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Investigation of potential differences in the sex of siirt pistachio (*Pistacia vera* L. cv. Siirt) trees and saplings using morphological and physiological techniques

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Summary

Sex identification in *Pistacia* species is economically important for pistachio producers because their long juvenile period delays crop production and gains. Since there is no easy method to identify sex during the juvenile period of this plant, morphological and physiological methods are expected to help in sex identification at the juvenile stage of *Pistacia vera* L. cv. Siirt (siirt pistachio) to determine potential differences in the sex of siirt pistachio trees and saplings. In the present study, the physiological and morphological differences were compared between female and male trees. Sixteen saplings were grown in the same field and environmental conditions. We measured GSH, GSSG, GR enzyme activity, total soluble sugar and protein, proline, MDA, chlorophyll-a, chlorophyll-b, carotenoid, pH values, and stomatal density of the leaf samples randomly selected from the sixteen saplings, and five male and five female trees. While the average GSH, GSSG, and GR activity of male trees was 2.45, 0.66, and 9.72, respectively, it was 5.94, 1.54, and 5.53 in female trees. The stomatal density of female and male trees and saplings was determined as 8.33-12.33, 15.00-23.66, and 9.66-24.00, respectively. The pH value was measured between 4.83-5.40, 4.64-4.74, and 4.23-4.76, respectively, in female and male trees and saplings. According to the pH values, the acidity of saplings was higher than in male trees, whereas it was higher in male trees than in female trees. However, proline, malondialdehyde (MDA), total soluble sugar and protein, photosynthetic pigments, and carotenoid did not display any significant differences between female and male trees and saplings. Considering the results of GSH, GSSG, and GR enzyme activity and stomatal density, which differed significantly between trees and saplings, S7 showed similarity to female trees, whereas S13 showed similarity to male trees.

Keywords: Siirt pistachio, saplings, stomatal density, reduced glutathione, pH

Introduction

The technique to determine sex in economically important plants at juvenile stages is a very significant problem for producers, especially when all higher parental selections are of unknown sex. It would help to reduce the efforts of crop producers and cultivators in saving field space, time, and other useful resources that are wasted in continuing unwanted plants at the juvenile stage (Agrawal et al., 2007). Pistacia species are among the species with unknown sex at the juvenile stage.

The *Pistacia* genus is a part of the Anacardiace family, and this genus includes nine species and five subspecies (Al-Saghir and Porter, 2012). Pistacia species are usually drought-tolerant, dioecious resin-bearing shrubs or trees. *Pistacia vera* is a plant species in the *Pistacia* genus, grown in the southeast of Turkey, the south of Egypt and Saudi Arabia, and the east of Tajikistan, Afghanistan, Pakistan, and Nepal (Al-Saghir and Porter, 2012). It is mainly grown as a horticultural plant for pistachio nuts. The *Pistacia* genus of the Anacardiaceae family represents important crops whose kernels are consumed for their nutritional and sensorial qualities (Grace et al., 2016).

Sex identification is a major problem in dioecious plants, and the sex identification mechanism is not fully understood. Therefore, in nature, it is not easy to separate male or female individuals of such economically important plants just by their morphology prior to flowering. Flowering in dioecious plants takes five to ten years, depending on the species. *Pistacia* members have a juvenile period of approximately 5 years (Ferguson et al., 2005). To overcome this obstacle, all saplings must be grafted to convert female and male individuals at a certain rate. Grafting takes a long time, is demanding, and delays crop production and gains. If the sex of saplings produced from pistachio seeds can be identified, the grafting obligation of saplings can be removed in this case.

Several approaches have been tried to solve the problems associated with sex distinction. One of such approaches is developing a marker system to recognize sex at the juvenile stage of the plant. A number of marker types, such as morphological, physiological, biochemical, and molecular markers, have been developed and identified to a particular extent and have been shown to be useful in separating male from female plants in various dioecious plants (Heikrujam et al., 2015). Furthermore, a wide range of molecular markers have been identified to differentiate male and female plants (Putri et al., 2013), but they are very expensive, time-consuming, and not very useful to field researchers. Molecular markers are also not highly reliable. Therefore, the development of a reliable, cheap, and easy new method to distinguish between female and male plants using morphological and physiological techniques is needed. Some studies have been conducted to analyze mostly morphological and physiological similarities between female and male plants (Gaur et al., 2017). Reports regarding various physiological similarities in the photosynthetic activity, respiration rate, transpiration rate, water efficiency, and phenolic contents (Heikrujam et al., 2015), the specific activity of peroxidase (Bekheet et al., 2008), catalase, total soluble protein (Truta et al., 2002), GSH (Li et al., 2016), proline, chlorophyll a-b, carotenoid and total soluble sugar (El-Yazal, 2008) contents between male and female individuals have been widely published.

