis of male trees of *Pistacia vera* L. was investigated to determine the sex chromosomes. For karyological determination, root tips were obtained from in vitro derived shoots. Afterwards fifty plates of mitosis were used for the karyotyping, the five best metaphase plates were measured for karyotype analysis, karyogram and idiogram. The conclusions obtained from the chromosome studies illustrated that the chromosome complement of *P.vera* L. has 2n = 30 chromosome pairs with metacentric, submetacentric, subtelocentric and telocentric centromeres. Karyotype analysis revealed that male trees of *P.vera* L. have heteromorphic sex chromosomes (Xy). The largest pair of chromosomes have different physical features from the other pairs. Neither polyploidy nor satellite was detected on any of these chromosome plates.

Keywords: Male trees of *Pistacia vera* L., in vitro, rooting, sex chromosome, karyotype, karyogram, idiogram.
Abbrevations: BA- 6–benzylaminopurine; GA_3-Gibberellic Acid; IAA- indole–3–acetic acid.

Introduction

The family of *Anacardiaceae* Lindl., the cashew family, includes more than 700 species in 82 genera, including mangos (*Mangifera indica* L), pistachios (*Pistacia vera* L.), cashews (*Anacardium occidentale* L.), and pink peppercorns (*Schinus terebinthifolia* L.) (Pell 2004). *Pistacia vera* L. species are cultivated widely in the Mediterranean regions of Europe and North Africa, the Middle East, China and California (Onay et al., 2004). The economic value of pistachio exports to 66 countries is about one billion dollars/per year, ranking second among each nation's sources of income after oil (Moghadam and Hokmabadi, 2010). The nuts are delicious and nutritious, as they contain proteins, carbohydrates, minerals (such as potassium, phosphorus and iron) and vitamins (A and B1) (Tekin et al., 2001). Located in an area where pistachio cultivation has occurred for centuries, many different cultivars continue to be grown today as well. The most popular pistachio cultivars: Uzun, Kırmızı, Siirt and Halebî are all grown in Turkey. In some countries, these species are used mainly as rootstock for *P.vera* and, rarely, for oil extraction (tannins) (Kafkas and Perl-Treves, 2002). Pollination in pistacia species is mediated by wind (Ozeker et al., 2006). Among pistachio, all species can pollinate and fertilize each other. Wild pistachio species can be used as pollinators for *P. vera* (Ak, 1992). Due to cross pollination and the easily formed interspecific hybrids, in pistachio plants, genetic diversity occurs on a large scale. A better understanding of the genetic diversity of these dioecious plants is also needed for genetic studies concerning sex chromosomes. First reports from cytogenetic studies on *P.vera* L. were limited only to chromosome numbers (Barghchi and Alderson, 1983; Fasihi Harandi et al., 1996; Fasihi Harandi and Behboodi, 1997; Ila et al., 2003, Ehsanpour et al., 2008; Ayaz and Namli, 2009). Molacular and cytogenetic techniques could also be important for clarifying differentiation of sexual chromosomes and for the sex determinations of the pistacia species. A few molacular studies have been performed to demonstrate sex determination of this genus (Hormaza and Herrero, 1998; Kafkas et al, 2001; Ehsanpour et al., 2008). The pistachio tree is a dioecious plant with male and female flowers that grow on separated trees (Acar and Ak 2001). Currently there is no method for distinguishing between male and female pistachio seedlings before flowering. Thus, in this study different rooting tests were carried out using *in vitro* conditions on a male trees of pistachio. The main objective of this study was to develop a genetic approach for *P.vera* L. and to identify its sex chromosomes.

