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Optimizing the relative water protection (RWP) as novel approach for monitoring drought tolerance in Iranian pistachio cultivars using graphical analysis

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Abstract

Pistachio (*Pistacia vera* L.) is the most important agricultural crop in arid and semi-arid regions of Iran. In order to optimize and evaluate the ability of relative water protection (RWP) for screening drought-tolerant pistachio cultivars, twenty-one cultivars with wide range of tolerance to drought stress were collected from across the orchards of Rafsanjan (Iran's center of pistachio cultivation) and were used in a randomized complete block design with three replications under two environmental conditions (normal and drought stress) in 2011-2012. Four different withering times (4h, 6h, 8h and 10h) for measuring RWP were compared to determine the best time for screening tolerant cultivars. The results showed that the RWP increased significantly ($P < 0.05$) under drought stress, but the increase was not significant in susceptible cultivars. The highest RWP was observed in tolerant cultivars and the lowest rate was detected in susceptible. The visualizing graphics of different statistical methods revealed that the diversity among the cultivars was increased when the withering time to measure RWP was longer (4h to 10h). Three groups of cultivars including tolerant, intermediate and susceptible were not clearly separated from each other at the times 4h and 6h. As a result, the measuring RWP at these withering times are not reliable to identify drought-tolerant pistachio cultivars. The time 8h was suitable to separate drought tolerant cultivars from susceptible, but this time was not able to screen cultivars with high tolerance from intermediate under drought condition. The findings adopted the RWP measured at the withering time 10h as ideal time for classification and identification of drought tolerant cultivars. In the present study, Kale Ghoochi 1 and Ancient Badami were selected, respectively, as the most tolerant and susceptible pistachio cultivars.

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Introduction

Pistachio (*Pistacia vera* L.) is one of the most popular nuts for people in the world. It contains only 3-4 calories per nut, and offer more than 30 different vitamins, minerals and beneficial phytonutrients making it a great dry fruit (Phillips *et al*., 2005; Siahnouri *et al*., 2013). The genus Pistachio belonged to family Anacardiaceae is a xerophytes plant and Iran is one of its origin and diversity centers (Arzani *et al*., 2013). The pistachio industry has played a major role for the Iranian economy being one of the biggest non-oil exports in the country (Barbe *et al*., 2011). Rafsanjan is one of the world's more important pistachio production areas which are located in the north-west of Kerman Province in the south-east of Iran. Pistachio orchards in Rafsanjan are irrigated during dry and stable weather conditions. The orchards are mostly homogeneous—only one cultivar is used—and they are pruned to have a globular, dense and regular canopy (Bagheri *et al*., 2012). Pistachio is a desert plant and has high tolerance to drought. Sepaskhah and Maftoun (1981) also demonstrated that pistachio has wide genotypic variability for drought and salt tolerance. But, it does not mean that pistachio trees require less water for optimal performance. The drought tolerance of the pistachio refers to its ability to survive under severe water stress conditions (Goldhamer, 1995; Sepaskhah and Karimi-Goghari, 2005). Increased establishment of irrigated pistachio orchards during the last two decades in Iran has decreased the availability of underground water resources and prolonged drought periods are the major concern for the pistachio producers (Bagheri *et al*., 2012). Thus, improvement of pistachio production for drought tolerance is one of the most important objectives in plant breeding programs for these regions. To achieve high yield and yield stability through breeding, breeders have to select high yielding pistachio cultivars with significantly improved tolerances to drought and salinity. Challenges then arise from the fact that high yield, drought tolerance and salt tolerance are all complex traits controlled by polygenes, possible negative associations of pistachio drought or salt with high yield, and different genetic and physiological mechanisms of the same traits at different developmental stages (Goldhamer, 1995).