This study analyzed the differences in GSH, GSSG, GR enzyme activity, total soluble sugar and protein, proline, MDA, chlorophyll-a, chlorophyll-b, carotenoid, pH values, and stomatal density between female and male saplings grown in the same field and environmental conditions, mainly using the metabolite approach to provide a reference for a comprehensive understanding of morphological or physiological differences between female and male trees and saplings of siirt pistachio.

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Materials and methods

Plant material

Female and male trees were grown in the Siirt Pistachio Producers Association's garden (37°56'28"N 41°57'42" E). Saplings were obtained from the germinated siirt pistachio seeds in this garden. The age of female (named F1, F2, F3, F4, F5) and male (named M1, M2, M3, M4, M5) trees was about fifteen years, and saplings (named S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16) were six months old. Leaf samples, at least ten, were taken separately from trees and saplings during the leafy period in June 2020, and these leaves were crushed in liquid nitrogen and used for analysis in Batman University Biology Research Laboratory.

Determination of biochemical parameters

For pH measurement, fresh leaves were taken from each plant and divided into approximately 1 mm diameter disks and mixed with demineralized water in an Eppendorf tube (volume ratio 1:8). After the mixture was shaken at 250 rpm for 1 hour and centrifuged at 12879 g for 5 min, the pH of the supernatant was measured with a pH meter with a fine-tipped electrode (Cornelissen et al., 2011).

The chlorophyll (chl) and carotenoid contents were extracted using 80% acetone from the fresh leaves of plants. The absorbance of the extracts was measured with a spectrophotometer at 480, 663, and 645 nm and recorded. Chlorophyll a, chlorophyll b, and carotenoid contents were calculated as previously described (Arnon, 1949).

The total soluble sugar content was measured using the phenol-sulphuric acid method (Dubois et al., 1956). 100 mg of fresh leaf samples were homogenized in 2 ml of 80% ethanol in mortar. The homogenate was incubated for 30 minutes in a water bath at 75 °C and then centrifuged at 2236 g · 1 ml of the supernatant, 1 ml of 5% phenol, and 5 ml of concentrated sulphuric acid (H_2SO_4) were added to the test tubes and mixed by vortexing. The absorbance of the reaction mixture was read by a spectrophotometer at a wavelength λ = 490 nm, and the total soluble sugar content was expressed as mg g^{-1} dry weight.

The proline content was determined spectrophotometrically by the acid-ninhydrin method (Bates et al., 1973; Ghoulam et al., 2002). 100 mg of the plant sample was homogenized with 2 ml of 40% methanol. 1 ml of the homogenate was taken, 1 ml glacial acetic acid was added, and then 6 M phosphoric acid (3:2 v/v) was added to this mixture. 25 mg of ninhydrin was added to the prepared reaction mixture, and the mixture was incubated at 100 °C for 1 hour. The tubes containing the reaction mixture were then cooled, 5 ml of toluene was added, and two different phase formations were observed after proper mixing. The absorbance of the upper phase was measured spectrophotometrically at a 528 nm wavelength to determine the proline content. The proline content was determined with the graph using the L-proline standard.

The MDA content (malondialdehyde), which is the measure of lipid peroxidation, was found according to the method described by Ohkawa et al. (1979). 0.1 g of plant samples were homogenized by adding 2 ml of 5% trichloroacetic acid (TCA). The prepared homogenate was centrifuged at 25 °C and 12879 g for 20 minutes. After this procedure, 0.4 µl of the supernatant was taken, and 0.4 µl of 20% TCA solution containing 0.5% thiobarbituric acid (TBA) was added. The reaction mixture was kept in a hot water bath at 95 °C for 1 hour and then placed in an ice bath to stop the reaction. In the next step, the reaction mixture was centrifuged at 8944 g for 10 minutes, and the absorbance values of the mixtures were read against blind at a 532 nm wavelength with a UV-Vis spectrophotometer. The MDA content was calculated using the equation obtained from the standard 1,1,3,3-tetraethoxypropane (MDA) graph.