Materials and methods

Explant cultivation

This study was conducted using rootings of *P.vera* L. micropropagated from apical tips in the laboratory of biotechnology at Dicle University, Diyarbakır, Turkey. The mature male material (apical shoot tips) was obtained from trees growing at the Pistachio Research Institute, in Gaziantep, Turkey. To establish *in vitro* shoot cultures, young offshoots were cut 10-15 cm in length. These explants were washed with tap water for 5–10 min. Then, they were dipped in 70% ethanol for 30 seconds, surface-sterilized by shaking successively at 150 rpm in a 15% (w/v) commercial bleach solution (NaOCI) for 40 min, and rinsed five times with sterile distilled water (5 min per rinse). Prior to being cultured, the cutting explants were excised and individually placed in a MS basal medium (Murashige & Skoog, 1962)

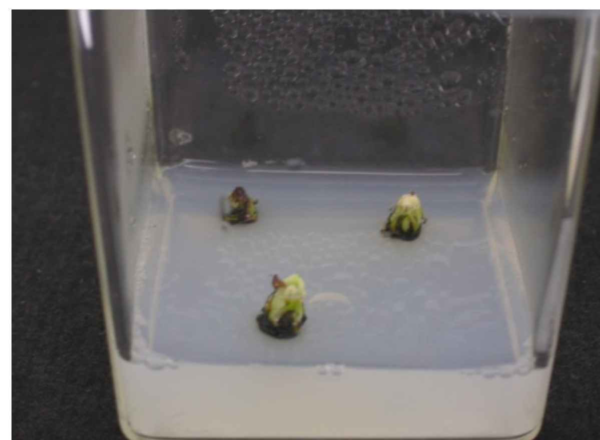
supplemented with 1.0 mg/l^{-1} BA (benzylaminopurine), 30 gr/l sucrose and agar (7 g/l) for micropropagation. The pH of the medium was adjusted to 5.8 with 0.5 N NaOH before sterilization by autoclaving at 121°C for 25 min. After inoculation, cultures were maintained at 25 ± 2°C with a 16 h photo period (40 µmol m^{-2} s^{-1}) provided for by mercury fluorescent lamps. *In vitro* regenerated shoots proliferated by subculturing every four weeks on a shoot multiplication medium: MS salts supplemented with 1 mg/l^{-1} BAP and 0.5 $mg/l^{-1}GA_3$. *In vitro* multiplied shoots were cultured on a rooting medium. For root induction, the basal ends of the *P.vera* L shoots were washed with sterile distilled water and then dipped in solutions that contained different concentrations of IBA (0.5, 1, 1.5, 2.5 and 10gr/l^{-1}) for different times (10, 20, 30, 40 ve 50s) for the rooting of the *P.vera* L. shoots. The dipped shoots were then cultured on a half-strength MS of 20 gr/l sucrose(w/v) and 7 gr/l agar (w/v, Agar-Agar). These were then incubated under a light and temperature regime as shoot multiplication cultures. Data on the rooting percentage were recorded after 15 days of transfer to the rooting medium. The Chi-square test was used to test rooting levels according to treatments and the obtained values are given as tables.

Cytological analysis

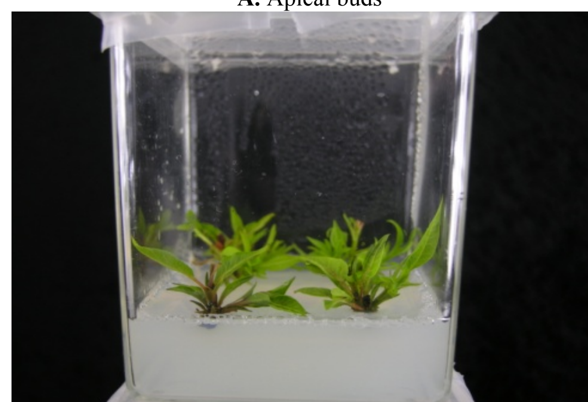
To obtein metaphase plates, root-tip meristems were immersed in paradichlorobenzen at 4°C for 4 h, and then fixed in ethanol: acetic acid (3:1, v/v) at 4°C for 16–24 h and stored in 70% ethanol at 4°C. Finally, these were hidrolyzed in 1N HCl 60°C for 6–8 min. To observe metaphase chromosomal, meristematic tissue was stained with Feulgen. To achieve staining, root-tips were put on Feulgen at room temperature for 2 h. The roots were then gently squashed in a drop of 45% acetic acid. For the cytological investigation, images were captured with a Nikon E 8400. To obtain the karyotype analysis, chromosomes from five metaphase plates of *P.vera* L. were measured, identified, and ordered according to their long-arm length(l), short-arm length(s), total length (C), relative length (RL), and ratio (r). The average of these five plates was presentation as a table and idiograms were drawn from the mean value. The arm-ratio was used to determine the centromere position of each chromosome according to Levan et al., (1964) and the symmetry was studied according to Stebbins (ST) (1971). Karyological features were evaluated as the number of pairs and total length, where C = s+ l; the relative length of each chromosome, where RL = C/ total length of all chromosomes in the genome x100; and the centromeric index, where CI =s /C x 100. The chromosomes of the photographed cells were numbered from 1 (the longest) to 15 (the shortest). To measure the chromosomes, a fine thread was laid over each chromosome in the photograph the length was marked and then measured in millimeters. Homologous pairs were arranged by comparing centromere positions and the presence of secondary constrictions. The idiogram and karyogram were based on averages for relative lengths and arm ratios.