Drought is one of major factors among abiotic stresses that limit plant performance, growth and productivity. The changes in the climatic conditions all over the world under the influence of global warming have recently made the situation more serious (Golestani and Assad, 1998; Hasheminasab *et al*., 2013). It induces many morphological, physiological, biochemical and molecular responses on plants, such as stomatal closure and reduced transpiration rates which contribute towards adaptation to such unavoidable environmental constraints (Sairam, 1994; Mohamed and Ismail, 2009). Although plant yield is the principle selection index used under drought stress conditions, breeding for drought tolerance by selecting solely for yield may not be successful, because the heritability of grain yield under drought conditions is controlled at independent genetic loci. Thus, plant breeders have always looking appropriate and repeatable indicators to screen germplasms for drought tolerance (Zobel *et al*., 1988; Hasheminasab *et al*., 2014). Physiologists have often suggested that the detection and selection of physiological traits related to plant water status are reliable methods to breeding for higher yield, and could be a valuable strategy for use in conjunction with normal methods of plant breeding (El Jaafari *et al*., 1993; Blum, 2005). Relative water protection (RWP) is an important physiological index in assessing the degree of water stress. It is indicating plant water status related to water stress as well as reflecting the metabolic activity in tissues. RWP is also among the main physiological criteria that influence plant water relations and have been using for screening drought tolerant genotypes (Hasheminasab *et al*., 2012a). Although Iran is one of the main diversity centers and origins of pistachios in the world, there are few works to identify Iranian drought-tolerant pistachio cultivars. Therefore, the main objectives of the present study were to optimize and develop the RWP as new selection index for screening drought tolerant cultivars in pistachio.

Materials and methods

Plant materials and experimental conditions

In order to evaluate and optimize the RWP for screening drought-tolerant pistachio (*Pistacia vera* L.) cultivars, twenty-one cultivars with wide range of tolerance to drought stress listed in Table 1 were collected from across the orchards of Rafsanjan (It is Iran's center of pistachio cultivation, Fig. 1) and were used in a randomized complete block design with three replications under two different environments (normal and water stress) at the Experimental Orchard in the City of Rafsanjan, Kerman, Iran (30° 24′ 24″ N latitude, 55° 59′ 38″ E longitude and 1469 m altitude) during 2011-2012. Climate in this region is classified as arid and semi-arid with mean annual rainfall of 100 mm and the annual temperature range is between –17°C to 42°C. Soil of the Experimental Orchard was clay-loam texture. For measurement of RWP, pinnately compound leaves of all cultivars at the nut filling stage were harvested and weighed.

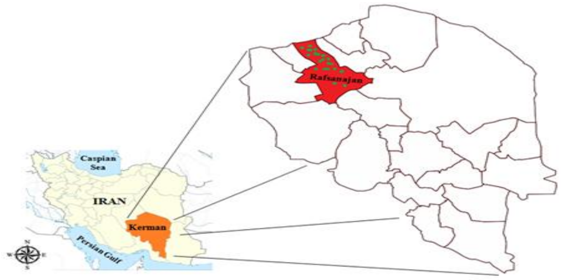

Fig. 1. The geographical locations of twenty-one pistachio cultivars collected in this study.

Relative water protection (RWP)

To determine the ideal withering time for measuring RWP, four different withering times were compared to identify drought-tolerant pistachio cultivars. RWP was calculated using the formula suggested by Hasheminasab *et al.* (2012a). Ten randomly selected pinnately compound leaves were taken and weighed for fresh weight (F_W). The leaves were then wilted at 25°C for 4, 6, 8 and 10h (This time can be different for various plant species.) and weighed again, respectively (Withering weight, W_W). Finally, the samples were oven dried at 70°C for 72h and reweighed (Dry weight, D_W). This index is indeed the proportion of water that is protected and not evaporated from the leaves after drying.

$$RWP = \frac{W_W - D_W}{F_W - D_W}$$

Statistical analysis of data

The measured data of RWP across two environmental conditions were analyzed by the statistical methods including descriptive statistics, principal component analysis (PCA), biplot analysis, scatter plot, discriminant analysis and canonical discriminant functions analysis using SPSS software packages 16.0 (SPSS, 2007), Minitab version 14 and Microsoft Office Excel (2007).