Determination of enzyme activities

To determine the GR enzyme activity and total soluble protein content, 1 g of fresh leaf samples was taken. Leaf samples were homogenized with a chilled pestle and mortar in 5 mL of extraction buffer (0.1 M phosphate buffer, pH 7.0), containing 10 mM KCl, 1 mM $MgCl_2$, 10 mM Na_2EDTA, and 1% polyvinyl poly pyrrolidone (PVPP), and centrifuged. After centrifugation of the homogenate, the supernatant phase was taken for protein content determination and enzyme assay. The protein content was found using a standard curve prepared with Bovine Serum Albumin (BSA) and expressed as µg g^{-1} fresh weight (Bradford, 1976). Of the fresh plant sample, 0.5 g was homogenized in 100 mM phosphate buffer (pH 7.0) and centrifuged at +4 °C and 17530 g for 20 minutes. 20 µl of the supernatant was taken, 480 µl of distilled water and 5000 µl of Bradford solution were added to it, respectively, and the absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 595 nm. For GR activity, 120 mM K-PO_4 buffer, 15 µM Na_2EDTA, 65 µM GSSG, 9.6 mM NADPH, and enzyme extract were measured at 340 nm. The enzyme activity was computed using the molar extinction coefficient of NADH 6.23 mM^{-1} cm^{-1} and expressed as 1 mmol ml^{-1} GSSG (Goldberg and Spooner, 1983).

For GSH and GSSG extraction, 0.5 g of fresh leaves were weighed and extracted in 5 ml of ice-cold 5% metaphosphoric acid containing 1 mM EDTA. The homogenates were centrifuged at 12879 g for 15 min at a temperature of 4 °C. Total glutathione (GSH + GSSG) was measured using the reaction mixture, containing 0.25 M K-P buffer (pH 7.5), 200 µM NADPH, 600 µM DTNB, 25 µl extract, and 0.3 U GR. The reaction mixture was incubated at 37 °C for 15 minutes, and the change in absorbance at 412 nm was measured. The oxidized glutathione (GSSG) content was measured by the reaction of 2-vinyl pyridine to remove GSH. Standard curves were drawn for the final calculation, and calculations were made using the obtained equation. The GSH content was obtained after subtracting GSSG from total glutathione (Sahoo et al., 2017).

Microscopy

Fresh leaves of female and male trees and saplings were collected, washed, and dried before removing epidermal layers. The epidermal layer method was applied according to TIEE (Grant and Vatnick, 2004). Stomata were counted in the microscopic (Leica DM 4000 B, X 40 objectives) field image on the lower epidermal layer of each leaf in 3 different field images. The image size was 0.246×0.185 cm, and the image area was 0.04551 cm^2 or 4.551 mm^2. Stomata, at least 2/3 of which were in the image area, were counted.

Data replication and statistical analysis

Each experiment was repeated twice with at least 20 explants. The shoot number per explant, shoot length, shoot proliferation percentage, and rooting percentage (%) were calculated after four weeks from the beginning of the experimental stage. The data were analyzed by employing the standard analysis of variance (one-way ANOVA) procedure. The mean separation was checked by Duncan's multiple range test. The significance level was set at $P< 0.05$. The results were expressed with standard error (SE). Statistical analysis was performed by using the SPSS version 16.0 for Windows.

Results

While measuring the pH values of female and male trees and saplings, we found that the pH value of female trees (between 4.83 and 5.40) was always higher compared to male trees (between 4.64 and 4.74) (Tab. 1). Moreover, according to pH values, it was observed that trees generally had higher pH values than saplings. The pH value of saplings varied between 4.23 and 4.76. According to these re-

Tab. 1: pH value, Proline, MDA and Protein contents in siirt pistachio leaves.