Results

This study was performed in two stages including rooting and cytogenetic studies for the determination and the assessment of sex chromosomes at the root tips of the *P.vera* L. In the rooting stage, shoot proliferation occurred in the presence of



A. Apical buds



B. Culture initiation

C. Shoot proliferation

D. Rooting of the shoots

Fig 1. The stages of micropropagation of mature male trees of *P.vera* L.

Table 1. Effect of dipping in IBA solutions for rooting of *P.vera* L. shoots

Concentration of IBA (gr/l)	Applied time (s.)	Obtained root (%)
Control	-	0
0.5	20	40
1	20	80
1.5	20	50
2	20	30
5	20	5
10	20	-
χ^2(7df)		P < 0.05

Table 2. Effect of different immersion time for rooting of *P.vera* L. shoots

Treatment	Applied time (s.)	Obtained root (%)
Control	-	-
1gr/l IBA	10	30
1gr/l IBA	20	90
1gr/l IBA	30	75
1gr/l IBA	40	60
1gr/l IBA	50	50
χ^2(5df)		P < 0.05

Table 3. The techniques for obtaining chromosome plates of *P.vera* L

Phases	Steps	Temp. (°C)	Period of waiting
First treatment	P- Dichlorobenzen	+4	4 h.
Fixation	Farmer solution	+4	16–24 h.
Storing	Ethyl alcohol 70%	+4	Max. 1 week
Hydrolyze	1N HCl	+60	6–8 min.
Staining	Feulgen	+25	2 h.

cytokinin with particular reference to BA and GA_3. Subculturing of the shoots for multiple shoot production on the same medium induced multiple shoots. Though BA and GA_3 were known to promote shoot proliferation, they were proved to be better in the present study for *P. vera* L. (Fig. 1.A-B-C). For root induction, the basal portion of the cultured shoots was washed by shaking it in sterile water, and then dipping it into different concentrations of IBA solutions (0.5, 1, 1.5, 2.5 and 10 gr/l^{-1}) at different times (10, 20, 30, 40 and 50 s) with a control group. It was determined that the optimum dipping concentration and time is l gr/l^{-1} and 20 s for rooting studies conducted by dipping the basal-cut-ends of in vitro micropropagated shoots into a dense IBA solution (Table 1 and Table 2). The shoots dipped in IBA showed a high rate of rooting (90%) within 28 days (Fig. D) Significance was determined by an analysis of variance (ANOVA) and data presented in percentages were subjected to Chi-square (χ2) analysis wherever appropriate. In cytogenetic studies, the techniques for obtaining chromosome plates of *P.vera* L were developed for root tips propagated *in vitro* (Table 3). The conclusions obtained from the morphological analysis of the chromosome studies determined the chromosome complement as 2n = 28+Xy with metacentric, submetacentric, subtelocentric, and telocentric pairs (Table 4). The analysis of 50 somatic cells of *P.vera* L. indicated that the species exhibits 2n = 30 chromosomes (Fig. 2).