Results and discussion

Fig. 2 demonstrated four different methods to measure relative water protection (RWP) as new physiological trait related to plant water status for screening drought-tolerant pistachio cultivars. The results showed that considerable variations among pistachio cultivars for RWP were observed when grown under normal and drought stress conditions. The genetic variability of these cultivars in response to water deficit was indicated by the results could help in identifying possible drought -tolerant cultivars and also suitable indicators for screening these cultivars (Razi and Assad, 1999; Hasheminasab *et al.*, 2012a; Farshadfar *et al.*, 2013). As seen in Fig. 2a-d, the diversity among the cultivars was increased when the withering time was longer (4h to 10h). Three groups of cultivars including tolerant, intermediate and susceptible were not clearly separated from each other at the times 4h and 6h. As a result, the measuring RWP at these withering times are not reliable to identify tolerant cultivars. According to Fig. 2c, the time 8h was suitable to separate drought tolerant cultivars from susceptible, but this time was not able to screen cultivars with high tolerance from intermediate under drought condition. The results revealed that measurement of RWP in the time 10h was the most effecent for screening all the three groups of cultivars. The ideal time for wheat cultivars was adopted at 6h after harvest leaves (Hasheminasab *et al.*, 2012a; Hasheminasab *et al.*, 2013). These differences could be related to tissue of plants and the ability to maintain leaf water. Almost all of the water lost from leaves is lost by diffusion of water vapour through the tiny stomatal pores. The stomatal transpiration accounts for 90 to 95% of water loss from leaves. The remaining 5 to 10% is accounted for by cuticular transpiration. The important factor governing water loss from the leaf is the diffusional resistance of the transpiration pathway, which consists of two varying components: 1) the resistance associated with diffusion through the stomatal pore and 2) the resistance due to the layer of unstirred air next to the leaf surface through which water vapor must diffuse to reach the turbulent air of the atmosphere (Canny, 1998; Vince and Zoltán, 2011). RWP increased significantly (P < 0.05) under drought stress, but the increase was not significant (P < 0.05) in susceptible cultivars (Fig. 2). The highest RWP was observed in tolerant cultivars Badami (17), Ancient Badami (16), Jafari (19), Badami-Male (15) and Kale Ghoochi-Male (20), and the lowest rate were detected in susceptible cultivars Kale Ghoochi 1 (1), Akbari 2 (5), Akbari 3 (6) and White Akbari (7) under both drought stress and non-stress conditions. Intermediate values were observed in Ancient Kale Ghoochi (8), Kale Ghoochi-Bargi (9), Ahmad-Aghaei (11), White Ahmad-Aghaei (12) and White Ohadi (14). The results clearly indicated that RWP measured at the withering times 8h and 10h provides a reliable method for identifying drought tolerant cultivars and quantifying water stress response. Dong *et al.* (2008) and Hasheminasab *et al.* (2013) in wheat, Yousfi *et al.* (2010) in alfalfa and Turkan *et al.* (2005) in bean reported that under stress conditions, higher leaf water retention capacity was a resistant mechanism to drought which the result was a reduction in stomatal conductance and transpiration rate. Loveys (1984) and Gowing *et al.* (1993) reported that under drought conditions, abscisic acid (ABA) is increased in plant tissue and this causes a variety of physiological effects, including stomata closure in leaves. By opening and closing stomata, the guard cells controlled transpiration to regulate water loss or retention. They also stated that tolerant plants had higher rates of ABA as compared with susceptible.

Table 1. Characteristics of investigated pistachio cultivars.

Cultivar	Code	Origin	Location	Predicting reaction to drought
Kale Ghoochi 1	1	Iran	Rafsanjan-Lotf Abad	susceptible
Kale Ghoochi 2	2	Iran	Rafsanjan-Anar	Susceptible
Kale Ghoochi 3	3	Iran- Rafsanjan-Nough	Rafsanjan-Nough	susceptible
Akbari 1	4	Iran	Rafsanjan-Nough	susceptible
Akbari 2	5	Iran	Rafsanjan-Kashkoieh	susceptible
Akbari 3	6	Iran	Rafsanjan-Kashkoieh	Susceptible
White Akbari	7	Iran-Rafsanjan- Nough	Rafsanjan-Nough	susceptible
Ancient Kale Ghoochi	8	Iran-Rafsanjan	Rafsanjan	Intermediate
Kale Ghoochi-Bargi	9	Iran-Rafsanjan-Kashkoieh	Rafsanjan-Kashkoieh	Intermediate
Fandoghi	10	Iran-Rafsanjan- Nough	Rafsanjan-Nough	Intermediate
Ahmad-Aghaei	11	Iran	Rafsanjan-Nough	Intermediate
White Ahmad-Aghaei	12	Iran-Rafsanjan- Nough	Rafsanjan	Intermediate
Ohadi	13	Iran-Zarand	Rafsanjan	Intermediate
White Ohadi	14	Iran-Rafsanjan- Nough	Rafsanjan-Anar	Intermediate
Badami-Male	15	Iran	Rafsanjan-Kashkoieh	Tolerant
Ancient Badami	16	Iran-Rafsanjan-Kashkoieh	Rafsanjan-Lotf Abad	Tolerant
Badami	17	Iran-Rafsanjan-Davaran	Rafsanjan-Davaran	Tolerant
Badami-Self-Female	18	Iran-Rafsanjan-Kashkoieh	Rafsanjan-Kashkoieh	Tolerant
Jafari	19	Iran-Rafsanjan	Rafsanjan-Kashkoieh	Tolerant
Kale Ghoochi-Male	20	Iran	Rafsanjan-Kashkoieh	Tolerant
Ancient Fandoghi	21	Iran-Rafsanjan- Nough	Rafsanjan-Lotf Abad	Tolerant