	pH	Proline (mmol g^{-1} FW)	MDA (µmol g^{-1} FW)	Total soluble Protein (mg g^{-1} FW)
F1	5.40±0.01^{a}	0.67±0.06^{fgh}	12.65±0.40^{hij}	2.38±0.39^{hij}
F2	5.09±0.02^{b}	0.58±0.06^{ijkl}	11.05±1.44^{lm}	2.55±0.06^{g}
F3	4.83±0.00^{c}	0.56±0.04^{jkl}	8.77±0.73^{no}	2.56±0.11^{g}
F4	4.87±0.02^{c}	0.75±0.04^{f}	10.43±0.67^{m}	2.52±0.10^{gh}
F5	4.84±0.01^{c}	0.50±0.04^{l}	11.36±0.30^{kl}	1.97±0.01^{k}
M1	4.64±0.08^{f}	0.93±0.08^{de}	9.39±0.41^{n}	2.75±0.12^{f}
M2	4.68±0.01^{e}	0.87±0.08^{e}	15.13±0.79^{c}	2.42±0.06^{ghi}
M3	4.73±0.03^{d}	0.66±0.04^{ghi}	8.65±0.02^{no}	3.16±0.14^{c}
M4	4.74±0.01^{d}	0.96±0.08^{d}	8.29±0.51^{o}	2.24±0.10^{j}
M5	4.71±0.04^{fg}	0.61±0.02^{hij}	10.87±0.47^{lm}	2.49±0.00^{gh}
S1	4.42±0.01^{i}	0.52±0.06^{kl}	13.44±0.24^{fgh}	4.19±0.16^{a}
S2	4.50±0.02^{h}	0.96±0.08^{d}	14.73±0.15^{cd}	2.46±0.20^{gh}
S3	4.59±0.02^{g}	0.70±0.02^{fg}	12.13±0.22^{ijk}	1.97±0.01^{k}
S4	4.23±0.04^{n}	0.60±0.04^{hijk}	15.01±0.18^{cd}	2.27±0.03^{ij}
S5	4.26±0.02^{mn}	0.64±0.06^{ghij}	11.10±0.39^{lm}	1.38±0.09^{n}
S6	4.47±0.01^{h}	1.69±0.06^{b}	13.88±0.46^{ef}	2.27±0.17^{ij}
S7	4.33±0.04^{jkl}	1.95±0.04^{a}	13.00±1.20^{gh}	3.24±0.10^{c}
S8	4.29±0.01^{lm}	1.16±0.02^{c}	12.91±0.86^{ghi}	1.68±0.05^{lm}
S9	4.48±0.05^{h}	0.75±0.04^{f}	14.63±0.83^{cde}	1.56±0.02^{m}
S10	4.29±0.04^{lm}	0.61±0.02^{hij}	8.24±1.09^{o}	1.00±0.03^{o}
S11	4.35±0.01^{jk}	0.93±0.04^{de}	20.58±0.70^{a}	2.97±0.12^{de}
S12	4.31±0.01^{kl}	0.70±0.02^{fg}	13.53±0.23^{fg}	3.12±0.02^{cd}
S13	4.76±0.01^{d}	1.01±0.06^{d}	9.08±0.75^{no}	2.90±0.02^{e}
S14	4.42±0.00^{i}	0.64±0.06^{ghij}	11.95±0.25^{jk}	1.73±0.03^{l}
S15	4.37±0.01^{j}	1.97±0.10^{a}	14.25±0.11^{def}	3.20±0.15^{c}
S16	4.33±0.00^{jkl}	0.58±0.06^{ijkl}	18.37±0.80^{b}	3.94±0.09^{b}

Values expressed as means ± S.D. of three parallel measurements. F: Female, M: Male, S: Sapling, FW: Fresh Weight.
*Means followed by the different lowercase letter in the column of each explant are significantly different at P< 0.05 according to the Duncan's Multiple Range Test.

Tab. 2: Glutathione reductase (GR) enzyme activity, reduced glutathione (GSH), oxidized glutathione (GSSG) content and GSH/GSSG in siirt pistachio leaves.

	GR (Unit mg^{-1} protein)	GSH (µmol g^{-1} FW)	GSSG (µmol g^{-1} FW)	GSH/GSSG
F1	5.34±0.39^{jk}	4.92±0.17^{k}	1.52±0.36^{g}	3.30±0.66^{bcdefg}
F2	6.40±0.29^{hi}	7.76±0.44^{fg}	1.89±0.32^{ef}	4.12±0.46^{bc}
F3	4.06±0.06^{l}	5.43±0.53^{jk}	1.40±0.20^{gh}	3.88±0.18^{bcde}
F4	6.22±0.26^{hij}	4.92±0.35^{k}	0.98±0.12^{ij}	5.07±1.03^{a}
F5	5.66±0.64^{ijk}	6.69±0.89^{h}	1.92±0.52^{ef}	3.67±1.05^{bcdef}
M1	12.56±0.62^{c}	1.14±0.17^{n}	0.49±0.13^{l}	2.36±0.27^{hi}
M2	11.67±1.79^{cde}	2.84±0.26^{m}	0.81±0.07^{ijkl}	3.48±0.01^{bcdef}
M3	6.55±0.03^{hi}	3.73±0.62^{l}	0.73±0.05^{jkl}	5.10±1.23^{a}
M4	6.88±1.14^{h}	1.52±0.17^{n}	0.51±0.07^{kl}	2.96±0.09^{fgh}
M5	10.98±0.55^{e}	3.03±0.17^{m}	0.80±0.09^{ijkl}	3.79±0.65^{bcdef}
S1	6.30±0.89^{hij}	6.44±0.35^{hi}	1.61±0.06^{fg}	3.98±0.05^{bcd}
S2	6.99±1.12^{gh}	7.51±0.44^{g}	2.08±0.21^{de}	3.62±0.59^{bcdef}
S3	12.36±0.79^{c}	8.20±0.35^{ef}	2.09±0.40^{de}	3.96±0.60^{bcd}
S4	7.85±0.31^{fg}	11.22±0.17^{b}	3.46±0.04^{a}	3.24±0.05^{cdefg}
S5	15.78±0.24^{b}	3.10±0.26^{m}	0.96±0.04^{ij}	3.21±0.41^{defg}
S6	6.21±0.44^{hij}	5.74±0.26^{j}	2.31±0.32^{bcd}	2.50±0.24^{ghi}
S7	6.19±0.28^{hij}	5.87±0.08^{ij}	1.42±0.17^{gh}	4.16±0.57^{b}
S8	6.21±0.91^{hij}	2.53±0.53^{m}	0.77±0.02^{ijkl}	3.29±0.79^{bcdefg}
S9	11.03±0.18^{de}	8.58±0.71^{de}	2.48±0.41^{bc}	3.53±0.59^{bcdef}
S10	16.26±1.42^{b}	3.73±0.26^{l}	1.11±0.14^{hi}	3.56±1.16^{bcdef}
S11	8.60±0.05^{f}	6.88±0.44^{h}	2.35±0.21^{bcd}	2.93±0.46^{fgh}
S12	6.84±1.50^{h}	5.36±0.62^{jk}	2.52±0.02^{b}	2.12±0.21^{i}
S13	11.94±0.35^{cd}	2.84±0.26^{m}	0.85±0.08^{ijk}	3.36±0.42^{bcdefg}
S14	17.99±0.74^{a}	8.96±0.53^{d}	2.18±0.04^{cde}	4.10±0.23^{bcd}
S15	5.73±0.04^{ijk}	10.03±1.33^{c}	3.33±0.32^{a}	3.05±0.59^{efgh}
S16	4.90±0.45^{kl}	12.04±0.62^{a}	3.16±0.05^{a}	3.80±0.26^{bcdef}