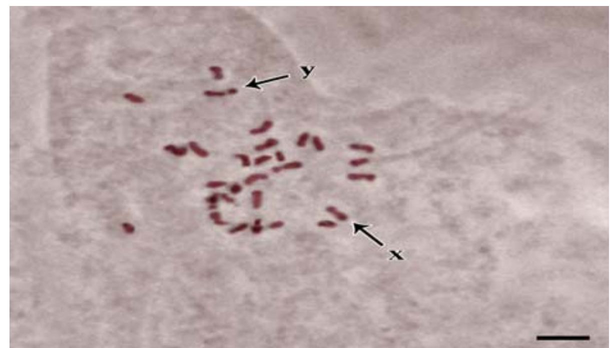


Fig 2. Metaphase plate at micropropagated of mature male trees of *P. vera* L. (bar: 38.64 µm)

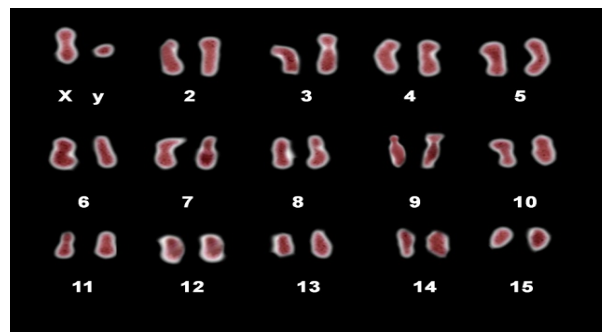


Fig 3. Karyogram of micropropagated mature male trees of *P. vera* L.

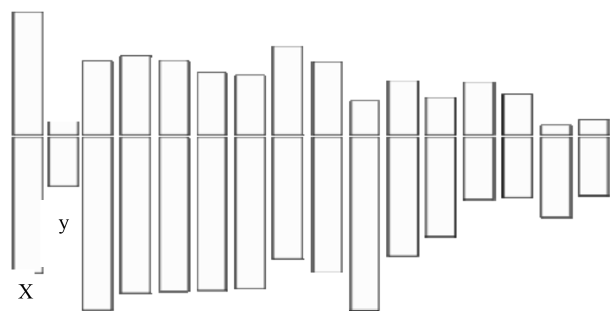


Fig 4. Idiogram of micropropagated mature male trees of *P. vera* L.

The karyotype formula for this species can be stated as: K(2n:28+Xy): 8sm+3m+2st+1t+Xy, a karyogram and a idiogram were prepared as a result of karyotype (Fig. 3. and Fig. 4.). Neither polyploidy nor satellite was detected on any of these chromosome plates. It was found that the biggest chromosome pairs on the complement have metacentric centromeres, and these chromosome pairs were heteromorph in the male metaphase plates with metacentric and subtelocentric centromeres. This evidence showed that they are the sex chromosomes for *P.vera* L. Even where sex is genetically determined, the mechanisms vary enormously, with clearly distinguishable sex chromosomes being very rare (Weibel et al., 1999; Volff and Schartl, 2001). This research describes a new system suitable for the karyotype analysis of *Pistacia*.

Discussions

In rooting stage, a wider survey of the existing literature suggests that BA is the most reliable and useful cytokinin for the shooting of the pistacia genus (Onay, 2000; Tilkat, 2006; Tilkat et al., 2008; Tilkat et. al., 2009 a,b). These workers

Table 4. Karyotype analysis mature male trees of *P. vera* L.(µm)