Principal component analysis (PCA)

PCA is a multivariate statistical method which transforms a number of possibly correlated variables into a smaller number of variables called principal components (Gabriel, 1971; Dong *et al.*, 2008; Saed-Moucheshi *et al.*, 2013). From Fig. 3, it was observed that an increase in the number of the components was associated with a decrease in eigenvalues, which is an important indicator in general genetics and very valuable for evaluating drought tolerant cultivars and also efficient indicators for screening these cultivars. The trend reached its maximum for two components. Thus, it is reasonable to assume that the PCA divided total estimated variables into two main components. Data presented in Table 2 showed that two main components together explained 84.271% (PC1 = 61.394 and PC2 = 22.877) of the total variation, which, in conventional analyses. Data obtained of PCA were graphed in a biplot analysis, so that the eigenvalues of PC1 were plotted against PC2 for both the cultivars and the different methods of measuring RWP (Fig. 4). The biplot is a helpful tool for revealing clustering, multicollinearity, and multivariate outliers of a dataset and it can be also used to display Euclidean distances, variances and correlations of variables of large datasets (Gabriel, 1971; Kohler and Luniak, 2005). The coefficient alpha or Cronbach's alpha was used to check the reliability of the biplot (Table 2). While coefficient alpha has values from 0 to 1.0, the PC is considered reliable (Field, 2009). Hence, this biplot certainly is reliable, since the alpha coefficients for PC1 and PC2 were 0.79 and – 0.124, respectively. In the biplot, the length of the lines approximates the variances of the different RWPs. The line is longer, its variance is higher. Accordingly, there were no differences among the various methods of measuring RWP. The correlation coefficient between any two variables is approximated by the cosine of the angle between vectors drawn from the origin to the trait. An angle of 0° or 180° degrees reflects a correlation of 1 or – 1, respectively, and an angle of 90° represents a correlation coefficient of 0 (Gower and Hand, 1996). The biplot indicated that the angles between withering times 4h with 6h and also 8h with 10h were acute (Fig. 4). Therefore, these times had significant and positive correlation and could be classified in the same group. As seen in the biplot, there was no relationship (nearly 90°) between withering times 4h and 6h with 8h and 10h. The distance from a cultivar to a variable name is an indication of the rank of that variable for that cultivar. Cultivars can be compared by determining their position relative to each other and to a variable name (Yan and Rajcan, 2002). Therefore, cultivars Badami-Male (15), Badami (17), Self-Female (18), Ancient Jafari (19) and Kale Ghoochi-Male (20) with both high PC1 and PC2 had the highest relationship with times 8h and 10h, and confirmed these cultivars are superior for drought condition. Cultivars Kale Ghoochi 1 (1) and Akbari 2 (5) were also selected as the most susceptible cultivars with lowest PC1. According to the biplot, a large number of cultivars were close to times 8h and 10h; it shows that these withering times are the best for monitoring of plant response to drought stress and evaluating tolerant rates of cultivars. According to PC-axis, the ranking of efficient withering times for screening drought tolerant cultivars were: 10h > 8h > 4h ≈ 6h.

Table 2. Principle component analysis of the different withering times for measuring RWP in pistachio cultivars under drought stress condition.

Dimension	Cronbach's Alpha	Variance accounted for		
		Eigenvalue	% of Variance	Cumulative %
1	0.79	2.456	61.394	61.394
2	-0.124	0.915	22.877	84.271
3	-2.116	0.387	9.664	93.936
4	-4.164	0.243	6.064	100
Total	1.000*	4	100	100

*: Total Cronbach's Alpha is based on the total Eigenvalue.

Table 3. Discriminant analysis of the different withering times for measuring RWP in pistachio cultivars under drought stress condition.