Values expressed as means ± S.D. of three parallel measurements. F: Female, M: Male, S: Sapling, FW: Fresh Weight.
*Means followed by the different lowercase letter in the column of each explant are significantly different at P< 0.05 according to the Duncan's Multiple Range Test.

sults, the leaf extracts of saplings displayed higher acidity than trees. According to the acidity values, the samples can be ranked as sapling >male>female.

While measuring the GSH values of female and male trees and saplings, we found that the GSH value of female trees (between 4.92 and 7.76) was always higher compared to male trees (between 1.44 and 3.73). Furthermore, the average GSH content of male and female trees was 2.45 and 5.94, respectively. The GSH value of saplings varied between 2.53 and 12.04. Considering the GSH content results, it was determined that saplings S5, S8, and S13 showed similarity to male trees and saplings S1, S6, S7, and S12 showed similarity to female trees (Tab. 2). While measuring the GSSG values of female and male trees and saplings, we found that the GSSG value of female trees (between 0.98 and 1.92) was always higher compared to male trees (between 0.49 and 0.81). When the GSSG content was examined, it was seen that the average GSSG content of male and female trees was 0.66 and 1.54, respectively. The GSSG value of saplings varied between 0.77 and 3.46. According to these GSSG values, saplings S1, S2, S3, and S7 showed similarity to female trees, and saplings S5, S8, and S13 showed similarity to male trees (Tab. 2) According to the GR enzyme activity of female and male trees and saplings, we found that the GR enzyme activity of female trees (between 4.06 and 6.40) was always lower compared to male trees (between 6.55 and 12.56). The average contents of GR enzyme activity results of male and female trees were 9.72 and 5.53, respectively. Furthermore, GR enzyme activity of saplings varied between 4.90 and 17.99. Concerning the GR enzyme activity results, saplings S6, S7, S8, S15, and S16 showed similarity to female trees, and saplings S9 and S13 showed similarity to male trees.

The leaf stomatal density of male trees, per 4.551 mm^2, was found to be approximately two times higher than that of female trees (Tab. 3, Fig. 1). Furthermore, it was observed that the leaf stomatal size of male trees was significantly greater than that of female trees (Fig. 1). The stomatal density of female and male trees and saplings was determined between 8.33-12.33, 15.00-23.66, and 9.66-24.00, respectively in the microscopic area (4.551 mm^2) (Tab. 3). Hence, it can be predicted that, according to the stomatal density of saplings, S1, S6, S7, S8, S14, and S16 displayed similarity to female trees and saplings S2, S3, S4, S5, S9, S10, S11, S12, and S13 showed similarity to male trees (Tab. 3, Fig. 1).

The total soluble sugar and protein, proline, MDA, photosynthetic pigments, and carotenoid contents displayed no significant differences between male and female trees and saplings of pistachio plants (Tab. 1, 3).