Chromosome pairs		Total length	Long arm length	Short arm length	Arm ratio	Sentromeric index	Relative length	Sentromer type
1	X	20,19±0,20	10,61±0,09	9,58±0,11	1,12±0,02	47,43±0,20	8,71±0,08	m
	Y	4,86±0,09	3,83±0,08	1,02±0,01	3,73±0,07	21,16±0,32	2,09±0,04	St
2		19,17±0,14	13,43±0,06	5,74±0,08	2,33±0,07	29,94±30,23	8,27±0,06	Sm
3		18,26±0,14	12,13±0,07	6,13±0,07	1,97±0,01	33,54±0,16	7,88±0,06	Sm
4		17,78±0,16	11,97±0,08	5,80±0,08	2,05±0,01	32,60±0,15	7,67±0,07	Sm
5		16,75±0,15	11,90±0,08	4,85±0,06	2,45±0,01	28,93±0,14	7,22±0,06	Sm
6		16,38±0,13	11,77±0,07	4,61±0,06	2,55±0,02	28,12±0,16	7,06±0,05	Sm
7		16,18±0,15	9,46±0,05	6,86±0,08	1,37±0,00	42,39±0,21	6,98±0,06	m
8		16,15±0,13	10,47±0,06	5,68±0,07	1,84±0,01	35,09±0,17	6,97±0,06	Sm
9		16,11±0,13	13,47±0,06	2,64±0,07	5,26±0,12	16,2±0,31	6,95±0,05	St
10		13,47±0,11	9,27±0,05	4,19±0,06	2,21±0,01	30,34±0,14	5,81±0,05	Sm
11		10,64±0,11	7,75±0,06	2,89±0,05	2,70±0,02	27,03±0,21	4,59±0,05	Sm
12		8,96±0,13	4,86±0,07	4,09±0,06	1,18±0,00	45,68±0,05	3,86±0,05	m
13		7,90±0,11	4,71±0,05	3,19±0,06	1,48±0,01	40,21±0,21	3,41±0,05	m
14		6,92±0,06	6,19±0,04	0,72±0,01	8,90±0,19	10,35±0,18	2,98±0,02	t
15		5,69±0,08	4,52±0,09	1,17±0,01	3,82±0,02	29,16±0,41	2,22±0,03	St
Total length of haploid complement: 231,59µm								

succeeded in their attempts for shoot proliferation by using BA plus GA_3. Al Barazi and Schwaba (1982), have reported that the dipping of pistachio under *in vivo* conditions resulted in rooting. Onay (2000), Ozden-Tokatlı et al., (2005) Tilkat (2006) and Tilkat et al., (2008) reported that IBA was more effective for rooting in the pistachio's culture. In these study the effect of various concentrations of auxin on the rooting of pistachio microshoots was investigated. In terms of chromosome number, our findings have also been cited by Fasihi- Harandi et al., (1996) Fasihi- Harandi and Behboodi (1997), Ila et al., (2003) and Ayaz and Namli (2009). Ayaz and Namli (2009) germinated seeds of the *P.vera* in *in vitro*, then obtained roots were pretreated with paradichlorobenzen and were fixed with acetic alcohol. After washing the roots were hydrolysed with 1N HCl and finally, were stained with feulgen. It was reported that according to the karyotype analysis in this study the diploid chromosome number was 2n=30 and the largest chromosome was median. Of the remaining chromosomes eight were submetacentric, three were metacentric, two were subtelocentric and one was telocentric. Apart from the largest chromosome pair (XX), the others exhibited similar findings in cocurrence with the present study. Hormaza and Herrero (1998), used RAPD techniques in finding the sex determination of the dioecious species, *P.vera* L. obtained band that was tightly linked to the gene(s) that control sex determination in pistachio. Kafkas et al., (2001) screened hundreds of primers in Pistacia in order to identify sex-specific polymorphisms, but found only a few putative sex-related bands. Ehsanpour et al., (2008) identified four female and male individual *Pistacia vera* cultivars (Akbari, Ahmad Aghaii, Fandoghi, Kaleh Ghochi) using nine Inter Simple Sequence Repeat (ISSR) primers. Two primers (AC)8CG and (AC)8TA were able to identify male and female plants by producing sex dependent DNA bands in female plants. Isolation of more plant sex-linked genes and their cytogenetic mapping with fluorescent in situ hybridisation (FISH) will lead to a better understanding of the processes driving sex chromosome evolution in the *Pistacia* genus. However, more research must be conducted in order to determine sex evolution. The most important point here is that the rooting and the karyotyping of mature male *P. vera* L. is a key step (a transition stage) to having adequate materials for use in further studies.

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