Variables	Wilks' Lambda	F	df1	df2	Sig.
4h	0.99	0.087	2	18	0.917
6h	0.934	0.634	2	18	0.542
8h	0.667	4.5	2	18	0.026
10h	0.538	7.733	2	18	0.004

Scatter plot

Fig. 5 showed a scatter plot of all three groups of cultivars including tolerant, intermediate and susceptible to drought based on the different methods of measuring RWP under drought stress condition. A scatter plot is a tool for analyzing relationships between two variables. One variable is plotted on the horizontal axis and the other is plotted on the vertical axis. The pattern of their intersecting points can graphically show relationship patterns. Most often a scatter plot is used to prove or disprove cause-and-effect relationships, while a scatter diagram can be used for screening datasets (Weisberg, 1985; Hasheminasab *et al.*, 2013). As seen in Fig. 5, scatter diagrams of withering time 10h (x-axis) on the other traits (y-axis) were efficient to screen drought tolerant cultivars. These diagrams revealed that the three groups can be separated from each other by this withering time, but there is a large amount of overlap among these groups when used of the RWPs measured at times 4h and 6h as drought tolerant indicator. The results revealed that the time 8h was more reliable for screening two groups of cultivars including tolerant and susceptible. Previous studies have demonstrated that the drought tolerant plants acclimated better than susceptible cultivar by maintaining higher water relations, low membrane injury, pigment photo-oxidation and chlorophyll degradation by inducing well-coordinated antioxidant defense, which results high photosynthesis and yield stability under stress environments (Yousfi *et al.*, 2010; Anjum *et al.*, 2011; Hasheminasab *et al.*, 2012b).

Table 4. Canonical discriminant function coefficients of the different withering times for measuring RWP under drought stress condition.

Variables	Function	
	1	2
10h	.967*	0.078
8h	.737*	0.509
4h	0.089	.928*
6h	0.273	.796*
Eigenvalue	.919a	.003a
% of Variance	99.7	0.3
Cumulative %	99.7	100

*: Significant at the 0.05 probability levels.

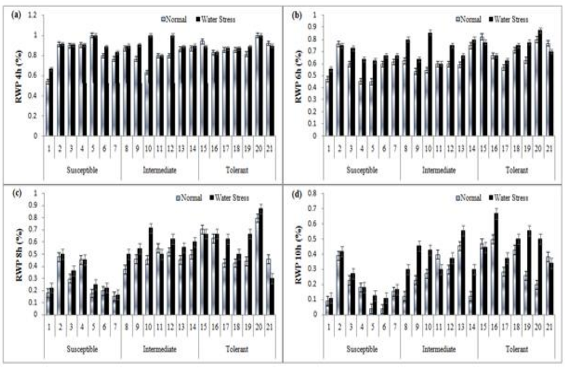

Fig. 2. Influence of drought stress on the measured RWP at the withering times 4h (a), 6h (b), 8h (c) and 10h (d) in pistachio cultivars. Numbers 1 to 21 are the codes of studied pistachio cultivars, respectively (shown in Table 1). Vertical bars indicate LSD ($P < 0.05$) for environmental conditions and cultivars (mean of three replicates ± S.E).

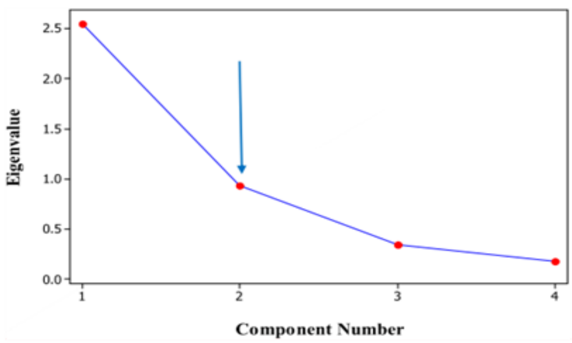

Fig. 3. Eigen values in response to number of components for different methods of measuring RWP.