The experimental results of this study showed that male and female *Pistacia* plants had significant distinctions in pH, stomatal density, GSH, GSSG, and GR enzyme activity values. These results have a potential for some saplings to separate as female and male. According to significantly different GSH, GSSG, and GR enzyme activities and stomatal density, it was found that sapling S7 displayed similarity to female trees, and S13 showed similarity to male trees.

Tab. 3: Stomatal density, Total soluble sugar and photosynthetic pigment contents in siirt pistachio leaves.

	Stomatal density	Total soluble sugar (mg g^{-1} FW)	Chlorophyll a (mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Carotenoid (µg g^{-1} FW)
F1	8.33 ± 0.57^{k}	1.19 ± 0.05^{ijk}	0.54 ± 0.03^{gh}	0.22 ± 0.01^{defg}	10.01 ± 0.16^{f}
F2	9.33 ± 0.57^{jk}	1.05 ± 0.06^{kl}	0.58 ± 0.01^{f}	0.31 ± 0.02^{abc}	11.35 ± 0.20^{d}
F3	9.00 ± 0.00^{jk}	1.11 ± 0.08^{kl}	0.61 ± 0.01^{e}	0.26 ± 0.05^{bcdef}	11.16 ± 0.58^{de}
F4	11.33 ± 1.52^{hijk}	1.05 ± 0.14^{kl}	0.50 ± 0.01^{ij}	0.22 ± 0.01^{defg}	9.65 ± 0.09^{fghi}
F5	12.33 ± 1.52^{fghij}	1.02 ± 0.06^{kl}	0.42 ± 0.01^{n}	0.18 ± 0.04^{g}	8.35 ± 0.27^{l}
M1	16.00 ± 1.73^{def}	0.61 ± 0.01^{m}	0.41 ± 0.03^{n}	0.20 ± 0.09^{efg}	9.71 ± 0.43^{fgh}
M2	15.00 ± 0.00^{efgh}	1.16 ± 0.04^{jk}	0.45 ± 0.06^{klm}	0.22 ± 0.05^{defg}	9.78 ± 0.23^{fgh}
M3	21.66 ± 0.57^{ab}	0.88 ± 0.10^{l}	0.41 ± 0.02^{n}	0.23±0.09defg	8.95 ± 0.17^{jk}
M4	22.66 ± 0.57^{ab}	0.89 ± 0.05^{l}	0.48 ± 0.07^{jk}	0.19 ± 0.02^{fg}	9.42 ± 0.10^{ghij}
M5	23.66 ± 0.57^{a}	2.70 ± 0.28^{ab}	0.30 ± 0.04^{o}	0.11 ± 0.01^{h}	6.63 ± 0.55^{m}
S1	12.33 ± 2.08^{fghij}	1.39 ± 0.13^{hi}	0.54 ± 0.06^{g}	0.28 ± 0.04^{bcde}	11.25 ± 0.58^{d}
S2	15.33 ± 2.08^{efg}	1.13 ± 0.09^{k}	0.45 ± 0.01^{klm}	0.21 ± 0.02^{defg}	9.11 ± 0.21^{ij}
S3	20.66 ± 2.88^{abc}	1.56 ± 0.03^{gh}	0.63 ± 0.01^{de}	0.28 ± 0.07^{bcde}	12.08 ± 0.78^{c}
S4	18.00 ± 2.00^{cde}	2.47 ± 0.27^{c}	0.68 ± 0.01^{bc}	0.31 ± 0.04^{ab}	13.09 ± 0.16^{b}
S5	19.00 ± 2.64^{bcd}	2.51 ± 0.39^{bc}	0.49 ± 0.02^{ij}	0.25 ± 0.02^{bcdefg}	10.16 ± 0.24^{f}
S6	10.66 ± 2.51^{ijk}	2.76 ± 0.05^{a}	0.46 ± 0.02^{k}	0.19 ± 0.01^{fg}	9.82 ± 0.62^{fg}
S7	13.66 ± 2.08^{fghi}	1.94 ± 0.11^{e}	0.43 ± 0.02^{lmn}	0.10 ± 0.01^{h}	8.36 ± 0.09^{l}
S8	12.00 ± 1.73^{ghijk}	1.62 ± 0.03^{fg}	0.64 ± 0.04^{d}	0.29 ± 0.04^{abcd}	11.89 ± 0.60^{c}
S9	23.33 ± 0.57^{a}	1.10 ± 0.06^{kl}	0.46 ± 0.08^{kl}	0.25 ± 0.07^{bcdefg}	9.24 ± 0.38^{hij}
S10	24.00 ± 4.35^{a}	1.21 ± 0.06^{ijk}	0.66 ± 0.03^{cd}	0.31 ± 0.06^{abc}	12.39 ± 0.81^{c}
S11	22.00 ± 2.64^{ab}	1.99 ± 0.10^{e}	0.70 ± 0.06^{b}	0.26 ± 0.04^{bcdef}	13.58 ± 0.51^{b}
S12	21.66 ± 3.51^{ab}	2.26 ± 0.12^{d}	0.51 ± 0.02^{hi}	0.23 ± 0.02^{cdefg}	10.69 ± 0.42^{e}
S13	20.66 ± 1.15^{abc}	2.78 ± 0.12^{a}	1.13 ± 0.01^{a}	0.36 ± 0.01^{a}	19.34 ± 0.53^{a}
S14	10.66 ± 2.51^{ijk}	2.48 ± 0.36^{c}	0.43 ± 0.01^{mn}	0.20 ± 0.06^{efg}	8.45 ± 0.48^{kl}
S15	14.33 ± 1.52^{fghi}	1.38 ± 0.03^{hij}	0.55 ± 0.03^{g}	0.22 ± 0.05^{defg}	11.02 ± 0.59^{de}
S16	9.66 ± 1.52^{jk}	1.79 ± 0.14^{ef}	0.30 ± 0.01^{o}	0.08 ± 0.01^{h}	5.81 ± 0.24^{n}

Values expressed as means ± S.D. of three parallel measurements. F: Female, M: Male, S: Sapling, FW: Fresh Weight.
*Means followed by the different lowercase letter in the column of each explant are significantly different at $P< 0.05$ according to the Duncan's Multiple Range Test.

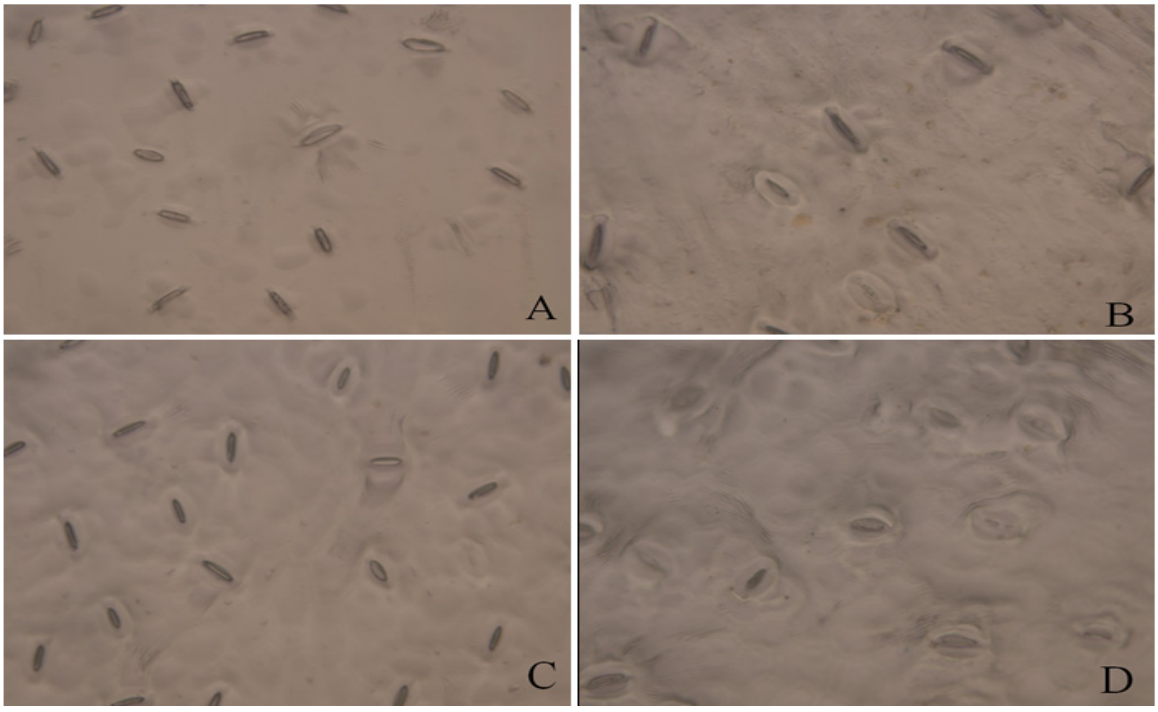

Fig. 1: Image of microscopic field of (Each microscopic field of view is 4.551 mm^2) lower epidermal layer stomata siirt pistachio trees leaf A; Male siirt pistachio trees leaf. B; Female siirt pistachio trees leaf. C; Any siirt pistachio saplings leaf (Estimated male siirt pistachio saplings). D; Any siirt pistachio saplings leaf (Estimated female siirt pistachio saplings).