Discriminant analysis

Discriminate Analysis is a powerful statistical method to investigate differences between groups on the basis of the variables of the cases, indicating which variables contribute most to group separation (Agresti, 2002). Table 3 provided a strong statistical evidence of significant differences among the three groups of cultivars for the withering times 8h (P < 0,05) and 10h (P < 0,01) with producing very high value F's, while the other times were not significant. Wilks' lambda in the Table 3 indicated the significance of the discriminant function and provided the proportion of total variability not explained (Agresti, 2002). The result showed that the times 8h and 10h unexplained 66.7 and 53.8% of variability among groups and 4h and 6h unexplained 99 and 93.4% of variability, respectively. The descriptive technique successively identifies the linear combination of variables known as canonical discriminant functions (equations) which contribute maximally to group separation (Agresti, 2002). The results of canonical discriminant functions analysis showed that the first two functions explain 100% of the total variation (Table 4). The first function explained 99.7% of the total data variation and had significant relationship with 10h (0.967*) and 8h (0.737*) under stress condition. Therefore, these times were the best for classification the cultivars. In order to better understand the ability of RWP for screening drought-tolerant pistachio cultivars, data obtained of canonical discriminant functions analysis were graphed in a biplot (Fig. 6). The results of the biplot revealed that a large number of susceptible cultivars were distributed around the central group 1 as well as many tolerant and intermediate cultivars together scattered around the central groups 2 and 3 (Fig. 6). Thus, it can be concluded that RWP was suitable to separate susceptible cultivars from tolerant and intermediate, but it was not able to screen cultivars with high tolerance from intermediate under drought stress condition. Farshadfar and Hasheminasab (2012) and Farshadfar *et al.*, (2013) reported that genetic gain in developing tolerance in bread wheat could be achieved through indirect selection of physiological indicators related to water status, because the additive genes mainly controlled these traits.

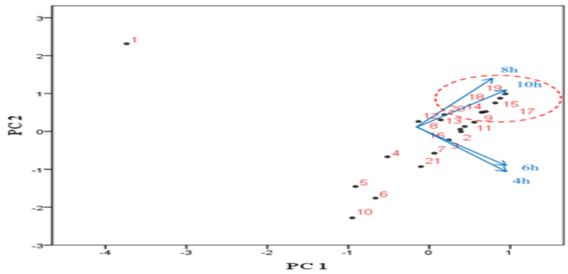

Fig. 4. Biplot of principal component analysis for the different withering times to measure RWP and twenty-one pistachio cultivars. Numbers 1 to 21 are the codes of studied pistachio cultivars, respectively (shown in Table 1).

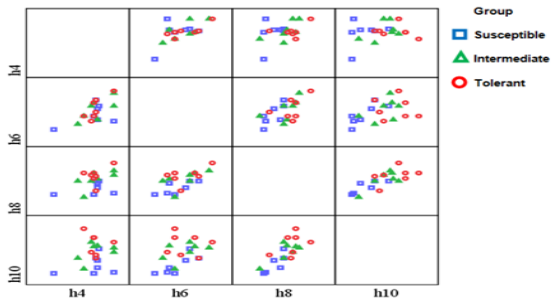

Fig. 5. Scatter plot the three groups of pistachio cultivars including tolerant, intermediate and susceptible to drought based on the different withering times for measuring RWP under drought stress condition.

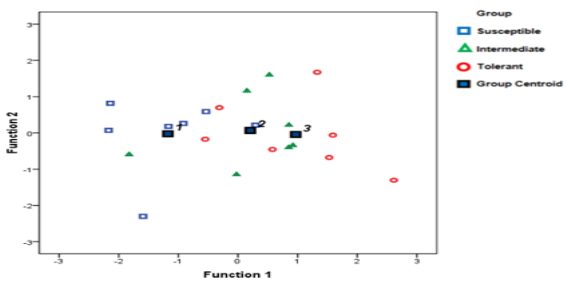

Fig. 6. Biplot of canonical discriminant functional analysis of the measured RWPs for classification of twenty-one pistachio cultivars.

Conclusion

The results of the present study demonstrated that there were significant differences among pistachio cultivars for RWP when grown under drought stress and non-stress conditions. The diversity among the cultivars was increased when the withering time to measure the RWP was longer (4h to 10h). Three groups of cultivars including tolerant, intermediate and susceptible were not clearly separated from each other at the times 4h and 6h. As a result, the measuring RWP at these withering times are not reliable to identify drought-tolerant pistachio cultivars. The time 8h was suitable to separate drought tolerant cultivars from susceptible, but this time was not able to screen cultivars with high tolerance from intermediate under drought condition. The results revealed that measurement of RWP in the time 10h was the most efficent for screening all the three groups of cultivars. The visualizing graphics of scatter plot, biplot of principal component analysis and biplot of canonical discriminant function analysis adopted the RWP measured at the withering time 10h as the best time for classification and identification of drought-tolerant pistachio cultivars.

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