Discussion

Distinctions in physiology have been documented between male and female plants of some species (Espirito-Santo et al., 2003). The study by El-Yazal (2008) found significant differences in the chemical constituents of chlorophyll a-b, total caroteniods, total carbohydrates, free proline, and total soluble protein between male and female date palms. Moreover, according to Zielewicz et al. (2020), the content of chlorophyll a-b and carotenoids in plants is very unstable and depends on numerous factors, and it was accepted that the content of pigments in plants would vary depending on the habitat conditions of the same species. Khukhunaishvili and Dzhokhadze (2006) identified differences in total soluble protein in the sex determination of *Actinidia chinensis* and *Actinidia kolomikta*. Li et al. (2016) reported that glucose and fructose contents in female *Pistacia* plants were two times higher than in male plants due to stronger sugar signaling activity in female plants than in male plants. Additionally, the study by Ma et al. (2012) demonstrated that the soluble protein and soluble sugar contents of male trees were higher than the leaves of female trees during the development period. Furthermore, it is known that the proline and MDA contents in plants increase and vary depending on the variability of environmental conditions (Ding et al., 2023). Some studies have reported that the proline and MDA contents differ between male and female plants (Gao et al., 2022). In our study, no significant differences were observed in terms of total soluble protein, total soluble sugar, proline, chlorophyll a-b, carotenoid, and MDA content between female and male trees and saplings.

Zhao et al. (1993) reported that the higher GSH content in female *Pistacia* plants than in male plants ensured the seed maturation and late aging of female plants. In another study, Li et al. (2016) stated that the GSH content in female leaves of *P. chinensis* was approximately five times higher than in male leaves of *P. chinensis,* and the GSSG content of female leaves of *P. chinensis* was almost two times higher than in male leaves of *P. chinensis*. The results of our study are consistent with the findings obtained by Zhao et al. (1993) and Li et al. (2016), who noted that the GSH and GSSG contents of female leaves in their study were at least two times higher than male leaves. The GSH and GSSG contents of female leaves in our study were almost two times higher than male leaves. Glutathione reductase (GR) represents an enzymatic antioxidant converting oxidized glutathione (GSSG) into reduced glutathione (GSH) through the ascorbate-glutathione cycle (Madhu et al., 2023). In our study, GR enzyme activity differed significantly between female and male trees, and some saplings exhibited similarity to either female or male trees. The results of the researchers (Zhu et al., 2019; Liu et al., 2021), who reported differences in GR enzyme activity between female and male trees, are parallel to our research findings.

Gaur et al. (2017) conducted a morphological study in *Commiphora wightii* plant and indicated that stomatal density on both sides of leaves was significantly higher in male plants compared to female plants. Moreover, Guangxiu et al. (2009) indicated that although the difference was insignificant, males had slightly higher stomatal densities than females in *Hippophate rhamnoides*. On the other hand, Dawson and Ehleringer (1993) determined that females of *Acer negundo* exhibited higher stomatal densities than males. In the current study, we found that stomatal density differed significantly between females and males. The stomatal density of males was nearly two times higher than that of females in the microscopic area (per 4.551 mm^2). The differences in stomatal density can be easily used as an indicator to separate female and male trees, and these stomatal differences of stomatal density can be used to separate saplings into two groups as female saplings and male saplings.

The pH parameter could not be discussed since there is no research on the relationship between pH values and sex differences in any plant. According to the pH values, trees can be easily separated as female and male because, while the pH value of female is 4.83-5.40 male is 4.64-4.74. The average pH value of saplings can be used to separate trees from saplings because the average pH value of saplings was 4.4, while the pH values of trees were 4.85.

Conclusion

In our study, potential similarities and differences between the sex of siirt pistachio (*Pistacia vera* L. cv. Siirt) trees and saplings were investigated by using simple measurement methods such as pH and stomatal density, together with some physiological analyses. The experimental results of this study showed that male and female *Pistacia* plants had significant distinctions in pH, stomatal density, GSH, GSSG, and GR enzyme activity values. Based on these differences, the sex of some saplings was estimated. Upon comparing the method of measuring pH and stomatal density to the method of determining GSH and GSSG, the method of measuring pH and stomatal density can be a very easy, reliable, and economical method to separate saplings from trees or female saplings or male saplings from each other grown under the same environmental conditions. In order to develop a more reliable marker for sex determination of *Pistacia* species, detailed research on pH and stomatal density parameters should be done by using a lot more plant samples. Therefore, the development of a pH or stomatal density indicator may be an important strategy in future studies in the sex determination of juvenile *Pistacia* species. In addition, our research will contribute to the literature for the sex determination of *Pistacia* species in the juvenile period and will guide to the researches to be done on this subject.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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